DEVELOPMENTS AND CLINICAL APPLICATIONS IN DIAGNOSTIC MOLECULAR MICROBIOLOGY

Cover design: Tim Schuurman and Ruud Karsten

Cartoons on cover by Bisytes Art (www.bisytes.nl)
Translation of cover cartoons to English

“Antoni’s discovery”
Lady: Is the soup tasty, darling?
Antoni: Yes, but apperently I’m not the only one enjoying it.

“Koch and Pasteur overwhelmed by practical time travel joke of Kary Mullis”
Kary Mullis: There you have it gentlemen, amplified DNA molecules.

“Star Trek”
Crewmember: And doctor…, have you made your diagnosis?
Spock: A low batery, I’m afraid.

Layout: Tim Schuurman

ISBN: 978-90-9023170-9

© 2008 Tim Schuurman.
All rights reserved. Save exceptions by law, no part of this publication may be reproduced or transmitted in any form, by any means, electronic, or mechanical, photocopying, recording, or otherwise, without the prior permission in writing from the author, or where appropriate, of the publisher of the articles.

Printed by: PrintPartners Ipskamp, Enschede, 2008

DEVELOPMENTS AND CLINICAL APPLICATIONS IN DIAGNOSTIC MOLECULAR MICROBIOLOGY

ONTWIKKELINGEN EN KLINISCHE TOEPASSINGEN IN DE DIAGNOSTISCHE MOLECULAIRE MICROBIOLOGIE

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 18 juni 2008 om 11.45 uur

door

Timothy Schuurman

geboren te Wormerveer

Promotiecommissie

Promotor: Prof.dr. A.F. van Belkum

Overige leden: Prof.dr. H.A. Verbrugh
Prof.dr. E.J. Kuipers
Prof.dr. J. Verhoef

Copromotor: Dr. A.A. van Zwet

The work described in this thesis was performed at the Department of Research & Development at the Laboratory for Infectious Diseases in Groningen.

Voor Kirsten, Jesper en Meike.

CONTENTS

CHAPTER 1

General Introduction

Diagnostic microbiology probably started in the late 17th century, when the Dutch scientist Antoni van Leeuwenhoek made microorganisms visible for the first time [124]. Since then, 3 major revolutions have taken place, all of which had a major impact on the field of clinical microbiology. The first revolution took place at the end of the 19th century after the development of solid culture media by Robert Koch [152]. This approach, aiming to obtain pure cultures of causative agents of bacterial infectious diseases, is nowadays still the cornerstone of clinical microbiology. Technological advances have resulted in automated culture and/or phenotypic identification systems that are available in essentially all modern clinical microbiology laboratories.

The second major revolution was the development of direct antigen or antibody detection in clinical specimens or serum, respectively, during the nineteen-sixties and -seventies, using techniques such as radio immunoassays, enzyme immunoassays (EIA) and enzyme linked immunosorbentassays (ELISA). This resulted in enhanced detection speed for many pathogens, or their immunological consequences, since cultivation could be surpassed. However, direct antigen detection tends to suffer from lack of sensitivity, specificity, elevated background titers, and poor negative predictive values [122, 173]. Furthermore, it detects antigen and not living organisms, and it does not facilitate anti-microbial susceptibility testing. The main problem associated with antibody detection is the window before antibodies appear in the serum. Although this antibody-negative window has been shortened with more sensitive modern technology, it still exists as it detects the host-response to infection and not the actual infection. Again, with antibody detection, no anti-microbial susceptibility information is obtained.

The third major revolution, nucleic acid (NA-) based detection of pathogens, initiated approximately 25 year's ago and is still ongoing. The following sections will focus on this latter molecular revolution.

DIAGNOSTIC MOLECULAR MICROBIOLOGY.

A Brief History.

From 1953, when Watson and Crick [175] elucidated the structure of DNA, it took another thirty years for the first molecular diagnostic tests to appear in the microbiology laboratory. These early molecular diagnostics consisted of probe-based detection of pathogens in clinical specimens by Southern hybridization [16, 18, 135, 181]. Unfortunately, the major drawback of these methods was the lack of sensitivity when applied directly to clinical specimens including tissues, cells, stools, and cerebrospinal fluid (CSF) [18, 135, 181].

Development of diagnostic molecular microbiology really speeded up in the late 1980s, when Kary Mullis and coworkers described the *in vitro* synthesis of specific DNA by a polymerase-catalyzed process; the polymerase chain reaction (PCR) [104, 137]. This PCR technology initiated a revolution in the diagnosis of infectious diseases. Indeed, spectacular results were obtained shortly after development of the PCR. It has facilitated the identification of new pathogens, including the causative agent of non-A, non-B hepatitis, now known as the hepatitis C virus (HCV) in 1989 [23]. Table 1.1. summarizes some early applications of PCR-based diagnostics.

TABLE 1.1. Early applications of PCR-based diagnostics.

Pathogen	Disease
Bartonella henselae	bacillary angiomatosis, cat scratch disease
Bordetella pertussis	pertusis
Chlamydia trachomatis	urethritis, cervicitis, pelvic inflammatory disease, ectopic pregnancy, tubal infertility and cervical neoplasia
enteroviruses	meningitis, gastroenteritis, respiratory infection
Hepatitis C virus (HCV)	hepatitis
herpes simplex virus (HSV)	meningitis, encephalitis, dermal infection, genital infection
human immunodeficiency virus (HIV)	aquired immunodeficiency syndrome
Mycobacterium tuberculosis	tuberculosis
Neisseria gonorrhoeae	pelvic inflammatory disease, chronic pelvic pain, ectopic pregnancy, neonatal conjunctivitis, infertility
Tropheryma whippelii	Whipple's disease

As with all new technologies, although exiting and promising results prevailed, also arose new problems for the clinical microbiology laboratory. Quality assessment programs showed that during the early 1990s sensitivity and specificity problems were surprisingly common among laboratories performing routine molecular diagnostic testing. Between 43-100% (13-23% of all negative specimens) and 55-100% (13-40% of all positive specimens)

of the participating laboratories reported false-positive and false-negative results, respectively [36, 87, 111, 126, 183]. In addition, with the enhanced sensitivity of the new molecular diagnostic tests, also questions regarding the interpretation of NA positive specimens were raised; should a positive NA result be interpreted in the same way as a positive culture result at all times?

We have come a long way since these early molecular diagnostic tests appeared in the clinical microbiology laboratory, and, at present, diagnostic molecular microbiology has become a permanent asset in the routine clinical microbiology laboratory.

CURRENT STATE OF MOLECULAR DIAGNOSTIC TESTING WITHIN THE CLINICAL MICROBIOLOGY LABORATORY.

Technological Developments; Assay Types.

In general, molecular diagnostic testing consists of three stages: i) making NA available for detection, ii) amplification (either organism, signal, or NA), and iii) identification of amplification products. However, the means to perform these three common stages and the sequence in which they are performed are subject to great diversity. The methods can be divided roughly into 3 main groups: i) probe hybridization assays (fluorescent in situ hybridization [FISH], colony blots), ii) signal amplification assays (branched DNA [bDNA], hybrid capture), and iii) NA amplification assays (PCR, nucleic acid sequence-based amplification [NASBA], transcription mediated amplification [TMA], strand displacement amplification [SDA], Qβ replicase amplification [QβA], ligase chain reaction [LCR], rolling circle amplification [RCA]) and many of their deviated methods. Figure 1.1 shows a graphical representation of signal amplification, and NA amplification assays.

Probe hybridization assays are non-amplification methods since the NA-probe hybrids are detected direct by fluorescent, radioactive, or non-radioactive (enzyme) labeling of the probe. However, to obtain sufficient sensitivity for clinical use, these assays are usually performed on cultured material, which indirectly amplifies the NA prior to the test. With FISH usually one of the rRNA genes is hybridized with a NA probe after permeabilization of the cell wall. Hybridized probes are subsequently detected using fluorescence microscopy. FISH can be performed with either oligonucleotide

or peptide nucleic acid (PNA) probes, both of which have shown excellent sensitivities and specificities when utilized on growth-positive blood cultures [120]. However, when used for direct detection of pathogens in clinical specimens, FISH suffers from the same lack of sensitivity as other microscopy procedures do, including Gram-staining. In a similar fashion, bacterial growth from an agar plate can be transferred to a nylon membrane, and, subsequently, the targeted organism can be identified by hybridizing this "colony blot" with a species-specific oligonucleotide probe.

In signal amplification assays, the released NA of the targeted organism is hybridized to a NA probe, which is subsequently detected by amplifying the probe's signal. The best known signal amplification assay is the bDNA assay (Fig. 1.1A). It owes its name to the branched complexes that are formed by the captured target (DNA or RNA), target or label extender probes, preamplifier probes (in the 3rd generation assays), and amplifier probes. These bDNA complexes amplify of the number of detector probe sites.

Hybrid capture (Fig. 1.1B) is another form of a signal amplification assay, based on the combination of hybridization of target specific single stranded (ss) RNA probes with target DNA, followed by capturing of the RNA:DNA hybrid with universal RNA:DNA-hybrid recognizing antibodies. The captured RNA:DNA-hybrids are subsequently detected by multiple alkaline phosphatase conjugated antibodies, resulting in signal amplification.

Several different approaches are available to physically amplify NA. Among these, especially PCR and to a lesser extend NASBA are the most widely used techniques to date.

PCR, which was first described by the group around Cetus scientist Kary Mullis in the second half of the 1980s [104, 137], consists of a 3-temperature stage process to amplify DNA (denaturing [± 95°C], annealing [± 50–65°C], and polymerization [± 72°C]), and can in theory amplify a single target molecule exponentially to 10^9 to 10^{14} copies over the course of 30–50 cycles. An interesting modification of PCR is the application of multiple primer pairs in a single reaction, therefore enabling simultaneous amplifications of multiple targets. This principle was described as early as in 1988 [21]. Although originally developed for amplification of DNA, RNA can also be amplified by PCR, after it is first transcribed to a complementary

copy DNA sequence (cDNA) using the enzyme reverse transcriptase (RT) [75].

NASBA (Fig. 1.1C) is a technique which mimics the *in vivo* process of retroviral replication [31, 59]. Several variations of this technology have been described under the names of transcription amplification system (TAS), self-sustained sequence replication (3SR), and transcription-mediated amplification (TMA) [49, 59, 81]. The basic principle of NASBA involves cycles of reverse transcription and RNA polymerase-mediated replication to generate anti-sense, single stranded RNA products. This process is mediated by 3 independent enzymes, RT, RNase H, and a T7 DNA-dependent RNA polymerase, which isothermally co-operate to exponentially form the single stranded RNA amplicons. Although NASBA is designed to amplify RNA, DNA can also be amplified using simple tricks such as heat denaturizing of the target sequence rendering it single stranded for direct RT-mediated primer extension. Increase of the efficiency of DNA NASBA, can be obtained by treatment of the extracted DNA with a restriction enzyme [38].

SDA (Fig. 1.1D) starts with target generation. An SDA-specific target primer (S1), containing a restriction site at its 5'-terminus, is elongated by a DNA polymerase lacking 5' → 3' exonuclease activity. Subsequently, a blocking or displacement primer (B1) that has hybridized upstream of primer S1 is elongated, displacing the synthesized product of S1. The displaced S1 product is the subjected to complementary strand syntheses with primers S2 and B2, forming a double stranded product that carries a restriction site. As the same process is repeated at the complementary strand, two copies of restriction site containing target are formed. The restriction sites are hemi-thiolated as one of the nucleotides in the dNTP mixture is replaced by its thiolated analog (dependent on the restriction enzyme used). When the restriction sites in the target are subsequently cleaved by the specific restriction endonuclease, a nick is created due to the hemi-thiolated structure of the restriction site. This starts the amplification cycle of SDA, where the nicked strand is elongated and thereby displacing the downstream strand, creating another double stranded target containing a hemi-thiolated restriction site.

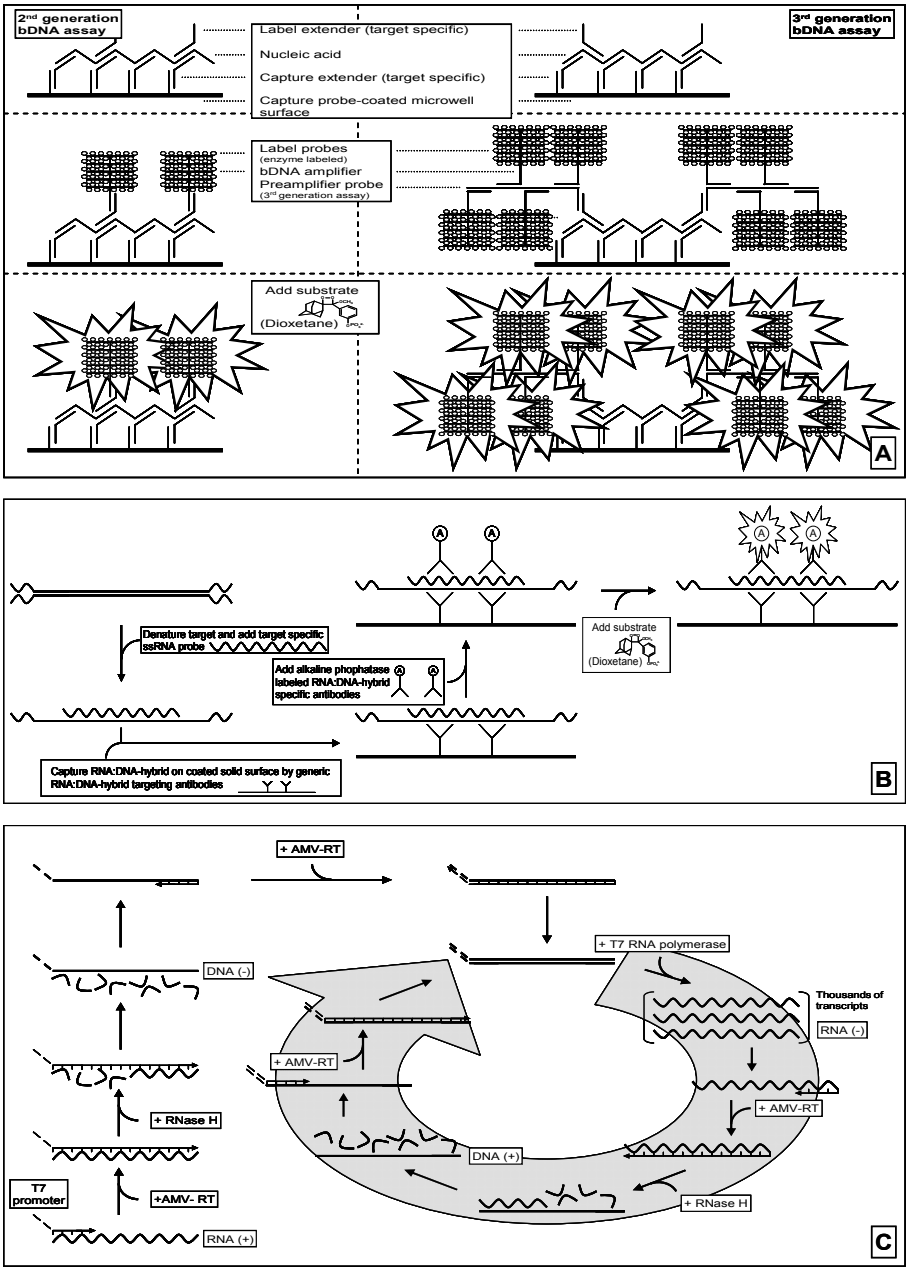
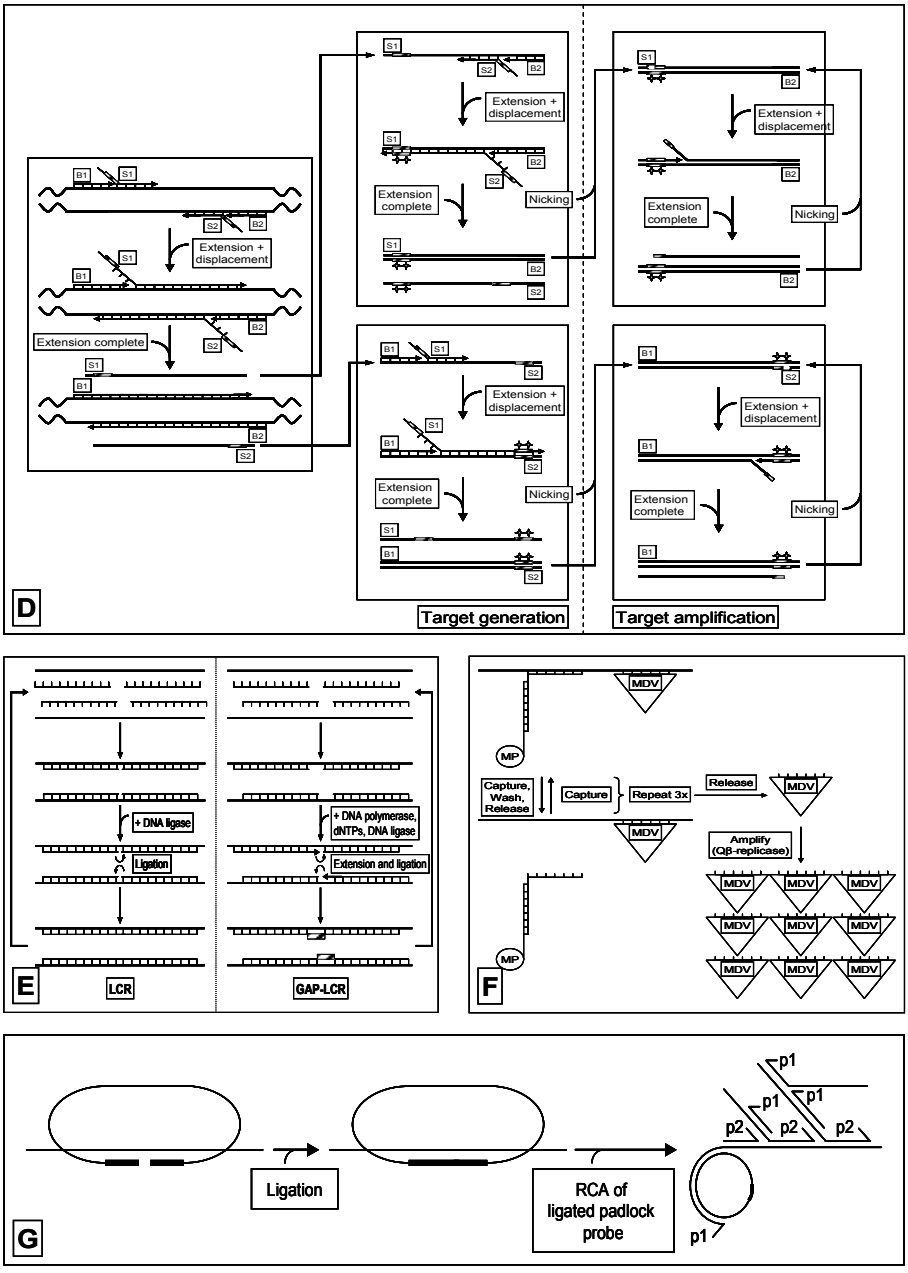


Figure. 1.1. Graphical representation of several signal and nucleic acid amplification assays widely used in diagnostic molecular microbiology.

A) bDNA; B) Hybrid-capture; C) NASBA; D) SDA, [S1/2, target-specific SDA primers containing a restriction sites at their 5'-terminus (striped box); B1/2, outer blocking/displacement primers; stars depict hemi-thiolated restriction sites introduced by DNA-polymerase mediated extension



using 1 thiolated nucleotide substitute in the dNTP mixture; E) (GAP-) LCR; F) QβA, [MP, magnetic particle; MDV, recombinant midivariant-1 RNA probe (target of Qβ replicase)]; G) RCA (modified from reference 181). See text for more details.

LCR (Fig. 1.1E) is quite similar to PCR, with as major distinguishing factor the lack of *de novo* DNA synthesis in LCR. It uses 2 sets of complementary probes that hybridize either directly adjacent to each other on the template sequence or 1-3 bases apart from each other (Gap-LCR). In the first case the probes are ligated by DNA ligase, whereas in the latter case DNA polymerase and dNTP's are used to fill in the gap, followed by the joining of both the extended probes by DNA ligase.

Q*β*A can be considered as a hybrid between signal and NA amplification, as the signal from a hybridized probe is amplified by RNA amplification. In Q*β*A (Fig. 1.1F), a recombinant probe containing a target specific sequence and a RNA substrate for the enzyme Q*β* replicase is hybridized to a captured target sequence. Subsequently the unbound probe is washed away or cleaved by RNase III, whereafter the enzyme Q*β* replicase amplifies the RNA substrate part of the probe at remarkable speed (10^6–10^9 $\times$ in 15 to 30 min.). A major drawback of Q*β*A is the observation that it is sensitive to "intrinsic" contamination [102].

RCA can be used as either a signal or NA amplification method, and has been described in two formats, linear or exponential amplification. In linear amplification a circular DNA molecule is amplified by polymerase extension of a complementary primer. As the template has to be a circular DNA, this method is restricted to circular viruses, plasmids and circular chromosomes. Exponential RCA uses a second complementary primer to the circular DNA to achieve isothermal, exponential amplification. With exponential RCA non-circular templates can be amplified using padlock probes (Fig. 1.1G), which hybridize to a specific linear template and are subsequently ligated to form a circular target molecule for RCA. Exponential RCA has been shown to be sensitive and highly specific, with 10^7-fold amplification in one hour under isothermal conditions [143].

Technological Developments; Detection of NA Amplification Products.

The most simple and direct detection method for amplification products is agarose gel electrophoresis in combination with ethidium bromide (EtBr) staining. However, this method is relatively insensitive and therefore other methods have been developed. These include Southern blotting with detection by (non-) radioactively labeled probes, solid- or liquid-phase hybridization (with detection by EIA, ELISA, reverse line blot (RLB), or

electrochemiluminescence (ECL)), micro-arrays, and mass-spectrometry. These methods have resulted in 10- to 100-fold increased sensitivities compared to conventional gel-based detection [3, 14, 69]. Furthermore, the PCR amplification and ELISA detection format has been automated in the COBAS AMPLICOR system (Roche Diagnostics). Unfortunately, however, this system does not allow use with in-house assays of similar chemistry [72].

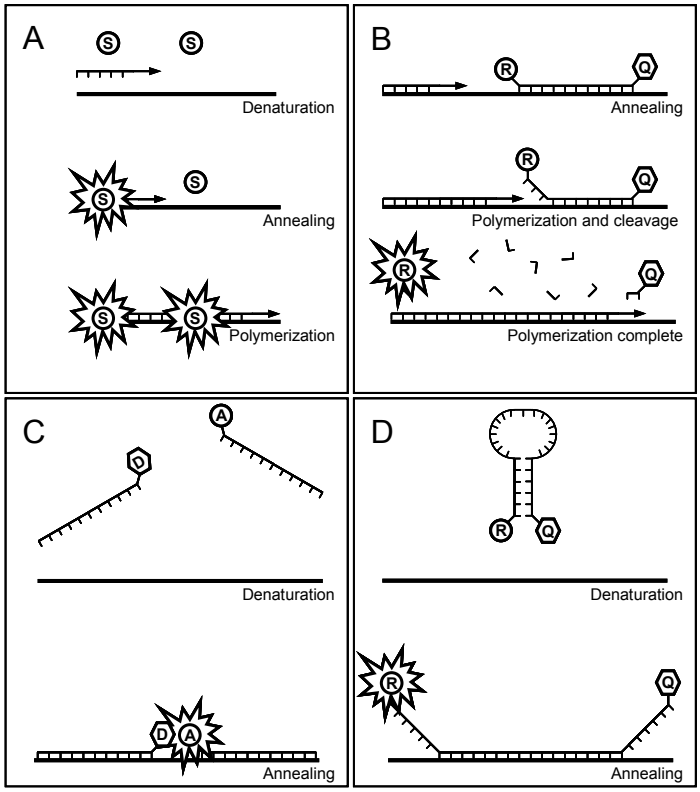


Figure 1.2. Graphical representation of the four most widely adapted real-time PCR chemistries.

A) SYBR green [S, SYBR green-I dye]; B) 5'-nuclease (hydrolysis, TaqMan) probes [R, reporter dye; Q, quencher dye]; C) FRET hybridization probes (HybProbes, LightCycler probes) [D, donor dye; A, acceptor dye]; D) Molecular beacons [R, reporter dye; Q, quencher dye].

With the development of fluorescent real-time detection methods, the need for subsequent handling of amplification products and the laborious detection methods have essentially been eliminated. Many formats are currently available for real-time detection of amplification products, but the

four most widely adapted chemistries are SYBR Green, 5'-nuclease probes (double-labeled oligonucleotide, hydrolysis, or TaqMan probes), fluorescence resonance energy transfer (FRET) hybridization probes (HybProbes or LightCycler probes), and molecular beacons. All these chemistries are suitable for PCR, whereas the molecular beacon is the only chemistry currently available for NASBA. Figure 1.2 shows a graphical overview of these four real-time detection chemistries, whereas other less commonly used real-time detection chemistries are reviewed elsewhere [94].

Table 1.2 summarizes some of the currently widely used real-time amplification instruments and their features.

Technological Developments; Sample Preparation.

One of the bottlenecks in molecular diagnostics is specimen preparation. Ideally, specimen preparation should be universal, constituting a single chemistry, which is applicable to both DNA and RNA, with equal affinity for ss and ds forms of NA. Furthermore, it should be applicable to a wide variety of clinical specimen types, have a high recovery rate (preferably 100%), should concentrate the NA from the specimen, should efficiently remove inhibitory substances, be insensitive to specimen-to-specimen cross-contamination, and be amendable to automation. In addition, it should be able to lyse all clinically relevant microorganisms.

For culture enriched specimens, a simple boiling step is usually efficient to release sufficient amounts of DNA to be detected by amplification methods without any noteworthy inhibitory effects, excluding the inhibitory effect caused by addition of excessive amounts of target DNA. However, when different clinical specimens need to be processed, simple boiling does not eliminate inhibitory substances present in some of these specimens (e.g. hemoglobin in blood, and complex polysaccharides, bilirubin and bile salts in stool).

Originally this was solved using organic NA extraction methods, but these procedures are nowadays considered to laborious and hazardous for laboratory staff due to the toxic and/or corrosive nature of the chemicals needed.

TABLE 1.2. Selection of currently available instruments for real-time PCR.

Manufacturer	Instrument[a]	Major chemistries supported[b]	Detection	Sample size	Remarks
Applied Biosystems (www.appliedbiosystems.com)	7300 RTPS	SYBR green, 5'-nuclease probe, molecular beacon	3 color-multiplex	96	Fast cycling available.
	7500 RTPS	SYBR green, 5'-nuclease probe, molecular beacon	4 color-multiplex	96	Fast cycling available.
	7900 HT RTPS	SYBR green, 5'-nuclease probe, molecular beacon	500-660 nm	96-384	Fast cycling available.
Bio-Rad (www.bio-rad.com)	MyiQ	SYBR green, 5'-nuclease probe, molecular beacon	Single color	96	
	iCycler iQ5	SYBR green, 5'-nuclease probe, molecular beacon	5 color-multiplex	96	
Cepheid (www.cepheid.com)	SmartCycler II	SYBR green, 5'-nuclease probe, molecular beacon	4 color-multiplex	16-96	Special sample containers. Independent modules.
	GeneXpert	SYBR green, 5'-nuclease probe, molecular beacon	4 color-multiplex	1-16	Combined sample prep and amplification in special sample containers. ASR use only.
Corbett Lifescience (www.corbettlifescience.com)	Rotor-Gene 6000	SYBR green, 5'-nuclease probe, molecular beacon	6 color-multiplex	36-72-100	Standard tubes.
	Rotor-Gene 3000	SYBR green, 5'-nuclease probe, molecular beacon	4 color-multiplex	36-72	Standard tubes.
Roche Diagnostics (www.roche-diagnostics.com)	LightCycler 1.5	SYBR green, Hybridization probes, 5'-nuclease probe, molecular beacon, SimpleProbe	3 channels	32	Special sample containers.
	LightCycler 2.0	SYBR green, Hybridization probes, 5'-nuclease probe, molecular beacon, SimpleProbe	6 channels	32	Special sample containers.
	LightCycler 480	SYBR green, Hybridization probes, 5'-nuclease probe, SimpleProbe	6 channels	96-384	
Stratagene (www.stratagene.com)	Mx3000p	SYBR green, 5'-nuclease probe, molecular beacon	4 color-multiplex	96	
	Mx3005p	SYBR green, 5'-nuclease probe, molecular beacon	5 color-multiplex	96	
	Mx4000	SYBR green, 5'-nuclease probe, molecular beacon	4 color-multiplex	96	

[a] RTPS, Real-Time PCR System; HT, High Throughput.

[b] Most widely used chemistries supported, additional less frequently described chemistries may also be supported.

To overcome the use of classical organic extraction, the NA binding properties of silica, or glass in the presence of chaotropic agents, such as guanidinium thiocyanate (GuSCN), guanidinium hydrochloride (GuHCl), sodium iodine (NaI), and sodium perchlorate ($NaClO_4$) can be exploited.

In 1990, the group of René Boom perfected this NA extraction chemistry for use with a large variety of clinical specimens [12]. Indeed, the “BOOM” chemistry, as it is also referred to, complies with nearly all the demands of the ideal specimen preparation procedure as mentioned above. Several large manufacturers of commercial NA extraction kits have therefore adapted this technology to serve as the basis of their extraction kits. Examples of these are Roche (High Pure), Qiagen (QIAamp, DNeasy), and bioMérieux (NucliSENS Basic Kit Isolation Reagents). Based on its performance, applicability and high overall usage, the “BOOM” chemistry is considered as the “gold standard” in NA extraction technology. The strength of the original “BOOM” chemistry lies in the generic nature of the extraction process, making it useful for the extraction of all forms of NA. The only drawback is that its lysis capability for Gram-positive bacteria, acid-fast bacilli, as well as fungi is insufficient [12]. Pretreatment strategies need to be incorporated, including lysis at 80°C [112], and enzymatic, or physical disruption [127].

Since sample throughput is increasing, automation of the NA extraction procedure is mandatory. Therefore, automated extraction systems have been developed. Nearly all these systems are again based on the “BOOM” chemistry. Some examples of these automated NA extraction platforms are the MagNA Pure LC and Compact systems (Roche), BioRobot series (Qiagen), ABI Prism 6100, 6700 systems (Applied Biosystems), m1000, m2000sp systems (Abbott), and NucliSENS Extractor, miniMAG (semi-automated), easyMAG systems (bioMérieux). These systems allow for medium to high-throughput NA extraction on a walk away basis with minimal hands-on time. Table 1.3 summarizes the most widely used automated NA extraction devices and their characteristics.

Technological Developments; Quality Control (QC).

Quality control is essential for assuring reproducible and confident diagnostic molecular microbiology test results. QC also plays a vital role in the accreditation of clinical microbiology laboratories: laboratories need to conform to the international standard ISO 15189 [110, 157].

TABLE 1.3. Selection of currently available instruments for automated NA extraction[a].

Manufacturer	Instrument	Chemistry	Sample size and throughput	Sample volume[b]	Elution volume[b]	Remarks
Applied Biosystems (www.appliedbiosystems.com)	ABI Prism 6100	Silica filter membrane	96 samples/ 30 min.	5-750 µl	75-200 µl	
	6700	Silica filter membrane	96 samples/ 75 min.	5-750 µl	75-200 µl	
bioMérieux (www.biomerieux.com)	NucliSENS miniMAG	Magnetic silica particles	12 samples/ 45 min.	≤ 1000 µl	≥ 10 µl	Generic extraction, IVD
	extractor	Silica filter membrane	10 samples/ 35-45 min.	10-2000 µl	20-70 µl	Generic extraction
	easyMAG	Magnetic silica particles	24 samples/ 60 min.	10-1000 µl	25-110 µl	Generic extraction, IVD
Cepheid (www.cepheid.com)	GeneXpert	NS	4-16 samples/ 30 min.	NS	NS	Combined sample prep and amplification, ASR use only.
Roche Diagnostics (www.roche-diagnostics.com)	MagNA Pure Compact	Magnetic glass particles	8 samples/ 30 min.	100-1000 µl	50-200 µl	
	LC	Magnetic glass particles	32 samples/ 105-135 min.	50-1000 µl	50-100 µl	
	COBAS AmpliPrep	Magnetic glass particles or specific probe capture	72 samples/ 150 min.	50-850 µl	65-75 µl	IVD applications available
Qiagen (www.qiagen.com)	BioRobot EZ1	Magnetic silica particles	6 samples/ 20 min.	200-350 µl	50-200 µl	IVD version available
	M48	Magnetic silica particles	48 samples/ 160 min.	200-400 µl	50-200 µl	
	MDx	Silica filter membrane	96 samples/ 150 min.	200-263 µl	91-200 µl	IVD version available

[a] NS, not specified; IVD, in vitro diagnostics; ASR, analyte specific reagents.

[b] Sample and elution volumes may be kit and/or application restricted by the manufacturer.

These guidelines include requirements for quality and competence, that clearly state that the laboratories have to independently establish the analytical and performance characteristics of all their assays, including in-house assays, analyte specific reagents (ASR), and CE marked assays.

In the early years when molecular diagnostic tests were only just introduced, several multicenter proficiency studies involving hepatitis B virus (HBV), HCV, HIV-1, and *M. tuberculosis*, during the early 1990s indicated problems surrounding sensitivity and specificity in many of the participating laboratories [11, 36, 87, 126, 183]. These proficiency studies, also shown in Table 1.4, focused the attention to the problems that needed to be addressed, and the quality control measures which should be implemented in today's diagnostic molecular microbiology laboratory. These quality control measures will be discussed in more detail in the following sections.

QC; Laboratory Facilities and Work-Flow.

One of the most critical requirements for successful molecular diagnostic testing is the physical separation of the processes, equipment, and reagents used. To facilitate this, the molecular diagnostic laboratory should be setup in at least 3 separate work areas, with a one-way directional workflow.

These separated work areas include the following: a reagent preparation/clean room, which is also in use for reagent storage (PCR room I), a specimen preparation area where NA are extracted and the NA are added to the amplification reactions (PCR room II), and the amplification and detection area (PCR room III), where preferably clean amplification and detection methodologies (i.e. closed tube amplification and detection), are separated from methodologies requiring further processing of amplified material (i.e. hybridization, EIA, ELISA, RLB, ECL, sequencing, etc.). Although with the current closed tube, real-time detection formats the separation of PCR room II and III may have become obsolete, most laboratories continue this separation to be on the safe side.

In accordance with this requirement for physical separation into different work areas, also all laboratory equipment should be dedicated for use in a single area, and for a single purpose. Also, the workflow of laboratory personnel should be directed from clean to dirty, or in other words from reagent preparation to post-amplification handling. All work areas should have very strict cleaning regimens with NA destroying solutions such as 10%

house-hold bleach solution. Laboratory coats and gloves should be changed for every transition in the workflow and also in between different process stages (i.e. between addition of infectious specimen to the NA extraction and the subsequent extraction process, or between NA extraction and adding the NA extract to the reactions).

The need for the separation of processes is evident, even with the high degree of automation nowadays. Although automation has greatly reduced the potential for contamination, it has not completely eliminated it. We should therefore not neglect the need for physical separation of the processes in molecular diagnostics.

QC; Prevention of False-positive Results.

To avoid false-positive test results, standard operating procedures should be followed for all different work areas, assays and other related procedures; laboratory staff should be highly trained; only aerosol-resistant tips should be used for pipettes; negative controls should be included for monitoring contamination (both specimen to specimen and reagent contamination); and only small numbers of low level positive controls should be used. Nested amplification should be avoided as much as possible, since it has been linked to an increased risk for carry-over contamination [5, 6, 83, 103, 116]. It is even highly questionable whether nested PCR should be used at all, as most modern amplification technologies are able to detect a single copy of the selected target gene.

The use of the Uracil-*N*-Glycosylase (UNG) system is recommended to prevent amplicon carry-over contamination. The UNG system utilizes the cleavage of *N*-glycosidic bonds by the enzyme UNG in uracil containing templates [92]. The UNG system is capable of a 10^6 to 10^{10}-fold reduction in amplicon amounts, which makes it a very effective amplicon carry-over contamination prevention method [92]. However, it should be emphasized that use of the UNG system does not prevent other contamination routes, and UNG utilizing methodologies, do not preclude all types of false-positive results [36, 85, 89, 97, 103, 111, 112, 113, 141, 142, 163].

Robotic automation has also been shown to reduce the risk of false-positive results by reducing the number of potential stages where contamination can occur and by its increased precision [162].

TABLE 1.4. Results for qualitative detection of pathogenic microorganisms in multicenter proficiency studies.

Target microorganism[a]	Year[b]	Lab's	Data sets	Datasets with false-[c] positives	Datasets with false-[c] negatives	Specimens tested false-[c] positive	Specimens tested false-[c] negative	Remarks and [reference[c]]
Viruses								
influenza virus A (A)	2006a	17	17	0%	76.5%	0%	25.5%	
influenza virus B (A)	2006a	17	17				11.8%	
PIV 1 (B)	2006b	15	15	0%	93.3%	0%	46.7%	
PIV 3 (B)	2006b	15	15				42.2%	
RSV (C)	2006c	16	16	0%	50.0%	0%	29.2%	
hMPV (C)	2006c	14	14		14.3%		7.1%	
hRV 16 (D)	2006d	11	11	9.1%	81.1%	9.1%	21.2%	Total of 94 different assays, 44% real-time PCR, 29% nested PCR, 27% other methodologies, one single commercial assay used in 4% of sub-panels and scoring overall second to last [158].
hRV 72 (D)	2006d	11	11				39.4%	
hRV 90 (D)	2006d	11	11				21.2%	
ADV 4 (E)	2006e	14	14	0%	71.4%	0%	16.7%	
ADV 7 (E)	2006e	14	14				35.7%	
hCoV 229E (F)	2006f	8[d]	8[d]	0%[d]	100%[d]	0%	25.0%	
hCoV OC43 (F)	2006f	7	7			0%	40.0%	
mixed A	2006a	17	17	NA	11.8%	NA	11.8%	
mixed B	2006b	15	15	NA	13.3%	NA	13.3%	
mixed E	2006e	7	7	NA	42.9%	NA	42.9%	
HCV	1993	31	31	29%*	39%*	11%	NS	All in-house, of which 61% nested PCR [141*, 163*, 183].
	1996	86	136	21%*	63%*	NS	NS	73% in-house, of which 81% nested [36, 141*, 163*].
	1999	57	80	2.5%	46.3%	1.3%	11.5%	78% commercial, of which 23% (semi-)automated [141].
	2000	81	123	1.6%	57.7%	0.8%	25.6%	81% commercial, of which 58% (semi-)automated [141].
	2001	17	17	7.8%	3.9%	4.2%	0.5%	88% commercial, of which 40% (semi-)automated [97].
enterovirus	1995	13	13	NS	100%	2.0%	39.2%	Roche AMPLICOR test only [85].
	1999a	11	13	7.7%	84.6%	2.6%	23.1%	69-75% in-house, of which 78-89% nested, 71% of
	1999b	11	12	25.0%	91.7%	11.1%	16.0%	False-positive results related to nested PCR [103].
	1999c	11	12	50.0%	66.7%	12.5%	10.2%	
	2001	59	70	NS	NS	3.8%	21.9%	77% in-house, of which 69% (semi-)nested [171].

TABLE 1.4. Continued.

Target microorganism[a]	Year[b]	Lab's	Data sets	Datasets with false-[c] positives	Datasets with false-[c] negatives	Specimens tested false-[c] positive	Specimens tested false-[c] negative	Remarks and [reference[c]]
HBV	1995	39	43	35%*	37%*	12.6%	12.6%	98% in-house, of which 24% nested [126, 141*, 163*].
	1999	42	57	8.8%	43.9%	5.3%	10.5%	56% commercial [163].
	2000	51	72	2.8%	45.8%	1.4%	17.4%	61% commercial [163].
HSV	1999	66	76	7.9%	68.4%	4.8%	21.9%	95% in-house [142].
	2000	71	78	14.1%	67.9%	6.0%	17.1%	88% in-house [142].
orthopoxviruses	2004	22	22	NS	NS	0.9%	22.7%	All in-house, real-time PCR better performance [108].
	2006	33	33	NS	NS	11.8%	15.0%	76% in-house, 70% real-time, both real-time and commercial kit related to better performance, false-positivity related to first time participants [107].
SARS-CoV	2004a	58	58	6.9%	72.4%	2.3%[e]	15.3%	Commercial real-time PCR kits (24%) positively influenced sensitivity, 2004b data from manufacturers only [44].
	2004b	4	4	0%	0%	0%	0%	
Lassa virus	2004	14	14	NS	NS	1.4%	35.7%	All in-house, real-time PCR better performance [108].
filoviruses	2004	14	14	NS	NS	0%	29.6%	All in-house, real-time PCR better performance [108].
denque virus	2004	13	15	40.0%	86.7%	42.9%	29.5%	87% in-house, nested in 83% of labs with false-positives [83].
HPV	2000	3	3	0%	100%	0%	2.8%	Single commercial assay (GMY-LB from Roche) [79].
HHV-8	1999	5	10	40.0%	90.0%	11.4%	30.0%	All in-house, 70% nested, false-positive results related to nested PCR [116].
Bacteria								
Chlamydophila pneumoniae	2001	9	16	18.8%	81.3%	18.8%	28.1%	All in-house, 63% nested, false-positive results in 67% related to nested PCR [5].
	2002	15	16	0%	56.3%	0%	13.4%	All in-house, 80% real-time [89].
	2002a	4	4	0%	100%	0%	41.7%	Single in-house nested PCR, DNA dilution series and artificial contaminated specimens extracted on-site with 3 NA extraction protocols tested [4].
	2002b	4	12	8.3%	16.7%	1.7%	10.0%	
	2002c	4	12	8.3%	NA	3.3%	NA	

TABLE 1.4. Continued.

Target microorganism[a]	Year[b]	Lab's	Data sets	Datasets with false-[c] positives	Datasets with false-[c] negatives	Specimens tested false-[c] positives	Specimens tested false-[c] negatives	Remarks and [reference[c]]
Chlamydophila pneumoniae continued	2004	17	18	5.6%	94.4%	5.6%	40.2%	94% in-house, 83% real-time [89].
Mycobacterium tuberculosis	1994	7	7	100%	100%	21.7%	39.5%	All in-house, of which 29% nested [112].
	1996	30	30	43.3%	76.7%	23.2%	31.5%	73% in-house, of which 18% nested [111].
	2001	77	85	NS	NS	4.3%	26.3%	62% commercial, of which 42% automated, 71% of false positive data sets related to in-house assays [113].
	2006a	5	5	40.0%	NA	5.6%	NA	4 in-house TaqMan assays, 3 targeting within a 84 bp region (but not identical) of the IS6110, the other 239 bp upstream of this region [138].
	2006b	5	5	0%	100%	0%	46.7%	
	2006c	2[f]	2[f]	0%	100%	0%	61.1%	
Bordetella pertussis	2005a	6	7	0%	85.7%	0%	23.6%	All in-house, improvement from 14% to 67% real-time, target gene highly effects specificity [105].
	2005b	9	9	88.9%[g]	55.6%	18.5%[g]	9.3%	
Mycoplasma pneumoniae	2002	12	13	15.4%	100%	4.9%	39.6%	92% in-house, 69% real-time [89].
	2004	15	18	0%	16.7%	0%	7.4%	94% in-house, 83% real-time [89].
Escherichia coli O157:H7	2004a	15	15	20.0%	46.7%	7.8%	24.2%	Single in-house gel-based assay, performance is mainly laboratory dependent [8].
	2004b	13	13	7.7%	53.8%	9.0%	30.5%	
Chlamydia trachomatis	2000	96	102	1.9%	34.3%	0.7%	6.3%	91% commercial [172].
Salmonella enterica	2003	16	16	25.0%	50.0%	4.0%	4.0%	Single in-house gel-based assay [96].

[a] PIV, parainfluenza virus; RSV, respiratory syncytial vrus; hMPV, human metapneumovirus; hRV, human rhinovirus; ADV, adenovirus; hCoV, human coronavirus; mixed A, influenza virus A and B; mixed B, PIV 1 and 3; mixed E, hCoV 229E and OC43; HCV, hepatitis C virus; HBV, hepatitis B virus; HSV, herpes simplex virus; SARS-CoV, severe acute respiratory syndrome-coronavirus; HPV, human papillomavirus; HHV-8, human herpesvirus 8.
[b] Year the proficiency panel was distributed, or year of publication of the results; 2006a, represents 1st sub-panel from a study with multiple sub-panels, and so on.
[c] *, data derived from other reference than original study, marked with * in references; NS, not specified; NA, not applicable.
[d] 1 laboratory only performed hCoV 229E PCR, and therefore this lab not included in the results for participant/datasets.
[e] Total number of false-positive reactions was not specified, but taking into account that every participant with false-positive results had at least 1 false positive result, the false-positivity rate among the 174 tests expected to be negative would be at least 2.3%.
[f] 3 of the 5 labs were excluded from the additional data due to cross-reaction of their TaqMan assays with the *Mycobacterium smegmatis* internal control, which was tested at 3 concentrations.
[g] The numbers represent both false-positives in buffer controls and detection of *Bordetella holmesii* and *B. brochiseptica*, when only the buffer controls were taken into account the numbers are 11.1% and 2.8% respectively; pretesting results for the organizing laboratory were not included.

The recent introduction of closed tube amplification and detection methodologies such as real-time PCR contributed significantly to the prevention of false-positive results [44, 107, 108]. This, among other reasons, has resulted in many laboratories to convert conventional molecular diagnostic tests to real-time PCR tests.

When a target sequence or gene is selected, it is important to check the specificity of the selected sequence for cross-reaction with other microorganisms that can be present in the same specimen. It has been documented for instance for *Bordetella pertussis* that detection of the IS481 insertion sequence can result in false-positive results due to *B. holmesii* and *B. bronchiseptica*. False-positive results with mycobacteria other then *M. tuberculosis* carrying *IS6110* homologues were also observed with a TaqMan assay targeting a region of the IS*6110* insertion sequence for *M. tuberculosis* located 239 bp upstream of three other TaqMan assays [138].

Finally, the use of commercially available tests has been shown to contribute to the avoidance of false-positive test results, because of enhanced test standardization. However, the use of commercially available tests does not completely guarantee optimal test performance [36, 85, 89, 97, 103, 113, 141, 158, 163, 172]. Furthermore, commercial tests are only available for a limited number of pathogens, also referred to as “commercially interesting targets” (Table 1.5) [109]. Therefore, there is still a wide range of clinically relevant pathogens for which in-house assays need to be developed.

TABLE 1.5. Examples of commercially interesting vs commercially non-interesting targets in clinical microbiology [H. G. M. Niesters, personal communication].

Commercially interesting targets	Important in-house targets to be developed	
HIV type 1	HIV type 2	Parvovirus B19
Hepatitis B virus	Epstein–Barr virus	Hepatitis A virus
Hepatitis C Virus	Cytomegalovirus	Hepatitis E virus
	Herpes simplex virus 1/2	Adenovirus
Chlamydia trachomatis	Varicella zoster virus	Astrovirus
Neisseria gonorrhoeae	Herpesvirus type 6	Norovirus
Methicillin-resistant	Herpesvirus type 8	Rotavirus
Staphylococcus aureus	Enterovirus	
	Parechovirus	*Bartonella henselae*
	Rhinovirus	*Bordetella pertussis*
	Respiratory syncitial virus	*Chlamydophila pneumoniae*
	Human metapneumovirus	*Legionella pneumophila*
	Influenzavirus	*Mycobacterium tuberculosis*
	Parainfluenzavirus	*Mycoplasma pneumoniae*
	Coronavirus	……
	West-Nile Virus	

Although the measures presented above are mostly sufficient for molecular diagnostic testing, there are some applications which require additional monitoring of the reagents used. This is especially true for broad-range bacterial and fungal PCRs and for assays targeting *Legionella* species. These assays are more prone to false-positive results due to reagent contamination. Such reagents provide an ignored source of contamination. Decontamination fluids, such as chlorhexidine solutions and Betadine iodine in a balance salt solution, have been reported to be contaminated with (large) quantities of bacterial DNA [55, 161]. Commercial NA extraction kits have also been reported to be contaminated with bacterial DNA [48, 119, 167], as are enzyme preparations [15, 34].

This problem remains difficult to solve, although several ingenious attempts have been described, including UV-irradiation (with or without psoralen), DNase-treatment, restriction endonuclease treatment and ultrafiltration [34, 99, 180, 181]. Unfortunately, most of these methods either result in loss of sensitivity [34], or need further validation to assess tes reproducibility [15, 181, Tim Schuurman and Richard de Boer, Laboratory for Infectious Diseases (unpublished data)]. This emphasizes the need for certified DNA-free reagents. Roche recently introduced such high quality reagents under the name of M^{GRADE} reagents, available for MagNA Pure NA extraction (microbiology kit) and several LightCycler PCR kits. If more of these reagents do not become

available from commercial sources, efforts should be made to produce reproducible protocols for effective cleaning or inactivation of contaminated reagents. Until then, thorough screening of reagents before use is the only method to prevent reagent-derived false-positive results.

QC; Prevention of False-negative Results.

Optimal sensitivity is still an actual problem, as is also clearly shown by the recent proficiency studies (Table 1.4). Within these proficiency studies the overall percentage of false-negative results is between 5 to 45%. Furthermore, the percentage of participants reporting false-negatives is also high in most of the studies (median 67.3%, range 0-100%). However, as these panels are frequently designed to assess the performance near the limit of detection, the overall false-negativity rate may be overated. On the other hand, as these panels do include samples that contain lower level of clinical loads, it may be concluded that in routine testing low level infections are not detected with the highest confidence.

Several aspects can contribute to a false-negative result. Recovery of NA may be low by ineffective lysis of the target organism or by ineffective extraction of the NA from the clinical specimen. Extracted NA may be degraded due to contamination of buffers with DNases and RNases. NA amplification can be inhibited by substances co-purified from the clinical specimen. And finally, sequence variants may be missed by the primers and/or probes used.

To limit the possibility of false-negative results as much as possible, several measures can be taken. First of all, to ensure optimal lysis of the target organism and efficient extraction of NA, the sample preparation procedure should be thoroughly validated. Furthermore, positive controls should be included at levels which are reproducibly detected, but also just above the detection limit to challenge the detection system. Testing should preferably take place in the specimen matrix for which the assay is intended [46].

To prevent contamination of buffers with DNase and RNase, good laboratory practice should be sufficient in combination with quality control of all reagents in use.

TABLE 1.6. Inhibition controls: Strengths and limitations[a]

Type of inhibition control	Strengths	Limitations
Endogenous		
Housekeeping gene (albumin, β-globulin, 18S/28S rRNA genes, β-actin, GAPDH).	-Controls for NA extraction and inhibition. -Simple use, only requires design of primers (and probe) for detection.	-Does not control for lysis, requires additional positive control(s). -Amount of control gene in sample is usually relatively high and also variable among different specimens → low to medium level inhibition may remain undetected. -Not suitable for cell free clinical specimens.
Exogenous		
Spiking a duplicate reaction with purified NA from target organism.	-Very simple control, no need for extra efforts.	-Only controls for inhibition. -Should only be used with assays for which other inhibition controls are not (yet) available.
Synthetic nucleic acid sequence, competitive or non-competitive.	-Easy to obtain (commercial synthesis) -Controls for NA extraction and inhibition. -Incorporation of multiple primer sites makes it applicable in multiple NATs (competitive)/ universal (non-competitive). -Can be produced for both DNA and RNA targeted assays.	-Does not control for lysis. -Stability of naked RNA preparations. -Additional primers may result in interaction and loss of amplification efficiency (non-competitive). -Only applicable to assays with relatively small amplicons (maximum 300 bp), due to maximal synthesis length. -(Commercial) synthesis may be prone to contamination originating from other previously synthesized NA.
Plasmid construct, competitive or non-competitive.	-Controls for NA extraction and inhibition. -Use of identical primers as wild type target minimizes interaction between primers. -Incorporation of multiple primer sites makes it applicable in multiple NATs (competitive)/ universal (non-competitive).	-Not applicable for RNA assays. -Does not control for lysis, requires additional positive control(s). -Requirements for safe construction of construct (cloning) and production are usually beyond the facilities of a diagnostic (community) laboratory.

TABLE 1.6. Continued.

Type of inhibition control	Strengths	Limitations
Plasmid construct, competitive or non-competitive, *continued*.	-Simple use, addition to the lysis stage of NA extraction.	-Additional primers may result in interaction and loss of amplification efficiency (non-competitive).
Armored DNA/RNA, competitive or non-competitive.	-Controls for lysis of virus-like particles, NA extraction and inhibition. -Incorporation of multiple primer sites makes it applicable in multiple NATs (competitive)/ universal (non-competitive). -Simple use, addition to the lysis stage of NA extraction.	-Does not control for lysis of bacteria, fungi, and parasites. -Requirements for safe construction of construct (cloning) required to produce an armored DNA/RNA are usually beyond the facilities of a diagnostic (community) laboratory. -Additional primers may result in interaction and loss of amplification efficiency (non-competitive).
Genetically engineered (similar) organism (i.e. *M. smegmatis* in *M. tuberculosis* assays).	-Controls for lysis, NA extraction, and inhibition. -Incorporation of multiple primer sites makes it applicable in multiple NATs for similar pathogens. -Simple use, addition to the lysis stage of NA extraction.	-Usually non-universal, so multiple controls need to be engineered, or multiple primer sites need to be incorporated. -Requirements for safe construction of construct (cloning) and production are usually beyond the facilities of a diagnostic (community) laboratory. -Use of genetically engineered organisms may be restricted by law.
Universal non-human virus (phocine herpes virus, phocine distemper virus, equine arthritis virus) or bacterium (*Synechococcus* spp.).	-Controls for lysis, NA extraction, and inhibition. -Universal. -Simple use, addition to the lysis stage of NA extraction. -Easily produced by diagnostic (community) laboratories (culture).	-Does not control for lysis of bacteria, fungi, and parasites (virus). -Additional primers may result in interaction and loss of amplification efficiency.

[a] NATs, nucleic acid amplification tests.

The most critical issue responsible for false-negative results is amplification inhibition. The first stage of prevention is the use of an NA extraction procedure which is highly efficient in removing PCR inhibitory substances from the clinical specimen, such as “BOOM” chemistry based extraction procedures. A second stage is the use of amplification facilitators such as bovine serum albumin or T4 gene 32 protein [2, 80] or the use of more resistant thermostable DNA polymerases [1]. Unfortunately, even with all these measures in place, inhibition can still occur, and when it does, it is of the utmost importance to detect it.

Detection of amplification inhibition requires the use of an inhibition control, commonly referred to as an internal control (IC). However, inhibition controls are not necessarily internal controls. What kind of inhibition control should be used depends strongly on the user’s preference and also on the type of application. The ideal IC should monitor all steps of the process, including lysis, NA extraction, and inhibition. Furthermore, it should mimic the target as closely as possible, with respect to the outer wall of the organism (lysis), genetic material (DNA, RNA, single or double stranded), and amplicon (length and primers).

The simplest inhibition control involves spiking of a duplicate reaction with target NA, although this only monitors inhibition and not sample preparation. In the end, this kind of control should only be used in preliminary testing of a new assay before a(n) (better) IC is available.

The use of endogenous controls is also not ideal, especially since the amount of control shows variance and competition for reaction ingredients between specimens.

Synthetic NAs and plasmids may be constructed in such a manner that they mimic the target amplicon, resulting in similar amplification characteristics. However, these controls do not monitor for adequate lysis. For viruses and Gram-negative bacteria this may be circumvented by packaging the construct in a protein coat, as is the case with armored DNA/RNA. For more resistant outer cell structures (i.e. Gram-positive, acid-fast, or fungal) genetically modified organisms (GMO) may be used, although the construction of such organisms may be out of the reach of most clinical microbiology laboratories, and furthermore, the storage and use of these GMO may be restricted by law.

Although the ideal IC should mimic the target as much as possible, this may also overcomplicate the process when multiplex amplification is performed, as for each target a separate control should be included. Therefore, universal controls with separate amplification primers and a detection probe are increasingly being used. Examples of this are non-human viruses (i.e. phocine herpes virus, phocine distemper virus, and equine arthritis virus) and non-human bacteria (*Synechococcus* spp. [marine cyanobacteria]). Table 1.6 summarizes the strengths and limitations of widely used inhibition controls.

Finally, to minimize the effects of sequence variance, conserved target sequences should be chosen when designing an assay. When the assay is in use, the primer and probe sequences should periodically be screened in public NA databases, to pick up any newly described sequence variants and to subsequently compensate for this.

QC; Reliable Quantitative Results.

Quantification of microbial loads has become more and more important over the past decade. Examples of clinical applications where quantification has become invaluable are viral load monitoring for HIV- and HCV-infected patients during treatment, and viral load monitoring for cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in transplant patients in order to initiate pre-emptive therapy. Although viral load monitoring is already common ground within the field of clinical virology, the importance of microbial load quantification has only recently come into the picture in bacteriology [117]. However, although the clinical relevance of accurate quantification has been proven, again the performance of several quantitative assays remains poor [162]. This is caused by several factors including: i) different types of assays, ii) different units to express quantitative results, and iii) lack of standardized reference reagents for validation.

Different types of assays can result in variation in quantitative data due to for instance the sensitivity to inhibition in a NA amplification based assay, compared to a signal amplification assay that does not suffer from interference of PCR inhibitors.

Also differences in target choice of assays utilizing the same assay format can result in discrepancies. An example of this was recently reported when 2 internally controlled, competitive quantitative PCRs (QPCR) were

used to study the concentration and state of CMV DNA in serum, EDTA-anticoagulated (EDTA-) plasma, and EDTA-whole blood. Both QPCRs were performed in the same assay format, both targeting the same region of exon 4 of the major immediate-early gene of human CMV, and using identical probes for detection in both assays. The only difference between both assays was in the size of the generated amplicons, 578 bp vs 138 bp respectively [13]. Up to 16-fold higher CMV DNA levels were detected by the 138 bp QPCR compared to the 578 bp assay, whereas only 4-fold differences could be attributed to the expected variance of both assays (i.e. 25%, [13]). The authors concluded that the differences could be attributed to large quantities of small CMV DNA fragments (i.e. < 600 bp, as determined by the size distribution) which could not be detected by the 578 bp QPCR, but would be detected with the 138 bp QPCR. This shows that quantitative assays with a nearly identical performance when challenged with intact DNA can provide totally different results when they are challenged with clinical specimens. The currently widely used real-time PCR format may circumvent this, as almost all these assays generate small amplicons (< 150 bp).

A second problem with quantification is the way the results are reported. This is nicely illustrated when one takes a look at the list of HCV reporting units from the literature [110, 162]. This list contains copies/ml, genome equivalents/ml, megaequivalents/ml, superquant copies/ml and finally the international unit/ml (IU/ml). The IU was introduced to standardize quantitative reporting, and has been adapted for use in nearly all HCV NATs. Unfortunately, 1 IU still does not equal 1 IU (when it is converted to copies) in every HCV assay, with conversion factors ranging between 2.0 and 5.2. This illustrates that the introduction of IU has not yet overcome the problem of results reporting standardization [110, 162]. Furthermore, although the IU has become accepted in HCV testing, it is still not accepted for other important quantification targets such as HIV and HBV [110, 162].

Introduction of well-characterized international standards should be a first stage to standardized reporting and it would provide a strong basis for international, inter-institutional assay validation [110, 162]. However, the currently available standards have not been generally accepted yet [110]. Furthermore, these standards are usually based on a single genotype, which can still result in discrepant performances of quantitative assays for other

genotypes. This was illustrated by 2 recent reports where the COBAS TaqMan 48 HCV assay (CTM48) was compared to the COBAS Amplicor HCV Monitor (HCM) [29], or VERSANT HCV 3.0 bDNA assay [58] for both type 1 and non-type 1 HCV-positive specimens. HCV type 1 results showed good concordance in viral loads, but for non-type 1 viruses, the CTM48 assay underestimated the viral loads by on average 0.5 to 0.8 $\log_{10}$ IU. In addition, the lower limit of detection of the CTM48 assay, as stated by the manufacturer, should be reconsidered for non-type 1 viruses [58]. In the end, these reports have forced the manufacturer to restrict the use of this test to only HCV genotypes 1 and 6. On the other hand, other studies comparing CTM48 and HCM focusing more on type-1 specimens have also shown that for this HCV type there is also lack of agreement between both tests (i.e. 2-fold lower to + 0.45 $\log_{10}$ higher for CTM48). However, NA extraction was not performed according to the manufacturers recommendations in these studies (MagNA Pure LC/BioRobot 9604 instruments vs AMPLIPREP/High Pure System) [7, 53, 78]. Recently a first evaluation of the CTM48 v2.0 test, which should overcome the problems associated with version 1, showed that the assay performance for genotypes 2 to 5 was significantly improved [30].

Another problem with acceptable standards is that they have, to date, only become available for the “commercially interesting” targets.

QC; Assessment of Quality by Participation in External QC Programs.

When an ISO 15189 compliant assay is in place, its quality needs to be maintained. The laboratory does so by monitoring its internal quality assurance specifications. However, to be able to compare the results of one laboratory to others, participation in external quality assessment is necessary. One of the best examples on how to organize this external proficiency testing is provided by the Quality Control for Molecular Diagnostics (QCMD, www.qcmd.org) initiative (and its predecessor the European Union Quality Control Concerted Action [EU-QCCA]). This organization has developed proficiency testing panels for participants from up to 20 countries, involving 42 to 97 laboratories that reported up to 123 datasets [162]. These panels consist of 8 to 13 well-characterized, simulated clinical specimens for several important infectious agents including HIV (detection of RNA and DNA, and drug resistance genotyping), HCV (RNA detection and genotyping), HBV (DNA detection and genotyping), herpes

viruses, enteroviruses, respiratory viruses, parvovirus B19, *M. tuberculosis* complex, *C. trachomatis*, *N. gonorrhoeae*, *Toxoplasma gondii*, and methicillin-resistant *S. aureus* (MRSA). Although the list of established QCMD panels is already long, there will probably be an increasing demand for other pathogens in the near future, as well as alternative panels to assess basic skills, such as the quality of NA extraction. The establishment of future panels is highly dependent on the interests of the diagnostic community.

APPLICATIONS IN CLINICAL MICROBIOLOGY; Virology, Bacteriology, Parasitology, and Mycology.

Over the past 2 decades, diagnostic molecular microbiology testing has had a major impact on the clinical microbiology services. A review of the recent literature confirms that there is probably not a single human pathogen for which a conventional or real-time NA assay has not been described [164]. Without doubt, the most impressive advancements have been made in clinical virology. The main reasons for this are that the diagnosis of viral infections is hampered by low sensitivity of viral culture, the slow growth of many cultivable viruses, and the fact that for several viruses no clinically useful culture systems are available. In addition, the use of cell culture systems is costly, laborious, and requires skilled laboratory personnel. Other diagnostic tests, such as serology, also have their drawbacks. Serology tends to suffer from the immunological window-phase before antibodies appear during the early disease stages, and furthermore it may be impossible to discriminate between active and past infection for several viruses. Therefore, the introduction of NA based testing has resulted in a major improvement in diagnostic virology, as will be shown in some of the following examples.

Virology; Central Nervous System Disease.

Detection of viral agents in central nervous system (CNS) disease includes a broad spectrum of different viruses. The main agents include herpesviruses (HSV, varicella zoster virus (VZV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV)), enterovirus, JC virus (JCV), and West Nile virus (WNV) [46].

HSV CNS disease can range from mild meningitis to severe necrotizing encephalitis [155]. Detection of HSV in cerebrospinal fluid (CSF), which is difficult using conventional culture methods, by NATs has been recognized early after the introduction of these tests and has resulted in NATs becoming the gold standard in the laboratory diagnosis of CNS disease [149, 155]. Typing of HSV DNA can be used for epidemiological purposes but also for patient management, since HSV-1 has been linked to more severe CNS disease than HSV-2 [155]. Detection of HSV DNA in conjunction with other herpes viruses, as well as other microbial targets involved in CNS disease is also important, due to the overlapping clinical presentation between the different pathogens associated with CNS disease. For instance, enteroviral meningitis is usually self-limiting and needs to be discriminated from HSV CNS disease, which requires accurate treatment to prevent mortality and morbidity [149]. Discrimination of bacterial and viral causes of CNS disease is also important with respect to the different treatment strategies required and prevention of unnecessary antibiotic treatment.

Virology; Respiratory Disease.

Important viral causes of respiratory infections are influenza viruses, parainfluenza viruses, respiratory syncytial virus, rhinoviruses, human coronaviruses, human metapneumovirus, and adenoviruses. These agents are a significant cause of morbidity and mortality in several patient groups, which include neonates, the elderly, and immunocompromised patients such as solid organ or hematopoietic stem cell recipients [46, 157]. Laboratory diagnosis of viral respiratory infections is classically performed by viral culture (in combination with immunofluorescence), antigen detection and serology, all of which tend to suffer from the disadvantages mentioned earlier. Therefore, molecular diagnostic testing is rapidly being implemented to facilitate diagnosis of viral respiratory infections. The main advantage of the molecular detection of respiratory viruses is the increased sensitivity (97–100% for PCR, compared to 50–78% for conventional methods [157]), which in its turn results in increased overall detection rates for these pathogens (increase of 24–225% compared to conventional methods [46, 157]). Other grounds for the transition to molecular detection of respiratory viruses are cost effectiveness (compared to labor intensive and expensive culture), and especially speed [151, 157].

Another specific example of the advent of molecular diagnostic testing in respiratory disease, was shown during the severe acute respiratory syndrome (SARS) outbreak. SARS causes a highly contagious atypical pneumonia with a high case fatality rate, resulting from an infection by a novel coronavirus (SARS-CoV). Serology, although currently the only diagnostic test with 100% sensitivity, is not useful in the rapid diagnosis of SARS at presentation, as IgM and IgG antibodies only appear after 1 week, and 20–26 days, respectively [24]. Although SARS-CoV can be relatively easily recovered and identified by cell culture, this methodology is also not appropriate for high-throughput routine clinical microbiology laboratories due to the risk of laboratory-acquired infections. Safe handling of SARS-CoV in cell culture therefore requires a biosafety level 3 facility, which is usually not available in community laboratories [179]. With molecular diagnostic testing, the risk of a laboratory-acquired infection is reduced, since only the initial patient specimen contains infectious material, and the virus itself is not multiplied in the laboratory. In addition, RT-PCR was also shown to be more sensitive compared to viral culture for every specimen type tested [22], although the overall clinical sensitivity has been estimated to range from 50% for first-generation assays to 80% for second-generation assays [24, 123], emphasizing that the use of molecular methods to rule out SARS is currently not recommended [43, 154].

Virology; Blood Borne Viruses.

Detection of blood borne viruses has also substantially benefited from the introduction of molecular methods within the clinical microbiology laboratory. HCV RNA can be detected starting 1–3 weeks post exposure, whereas antibodies are usually detectable only after on average 70 days. Furthermore, the use of qualitative molecular methods is also necessary to confirm active infection, as detection of antibody can not distinguish between active and resolved infection, and to establish sustained response or relapse in end of treatment/end of follow up samples from treated patients [39].

Molecular methods have also substantially improved the screening of blood donations for HIV and HCV by reducing the potentially infectious window period from 22 and 66 days to approximately 9 and 7 days [144]. These highly sensitive assays are also extremely useful when needle stick injuries or other accidental exposures to potentially infected material occur.

Virology; Viral Load Monitoring.

For several viral pathogens, including CMV, EBV, HBV, HCV, and HIV, viral load monitoring has become an integral part of patient management. Quantitative measurements can provide an assessment of disease severity, prediction of progression to more severe disease, and for following treatment success. For instance in solid organ transplant and stem cell recipients, CMV DNA detection and viral load monitoring has been implemented in the routine post-transplantation follow up, and allows for pre-emptive therapy prior to the onset of clinical disease [73]. In a similar fashion, EBV DNA viral load monitoring has been demonstrated to be beneficial in the prediction of and intervention in post-transplant lymphoproliferative disease [109, 169]. Unfortunately, as most of the viral load monitoring assays for CMV and EBV are in-house assays based on different chemistries and specimen types, and international standards are lacking for both targets, the standardization of viral load measurements for both EBV and CMV has been difficult. This has resulted in individual guidelines for separate laboratories and makes extrapolation of published results to other settings difficult [46]. This is an important issue which should receive a high priority to resolve. However, with quantitative results of samples from individual patients, it still allows for effective monitoring of viral disease.

Of course, the success of viral load monitoring during treatment in HBV, HCV, and HIV infected patients is well documented [20, 32, 74, 91, 100, 115, 134, 182]. For these infections, viral load measurements do not only provide prognostic information, but also can serve as indicators for lack of compliance to treatment or the emergence of a drug-resistant mutant during therapy. When the emergence of resistance is suspected by the absence of a virological response, it can subsequently be confirmed by genotyping [65, 109, 125, 159, 151].

Virology; Potential Viral Agents of Bioterrorism.

With the increasing awareness of potential terrorist attacks with biological agents after the 9/11 aircraft attacks on the New York World Trade Centre and the intentional release of anthrax spores in the U.S. mail system in 2001 [70], diagnostic molecular microbiological testing for potential agents of bioterrorism has gained special attention. The most important viruses to be used as potential agents of bioterrorism are variola major virus (smallpox), filoviruses, and Lassa virus [133]. In the case of a suspected event

of bioterrorism, rapid diagnostic testing is needed to confirm or rule out the possible biological agents involved. Therefore, rapid molecular diagnostic testing for these biosafety level 4 categorized viruses has been initiated in specialized reference laboratories in many countries. Community laboratories, with diagnostic molecular microbiology facilities, can play a role in ruling out other possible infections that may mimic the clinical presentation of a possible agent of bioterrorism. An example of this is skin rashes produced by HSV or VZV when smallpox may also be suspected. However, additional safety measures to prevent exposure for laboratory staff should be taken [27, 46]. The specimens can be preventively autoclaved, under controlled conditions, before testing the sample for the presence of viral target nucleic acids by real-time PCR, as autoclaving was shown to have no detrimental affect on the amplification of target DNA from HSV, VZV, and vaccinia virus [47].

Bacteriology; Fastidious Bacteria.

Within the field of clinical bacteriology, diagnostic molecular microbiology has focused primarily on the detection of fastidious and non-cultivable bacteria. One of the main reasons for this has been that conventional molecular diagnostic tests are still technically demanding. In addition, the time to final results could not compete with that of the well established culture-based detection methods for most easily cultivable, clinically relevant bacteria [46]. Furthermore, several clinical specimen types (e.g. feces, sputum etc.) screened by routine culture procedures, proved to be unsuitable for molecular diagnostic testing due the presence of intrinsic factors inhibiting the PCR. Some of the fastidious pathogens for which molecular diagnostic testing has been implemented within the field of clinical bacteriology will be discussed briefly.

In the diagnosis of sexually transmitted diseases, molecular detection of *C. trachomatis* and *N. gonorrhoeae* has gained widespread acceptance, and these methods have almost fully replaced conventional culture-based detection for these pathogens. Several assays, using different chemistries, are currently commercially available, including (COBAS) AMPLICOR CT/NG (Roche [PCR]), ProbeTec ET (Becton Dickinson [SDA]), LCx *C. trachomatis* assay and the LCx *N. gonorrhoeae* assay (Abbott Laboratories [LCR]), and APTIMA Combo 2 (Gen-Probe [TMA]). These systems have improved

sensitivities (85–99% [57, 93, 156, 168, 181]), compared to culture based detection (62–68% for *N. gonorrhoeae*, and 38–56% for *C. trachomatis* [71, 86, 93, 153]), while maintaining high specificity (> 95%). *N. gonorrhoeae* testing by AMPLICOR, however, requires confirmation of all preliminary positive results due to cross-reaction with several other *Neisseria* and *Lactobacillus* species [93].

The detection of the so-called atypical bacteria causing community-acquired pneumonia, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae* and *Legionella* species (especially *L. pneumophila*), has also benefited greatly of the transition from conventional (i.e. culture, antigen detection or serology) to molecular detection methods, with respect to sensitivity and speed [46, 157]. Sensitivities for these molecular detection methods have been reported in the range from 80–100%, compared to 43–100% for conventional methods [10, 11, 90, 157].

Whooping cough (pertussis) is an acute respiratory disease occurring exclusively in humans. Widespread vaccination has provided a high level of protection in children, but unvaccinated populations, and cohorts in whom vaccination-induced immunity has waned, remain at risk. Pertussis is hard to diagnose by culture, although this is still regarded to be the “gold standard”, with reported sensitivities of only 7–36%, as compared to serology or PCR [157]. Molecular detection of the bacteria involved in pertussis disease, *B. pertussis*, and *B. parapertussis*, is usually targeted at the IS481 and IS1001 insertion sequences [131, 157]. Detection of both IS481 and IS1001 can also facilitate the discrimination between *B. pertussis* and *B. parapertussis*, of which the latter is usually associated with milder disease [131]. Recently however, several reports have indicated that *B. holmesii*, and *B. bronchisceptica* also harbour IS481 and IS1001 sequence homologues [105, 131, 157], thereby complicating molecular diagnosis of pertussis. Although these *Bordetella* species have also been identified in patients with pertussis-like symptoms, their clinical relevance needs further study [105, 131].

Other examples of bacteria that can only be detected by molecular means as culture is either extremely difficult or impossible for the routine microbiology laboratory, or represents a significant occupational risk to the laboratory personnel, are *T. whippelii* in Whipple’s disease, *B. henselae* in cat scratch disease and bacillary angiomatosis, and *Coxiella burnetii* in Q-

fever. Further details on the molecular methods for the diagnosis of fastidious bacteria can be reviewed elsewhere [52].

In mycobacteriology, culture still remains the cornerstone of diagnosis, although molecular detection has potential in reducing the diagnostic time from weeks to days [121]. Several assays are commercially available, and include the (COBAS) AMPLICOR *M. tuberculosis* assay (Roche), Amplified *M. tuberculosis* Direct (AMTD2) assay (Gen-Probe), and BD ProbeTec ET Direct TB System (Becton Dickinson). Furthermore, many home-brew assays are in use for the detection of *M. tuberculosis* complex [46, 113, 138] and *Mycobacterium* genus [17, 56, 82, 146]. The major issue in molecular detection of *M. tuberculosis* is the lower sensitivity in specimens negative for acid-fast-bacilli (AFB) by microscopy (33–100%), compared to AFB smear-positive specimens (82–100%) [121]. Therefore, molecular methods are currently mainly used to confirm the diagnosis of AFB-positive smear results, and to simplify and speed up the identification of positive cultures of mycobacteria.

Bacteriology; Rapid Bacterial Diagnosis.

Rapid detection of bacterial infections is another field where molecular diagnostics is gaining acceptance within the clinical microbiology laboratory. A recent development is the introduction of molecular screening for MRSA directly in clinical specimens. MRSA have a major impact on patient management in health care institutions and give rise to an increase in health care costs. Furthermore, MRSA have recently disseminated into the general community as well. Several different strategies can be applied to (directly) screen clinical specimens for the presence of MRSA, the main procedures being; i) detection of a *S. aureus* specific sequence (*nuc*, *femA* or Sa442) and the *mecA* gene [50, 54, 170] in single or multiplex reactions, and ii) single-locus amplification of the *mecA* harboring SCC*mec* right extremity in conjunction with *orfX* gene located directly downstream of the SCC*mec* integration site [9, 35, 42, 67, 68]. Although direct screening of clinical specimens has been described for both approaches with clinical performances equal or better than culture, both methods have problems with false-positive results. The multi-locus amplification assays can not discriminate between methicilin-susceptible *S. aureus* and methicilin-resistant coagulase negative staphylococci when both species are present in a clinical specimen. In theory, the single-locus amplification assays do not suffer from this problem.

However, false-positives have also been reported for these assays as well [9, 35, 42, 67, 68, 136]. Therefore, culture confirmation of MRSA PCR-positives is still necessary. Despite these specificity problems, rapid MRSA screening by PCR has great potential, due to the high NPV (> 98%) of these assays with different clinical specimens [9, 42, 50, 54, 68, 170]. A recent Dutch survey estimated that a 44% reduction could be obtained within a low-endemicity setting [170].

Rapid group B streptococcus screening in pregnant women and neonates by real-time PCR has shown increased sensitivity compared to conventional culture-based approaches [37, 106], A recent systematic review of several rapid assays for group B streptococcus testing confirmed that real-time PCR is a candidate for rapid near patient intrapartum group B streptococcus screenig, but the authors concluded that before this method can be implemented a robust technology assessment of their accuracy, acceptability, and cost-effectiveness is still required [66].

Other examples where rapid bacterial diagnostics may have its impact are rapid detection of *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* in meningitis [33, 46, 151], and the detection of bloodstream infections [118].

Bacteriology; Potential Bacterial Agents of Bioterrorism.

In the event of a potential act of bioterrorism, rapid diagnostic testing is mandatory. The most critical bacteriological agents for use in a bioterrorist attack are *Bacillus anthracis, Yersinia pestis,* and *Francisella tularensis* [145, 150]. Opposite to the potential viral agents of bioterrorism, detection of these potential bacterial biological weapons will be conducted primarily by community laboratories as these agents are occasionally encountered in sporadic natural infections as well. Conformational testing will then be conducted by specialized reference laboratories. Although community laboratories currently use conventional methods for detection of the bacterial bio-warfare agents, rapid molecular diagnostic testing may become the method of choice since it obviates the need for culture, which is associated with an increased risk for laboratory infections. Furthermore, the use of real-time PCR tests has been shown to increase the speed of generating final results, with equal or improved sensitivity compared to culture [45, 46]. In addition, clinical specimens might first be autoclaved before being processed for molecular testing, thereby further reducing the risk for laboratory staff. As

stated earlier on, autoclaving does not seem to negatively influence the test sensitivity of molecular diagnostic procedures for *B. anthracis*, as well as for vaccinia virus, HSV and VZV [47, 51].

Bacteriology; Antibiotic Resistance.

Genotypic resistance profiling is not dependant upon variable phenotypic resistance expression. As an example of this recent development the rapid screening of MRSA carriage has been discussed above. Other relevant targets with infection control implications are vancomycin-resistant *Enterococcus* spp. (VRE), and extended spectrum β-lactamases (ESBL) producing bacteria such as *E. coli* and *Klebsiella pneumoniae*. Conventional susceptibility testing for ESBLs remains a problem due to the notoriously low sensitivities of easy-to-perform susceptibility tests, resulting in that as much as 33% of ESBLs may remain undetected [88]. Therefore, molecular detection methods are currently on the rise for these organisms as they can provide more rapid results with improved sensitivity compared to conventional isolation and susceptibility testing [46, 151], and in addition, molecular methods may be better suited for the high-speed, high-throughput screening required in outbreak situations.

Bacteriology; Universal Bacterial Detection and Identification.

Amplification of universal bacterial genes currently has 2 major applications. First and foremost, this technique is used to identify clinical isolates which result in ambiguous biochemical profiles [98, 177], or for which phenotypic identification has a long turn-around time due to the fastidious growth of the organism, as is the case for mycobacteria [60, 61]. The most widely used genetic target is the 16S rRNA gene, whereas other genes, such as the 23S rRNA gene, the 16S-23S rRNA gene internal transcribed sequence (ITS), housekeeping genes such as the *rpoB* gene encoding the ß-subunit of RNA polymerase, the *groEL* gene encoding the heat-shock protein, the *gyrB* gene encoding the ß-subunit of DNA gyrase, and the homologous recombination-encoding *recA*, can also be used.

Several public databases and web-based identification tools are available for the identification of 16S rRNA gene sequences, such as GenBank, Ribosomal Differentiation of Microorganisms (RIDOM), Ribosomal database project II (RDP-II), and Bioinformatics Bacterial Identification Tool (BIBI) [28, 40, 61, 62, 98, 176]. In addition, commercial databases are also available,

such as the MicroSeq (Applied Biosystems) and SmartGene IDNS (SmartGene) databases [60, 147]. However, some of these databases have their limitations. For instance, public databases such as GenBank have no (internal) quality control measures in place for (newly) deposited sequences. Although quality controlled, RIDOM lacks several important bacterial genera such as staphylococci and streptococci, making it of limited relevance and applicability. The RDP-II release 8.1 uses an outdated, non-maintained database, whereas in release 9 several analysis tools have been discontinued. Commercial databases are (partially) quality controlled, but this has, of course, its associated costs. Furthermore, for MicroSeq it has been shown that the limited number of strains included (2,000), combined with the fact that they only represent type strains, limit the identification capability [147]. Currently, BIBI offers a combination of a similarity search and phylogeny assessment in an open, internet-based format, using quality controlled databases, making it very attractive for sequence-based bacterial identification. Furthermore, besides the 16S rRNA gene database, it also contains databases for other conserved genes (*rpoB*, *hsp65*, *sod*, *gyrB*). These additional universal gene targets are useful since 16S rRNA sequences may lack sufficient variance to discriminate between certain species within a genus (e.g. *E. coli* and *Shigella* species, and *Bacillus anthracis* and *Bacillus cereus*).

The second application of universal bacterial PCR, is the detection and identification of bacteria in clinical specimens originating from normally sterile anatomical sites. Although this approach seems very attractive, and promising results have been reported [41, 63, 84, 129], its widespread use has been hampered by its susceptibility to contamination. This contamination does not only originate from specimen to specimen carry over, or post-amplification handling of amplicons, but also from the environment and the chemicals, enzymes and consumables used. As discussed earlier, this issue still remains to be effectively solved.

Parasitology, and Mycology.

Both parasitology and mycology are disciplines where molecular diagnostic testing has remained of limited relevance. One of the most advanced applications in molecular diagnostic parasitology testing is the detection of *Plasmodium* species, malaria parasites, in blood specimens.

Molecular detection of *Plasmodium* species is more sensitive than conventional microscopic examination of peripheral blood smears [46, 101, 178]. Another advantage of molecular detection of *Plasmodium* species is the capability to identify the species other than *P. falciparum*, and even mixed infections, with greater confidence. This has significant clinical impact for therapy and patient management. In addition, molecular diagnostic tests provide accurate results without interpretative subjectivity, whereas the accuracy of microscopy is dependent upon the training and experience of those that prepare and read the slides. However, despite the increased sensitivity of molecular-based malaria detection, the technique is currently not available in clinical settings in most regions of the developing world where malaria is endemic, due to the high start-up costs for equipment, as well as the need for highly trained laboratory staff and reliable power supply [46, 101].

Other areas where parasitological molecular diagnostics are emerging are for the diagnosis of visceral and cutaneous leishmaniasis, toxoplasmosis, *Trichomonas vaginalis* infection, and intestinal parasites [46, 139, 174]. Molecular detection has been shown to have equal or enhanced sensitivity compared to conventional diagnostic procedures for all of these parasites.

In mycology, the most relevant applications in diagnostic molecular microbiology are aimed at 3 fungal pathogens, namely *Aspergillus* species, *Candida* species, and *Pneumocystis jiroveci*. Although molecular detection of *Aspergillus* species in patients at risk for invasive aspergillosis has been shown to be more sensitive than culture, these methods have been shown to have problems with specificity [46, 64, 128]. Therefore, it has been suggested that molecular detection accompanied by galactomannan antigen detection currently provides improved screening for invasive aspergillosis [46].

Detection of *Candida* species has focused primarily on the identification of the most common species associated with (nosocomial) bloodstream infections, which is associated with a high mortality rate [46]. In general, molecular detection shows promising sensitivity (80-100%), compared to culture [46, 118]. In addition, several reports of rapid identification of *Candida* species directly from clinical samples have shown promise in the clinical management of high-risk patients [64, 165]. The rapid identification of the fungal

pathogen directly from clinical samples and ensuring early therapy may help to reduce the length of hospital stay and high overall costs associated with management of candidemia [132].

P. jiroveci (formerly *P. carinii* f. sp. *hominis*) can cause serious pneumonia (*Pneumocystis carinii* pneumonia; PCP) in HIV-infected and other immunocompromised patients. Classical detection of *P. jeroveci* by microscopy of stained smears is hampered by the low sensitivity and analytical expertise required. Molecular methods have provided increased sensitivity in HIV-infected patients, especially for PCP associated with low numbers of *P. jiroveci*. However, in non-HIV-infected patients, including immunocompetent individuals and patients suffering from chronic lung diseases, the applicability of molecular methods is still debated, as *P. jiroveci* can also colonize the respiratory tract in the abscence of pneumonia [46, 148, 151, 160].

Further details on diagnostic molecular microbiology applications in clinical microbiology not described in the current overview can be found in reviews elsewhere [19, 26, 46, 94, 109, 118, 122, 130, 151, 156, 164, 173, 181].

DIAGNOSTIC MOLECULAR MICROBIOLOGY IN THE NETHERLANDS.

Reports of the use of molecular diagnostic methods in The Netherlands started appearing shortly after the original description of the new molecular technologies and have been applied in both basic research and diagnostic applications [25, 76, 77, 166]. Over the last 2 decades, the use of molecular diagnostics has increased greatly, as can also be deduced from Table 1.7.

Compared to 1998, the number of targets for which molecular diagnostic tests are available has expanded tremendously (Table 1.7 [114, 140]). Although the data from Table 1.7 represents two individual snapshots in time, it does nicely illustrate some of the trends in diagnostic molecular microbiology during that period. In addition, it should, be stated that the 2004 data were submitted to the database on a voluntary basis, and therefore may represent only part of the testing program in The Netherlands. Furthermore, developments are ongoing, and the 2004 situation will most likely be outdated. Most pathogens are tested for on equal basis by both community and academic laboratories; however, there are also pathogens with a more setting-restricted scope.

TABLE 1.7. Diagnostic molecular microbiology testing in The Netherlands, 1998 and 2004[a].

Microorganism[b]	1998	2004	
		Community laboratories (n = 17)	Academic hospitals (n = 10)
Bacteria			
All bacteria		2 (11.8%)	2 (20.0%)
Borrelia burgdorferi	2 community and 2 academic laboratories	2 (11.8%)	1 (10.0%)
Bartonella henselae	2 community laboratories	4 (23.5%)	0 (0.0%)
Bordetella parapertussis		6 (35.3%)	2 (20.0%)
B. pertussis	1 community laboratory	8 (47.1%)	2 (20.0%)
Chlamydophila pneumoniae		6 (35.3%)	7 (70.0%)
C. psitacci	3 community and 1 academic laboratories	2 (11.8%)	2 (20.0%)
Chlamydia trachomatis		16 (94.1%)	6 (60.0%)
Helicobacter pylori		3 (17.6%)	0 (0.0%)
Legionella pneumophila		4 (23.5%)	3 (30.0%)
Legionella species		3 (17.6%)	1 (10.0%)
Mycoplasma pneumoniae		7 (41.2%)	5 (50.0%)
Mycoplasma genus		4 (23.5%)	4 (40.0%)
Mycobacterium tuberculosis	Several community and 2 academic laboratories	14 (82.4%)	4 (40.0%)
Mycobacterium species		8 (47.1%)	5 (50.0%)
Neisseria gonorrhoeae	Several community laboratories	14 (82.4%)	2 (20.0%)
Staphylococcus aureus mecA		12 (70.6%)	5 (50.0%)
Tropheryma whippelii		0 (0.0%)	3 (30.0%)
Others[d]	1 community and 5 academic laboratories	7 (41.2%)	5 (50.0%)
Parasites and Fungi			
Toxoplasma gondii	4 academic laboratories	0 (0.0%)	3 (30.0%)
Trichomonas vaginalis		2 (11.8%)	1 (10.0%)
Others[e]	2 academic laboratories	3 (17.6%)	4 (40.0%)
Viruses			
Adenovirus		2 (11.8%)	2 (20.0%)
Cytomegalovirus		6 (35.3%)	6 (60.0%)
Coronavirus (excluding SARS)		4 (23.5%)	3 (30.0%)
Epstein-Barr virus		5 (29.4%)	4 (40.0%)
Enterovirus	2 community and 3 academic laboratories	8 (47.1%)	6 (60.0%)
Hepatitis B virus[c]	1 community and 4 academic laboratories	2 [1] (11.8%)	5 [1] (50.0%)
Hepatitis C virus[c]	2 community and 7 academic laboratories	6 [2] (35.3%)	5 [3] (50.0%)
Herpes simplex virus-1/2		10 (58.8%)	6 (60.0%)

TABLE 1.7. Continued.

Microorganism[b]	1998	2004 Community laboratories (n = 17)	2004 Academic hospitals (n = 10)
Viruses continued			
Human herpesvirus-6		2 (11.8%)	3 (30.0%)
Human herpesvirus-8		1 (5.9%)	2 (20.0%)
Human immunodeficiency virus (HIV)-1[c]	1 community and 5 academic laboratories	6 [1] (35.3%)	5 [4] (50.0%)
Human metapneumovirus		1 (5.9%)	2 (20.0%)
Influeza virus	1 academic laboratory	3 (17.6%)	4 (40.0%)
Parainfluenza virus		1 (5.9%)	2 (20.0%)
Rhinovirus	1 academic laboratory	1 (5.9%)	4 (40.0%)
Respiratory syncytial virus		1 (5.9%)	4 (40.0%)
Varicella-Zoster virus	3 community and 2 academic laboratories	9 (52.9%)	5 (50.0%)
Others[f]	2 community and 5 academic laboratories	3 (17.6%)	3 (30.0%)

[a] Data are based on reference [140] (1998) and reference [114] (2004); Academic laboratories also include research laboratories within academic settings.

[b] Microorgansims are included in the table when they are tested by at least independent 3 laboratories in the 2004 dataset.

[c] Numbers between brackets for 2004 indicate laboratories that also have genotyping methods available in addition to qualitative and/or quantitative detection.

[d] Others include: Actinobacillus actinomycetemcomitans (2004, 1 academic), Bacteroides vulgates (2004, 1 academic), Bartonella. quintana (1998, 1 community; 2004, 1 community), Bartonella species (2004, 1 community, 1 academic), Bifidobacterium species (2004, 1 academic), thermophylic Campylobacter species (2004, 1 community), Chlamydophila species (2004, 1 community, 1 academic), Clostridium difficile (2004, 2 academic), Corynebacterium diphteriae (1998, 1 academic), Coxiella burnetii (1998, 1 academic; 2004, 1 community), Ehrlichia species (1998, 1 academic; 2004, 1 community), Enterobacteriaceae (2004, 1 academic), Escherichia coli (2004, 1 academic), Fusobacterium species (2004, 1 academic), Group A streptococci (1998, 1 academic), Haemophilus ducreyi (2004, 1 community), Helicobacter pylori resistance/virulence (2004, 1 community), Helicobacter species (2004, 1 academic), Listeria monocytogenes (2004, 1 academic), Listeria species (2004, 1 academic), Micromonas micros (2004, 1 academic), Mycobacterium avium (2004, 1 community, 1 academic), Mycobacterium leprae (1998, 1 academic), Mycobacterium tuberculosis drug resistance (2004, 2 community), Mycoplasma fermentans (2004, 1 academic), Mycoplasma hominis (2004, 1 academic), Neisseria meningitidis (1998, 1 academic; 2004, 1 community, 1 academic), Porphyromonas gingivalis (2004, 1 academic), Prevotella intermedia (2004, 1 academic), Staphylococcus aureus vancomycin resistance (2004, 1 academic), Streptococcus pneumoniae (2004, 1 academic), Streptococcus species (2004, 1 academic), Tannerella forsythensis (2004, 1 academic), Treponema pallidum (2004, 1 community), Vero toxin-producing E. coli (1998, 2 academic), Yersinia enterocolitica (1998, 1 academic), and Yersinina pestis (1998, 1 academic).

[e] Others include: All fungi (2004, 1 community), Candida albicans (2004, 1 academic), Cryptosporidium parvum (2004, 1 academic), Dermatophytes (2004, 1 community), Echinococcus species (1998, 1 academic), Entamoeba dispar/hystolitica (2004, 1 community, 1 academic), Giardia lamblia (2004, 1 academic), Leishmania species (2004, 1 academic), Microsporidian species (2004, 1 academic), Pneumocystis jeroveci (2004, 1 academic), Plasmodium species (1998, 1 academic), and Saccharomyces cerevisiae (2004, 1 academic).

f Others include: BK virus (2004, 1 community, 1 academic), Calicivirus (2004, 1 community), Coxsackie A virus (1998, 1 community, 3 academic), Dengue virus (1998, 1 academic; 2004, 1 academic), Ebola virus (1998, 1 academic; 2004, 1 academic), Hepatitis A virus (1998, 1 academic; 2004, 1 community), Hepatitis D virus (2004, 1 community, 1 academic), Hepatitis G virus (2004, 1 community, 1 academic), Human herpesvirus-7 (2004, 1 academic), HIV-2 (2004, 1 academic), Measels virus (1998, 1 academic; 2004, 1 academic), Norovirus (1998, 1 community; 2004, 2 academic), Parvovirus B19 (2004, 1 academic), Poliovirus typing (1998, 2 academic; 2004, 1 academic), Rabies virus (1998, 1 academic), Rubella virus (1998, 1 academic; 2004, 1 community), SARS coronavirus (2004, 2 academic), and Yellow fever virus (1998, 1 academic).

For example, academic-based laboratories have broader testing capabilities for viral agents, compared to community laboratories. This may be linked to the fact that most community laboratories have historically performed limited virological testing, whereas in most academic hospitals full scale virology laboratories were and still are available. Furthermore, for several viral infections, such as HIV, HBV, and HCV, as well as some distinct patient groups (i.e. solid organ and/or stem cell recipients), academic hospitals are the primary treatment centers, and therefore need dedicated diagnostic and (therapeutic) monitoring capabilities. However, with the introduction of molecular methods to virology, some targets have rapidly become relevant for community laboratory testing as well, such as viral CNS disease, and community acquired pneumonia.

Bacteria on the other hand, seem more restricted to the community-based laboratories. Examples of these are *N. gonorrhoeae*, *M. tuberculosis*, *B. pertussis*, and *C. trachomatis*.

AIMS AND OUTLINE OF THESIS.

The preceding paragraphs have reviewed both the technical and clinical developments in diagnostic molecular microbiology over the past 25 years. From this it is clear that molecular diagnostic methods have become the diagnostic standard in clinical virology testing, whereas with regard to bacteriology, parasitology, and mycology, their use has remained limited to fastidious, non-cultivable organisms, or to those pathogens where conventional diagnostic tests lack sensitivity. However, with recent technical advance, such as automated sample preparation and real-time PCR, molecular detection of pathogens for which conventional detection methods have long since been considered the “gold standard”, may provide an effective alternative.

The scope of this thesis was to explore new possibilities of molecular diagnostics within the setting of a community-based clinical microbiology laboratory. Broad-range PCR has been used to serve as a molecular petridish in a similar fashion as non-selective culture. However, due to the technical difficulties associated with contamination, it has remained a research, rather than a diagnostic tool. In **Chapter 2** we describe the

development and clinical validation of a broad-range bacterial PCR with subsequent sequencing in the diagnosis of bacterial meningitis.

Automation of analytical and pre-analytical processes is one of the recent developments in diagnostic molecular microbiology. Since the number of specimens processed by the laboratory increases rapidly, automation has become mandatory. In **Chapters 3 and 4** (semi-) automated NA extraction methods are compared with manual "BOOM"-based extraction methods. **Chapter 3** is aimed at some of the primary specimen types processed by the clinical laboratory (i.e. whole blood, serum, plasma and cerebrospinal fluid), whereas **Chapter 4** focuses on one of the most challenging specimen types; feces.

The detection of bacterial gastrointestinal pathogens in community cases of diarrhea has long since been performed by selective culture. This approach however, is labor intensive, and therefore, final results are usually available only after at least 3 days. For gastrointestinal parasites, conventional methods include microscopy and antigen detection. Molecular methods have been shown to increase sensitivity, and furthermore, may also reduce the number of specimens needed for diagnosis.

In addition, as most specimens are negative, rapid and sensitive molecular screening of stool specimens should enable a substantial reduction in the time to final result. However, although this approach has already been suggested for *Salmonella* screening in swine herds [95], in humans it has to date remained largely a research and epidemiological tool, rather than a primary diagnostic tool. This is mainly linked to the technical difficulties associated with stool processing.

In **Chapters 5** to **9**, real-time PCR methods have been developed and validated for several gastrointestinal pathogens. **Chapter 5** assesses the feasibility of a molecular screening method for *Salmonella enterica* and *Campylobacter jejuni* within a routine clinical microbiology laboratory. In **Chapter 6** real-time PCR assays for the detection of Shiga toxin-producing *Escherichia coli* (STEC) are described and validated for use in a multicenter study to assess the clinical burden of non-O157 STEC. **Chapter 7** describes the multicenter study using the methods developed and validated in **Chapter 6** to assess the prevalence of STEC in The Netherlands. **Chapter 8** describes a comparison of real-time PCR, rapid immunoassay, and

microscopy for the detection of *Giardia lamblia*. In **Chapter 9**, the applicability of multiplex real-time PCR for *G. lamblia*, *Cryptosporidium hominis*/*parvum*, and *Entamoeba hystolitica* is assessed in general practice patients.

Finally, in **Chapter 10,** the preceding chapters are discussed, accompanied by a perspective on the implementation of the described methods in routine clinical practice of a community-based microbiology laboratory. In addition, expected future developments within the field of diagnostic molecular microbiology are presented.

REFERENCES.

1. **Abu Al-Soud, W., and P. Rådström.** 1998. Capacity of nine thermostable DNA polymerases to mediate amplification in the presence of PCR-inhibiting samples. Appl. Environ. Microbiol. **64:**3748-3753.
2. **Abu Al-Soud, W., and P. Rådström.** 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. J. Clin. Microbiol. **38**:4463-4470.
3. **Altwegg, M.** 1995. General problems associated with diagnostic applications of amplification methods J. Microbiol. Methods. **23:**21-30.
4. **Apfalter, P., O. Assadian, F. Blasi, J. Boman, C. A. Gaydos, M. Kundi, A. Makristathis, M. Nehr, M. L. Rotter, and A. M. Hirschl.** 2002. Reliability of nested PCR for detection of *Chlamydia pneumoniae* DNA in atheromas: results from a multicenter study applying standardized protocols. J. Clin. Microbiol. **40:**4428-4434.
5. **Apfalter, P., F. Blasi, J. Boman, C. A. Gaydos, M. Kundi, M. Maass, A. Makristathis, A. Meijer, R. Nadrchal, K. Persson, M. L. Rotter, C. Y. Tong, G. Stanek, and A. M. Hirschl.** 2001. Multicenter comparison trial of DNA extraction methods and PCR assays for detection of *Chlamydia pneumoniae* in endarterectomy specimens. J. Clin. Microbiol. **39:**519-524.[
6. **Apfalter, P., U. Reischl, and M. R. Hammerschlag.** 2005. In-house nucleic acid amplification assays in research: how much quality control is needed before one can rely upon the results? J. Clin. Microbiol. **43:**5835-5841.
7. **Barbeau, J. M., J. Goforth, A. M. Caliendo, and F. S. Nolte.** 2004. Performance characteristics of a quantitative TaqMan hepatitis C virus RNA analyte-specific reagent. J. Clin. Microbiol. **42:**3739-3746.
8. **Birch, L., C. A. English, M. Burns, and J. T. Keer.** 2004. Generic scheme for independent performance assessment in the molecular biology laboratory. Clin. Chem. **50:**1553-1559.
9. **Bishop, E. J., E. A. Grabsch, S. A. Ballard, B. Mayall, S. Xie, R. Martin, and M. L. Grayson.** 2006. Concurrent analysis of nose and groin swab specimens by the IDI-MRSA PCR assay is comparable to analysis by individual-specimen PCR and routine culture assays for detection of colonization by methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **44:**2904-2908.
10. **Black, C. M., P. I. Fieds, T. O. Messmer, and B. P. Berdal.** 1994. Detection of *Chlamydia pneumoniae* in clinical specimens by polymerase chain reaction using nested primers. Eur. J. Clin. Microbiol. Infect. Dis. **13:**752-756.
11. **Boman, J., A. Allard, K. Persson, M. Lundborg, P. Juto, and G. Wadell.** 1997. Rapid diagnosis of respiratory *Chlamydia pneumoniae* infection by nested touchdown polymerase chain reaction compared with culture and antigen detection by EIA. J. Infect. Dis. **175:**1523-1526.
12. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495-503.
13. **Boom, R., C. J. A. Sol, T. Schuurman, A. van Breda, J. F. L. Weel, M. Beld, I. J. M. ten Berge, P. M. E. Wertheim-van Dillen, and M. D. de Jong.** 2002. Human cytomegalovirus DNA in plasma and serum specimens of renal transplant recipients is highly fragmented. J. Clin. Microbiol. **40:**4105-4113.
14. **Boom, R., C. Sol, J. Weel, Y. Gerrits, M de Boer, and P. Wertheim-van Dillen.** 1999. A highly sensitive assay for the detection and quantitation of human cytomegalovirus DNA in serum and plasma by PCR and electrochemiluminescence. J. Clin. Microbiol. **37:**1489-1497.
15. **Borst, A., A. T. A. Box, and A. C. Fluit.** 2004. False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy. Eur. J. Clin. Microbiol. Infect. Dis. **23:**289-299.
16. **Brechot, C., M. Hadchouel, J. Scotto, F. Degos, P. Charnay, C. Trepo, and P Tiollais.** 1981. Detection of hepatitis B virus DNA in liver and serum: a direct appraisal of the chronic carrier state. Lancet **2:**765-768.

17. **Broccolo, F., P. Scarpellini, G. Locatelli, A. Zingale, A. M. Brambilla, P. Cichero, L. A. Sechi, A. Lazzarin, P. Lusso, and M. S. Malnati.** 2003. Rapid diagnosis of mycobacterial infections and quantitation of Mycobacterium tuberculosis load by two real-time calibrated PCR assays. J. Clin. Microbiol. **41:**4565-4572.
18. **Brooks Jackson, J., and H. H. Balfour JR.** 1988. Practical diagnostic testing for human immunodeficiency virus. Clin. Microbiol. Rev. **1:**124-138.
19. **Carrino, J. J., and H. H. Lee.** 1995. Nucleic acid amplification methods. J. Microbiol. Methods. **23:**3-20.
20. **Centers for Disease Control and Prevention.** 1998. Guidelines for the use of antiretroviral agents in HIV-infected adults and adolescents. Morbid. Mortal. Weekly Rep. **47:**43-82. (Erratum, 47:619.).
21. **Chamberlain, J. S., R. A. Gibbs, J. E. Rainer, P. N. Nguyen, and C. Thomas.** 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res. **23:**11141-11156.
22. **Chan, P. K., W. K. To, K. C. Ng, R. K. Lam, T. K. Ng, R. C. Chan, A. Wu, W. C. Yu, N. Lee, D. S. Hui, S. T. Lai, E. K. Hon, C. K. Li, J. J. Sung, and J. S. Tam.** 2004. Laboratory diagnosis of SARS. Emerg. Infect. Dis. **10:**825-831.
23. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244:**359-362.
24. **Christian, M. D., S. M. Poutanen, M. R. Loutfy, M. P. Muller, and D. E. Low.** 2004. Severe acute respiratory syndrome. Clin. Infect. Dis. **38:**1420-1427.
25. **Claas, E. C., A. J. van Milaan, M. J. Sprenger, M. Ruiten-Stuiver, G. I. Arron, P. H. Rothbarth, and N. Masurel.** 1993. Prospective application of reverse transcriptase polymerase chain reaction for diagnosing influenza infections in respiratory samples from a children's hospital. J. Clin. Microbiol. **31:**2218–2221.
26. **Cockerill, F. R., and J. R. Uhl.** 2002. Applications and challenges of real-time PCR for the clinical microbiology laboratory, p. 3-27. *In* U. Reischl, C. Wittwer, and F. R. Cockerill (ed.), Rapid cycle real-time PCR methods and applications. Springer-Verlag, Berlin, Germany.
27. **Cockerill, F. R., and T. F. Smith.** 2004. Response of the clinical microbiology laboratory to emerging (new) and re-emerging infectious diseases. J. Clin. Microbiol. **42:**2359-2365.
28. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxynomy. Nucleic Acids Res. **31:**442-443.
29. **Colson, P., A. Motte, and C. Tamalet.** 2006. Broad differences between COBAS ampliprep total nucleic acid isolation-COBAS TaqMan 48 hepatitis C virus (HCV) and COBAS HCV monitor v2.0 assays for quantification of serum HCV RNA of non-1 genotypes. J. Clin. Microbiol. 44:1602-1603.
30. **Colucci, G., J. Ferguson, C. Harkleroad, S. Lee, D. Romo, S. Soviero, J. Thompson, M. Velez, A. Wang, Y. Miyahara, S. Young, and C. Sarrazin.** 2007. Improved COBAS TaqMan hepatitis C virus test (version 2.0) for use with the high pure system: Enhanced genotype inclusivity and performance characteristics in a multisite study. J. Clin. Microbiol. **45:**3595-3600.
31. **Compton, J.** 1991. Nucleic acid sequence-based amplification. Nature **350:**91-92.
32. **Consensus panel.** 2002. National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C: 2002—June 10-12, 2002. Hepatology **36:**S3-S20.
33. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, A. J. Fox, and E. B. Kaczmarski.** 2001. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR.J. Clin. Microbiol. **39:**1553-1558.
34. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, E. B. Kaczmarski, and A. J. Fox.** 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J. Clin. Microbiol. **38:**1747-1752.

35. **Cuny, C., and W. Witte.** 2005. PCR for the identification of methicillin-resistant *Staphylococcus aureus* (MRSA) strains using a single primer pair specific for SCCmec elements and the neighbouring chromosome-borne orfX. Clin. Microbiol. Infect. **11:**834-837.
36. **Damen, M., H. T. Cuypers, H. L. Zaaijer, H. W. Reesink, W. P. Schaapsberg, W. H. Gerlich, H. G. Niesters, and P. N. Lelie.** 1996. International collaborative study on the second EUROHEP HCA-RNA reference panel. J. Virol. Methods **58:**175-185.
37. **Davies, H. D., M. A. Miller, S. Faro, D. Gregson, S. C. Kehl, and J. A. Jordan.** 2004. Multicenter study of a rapid molecular-based assay for the diagnosis of group B streptococcus colonization in pregnant women. Clin. Infect. Dis. **39:**1129-1135.
38. **Deiman, B., F. Jacobs, S. Vermeer, C. Schrover, and P. van der Wiel.** 2005. Development of NucliSENS EasyQ herpes simplex virus 1/2 reagents. Abstract of the 8th annual ESCV meeting, Geneva, Switzerland, 27 - 30 April 2005.
39. **Desombere, I., H. Van Vlierberghe, S. Couvent, F. Clinckspoor, and G. Leroux-Roels.** 2005. Comparison of Qualitative (COBAS AMPLICOR HCV 2.0 versus VERSANT HCV RNA) and Quantitative (COBAS AMPLICOR HCV Monitor 2.0 versus VERSANT HCV RNA 3.0) Assays for Hepatitis C Virus (HCV) RNA Detection and Quantification: Impact on Diagnosis and Treatment of HCV Infections. J. Clin. Microbiol. **43:**2590-2597.
40. **Devulder, G., G. Perrière, F. Baty, and J. P. Flandrois.** 2003. BIBI, a bioinformatics bacterial identification tool. J. Clin. Microbiol. **41:**1785-1787.
41. **Dreier, J., M. Stormer, and K. Kleesiek.** 2004. Two novel real-time reverse transcriptase PCR assays for rapid detection of bacterial contamination in platelet concentrates. J. Clin. Microbiol. **42:**4759-4764.
42. **Drews, S. J., B. M. Willey, N. Kreiswirth,, M. Wang, T. Ianes, J. Mitchell, M. Latchford, A. J. McGeer, and K. C. Katz.** 2006. Verification of the IDI-MRSA assay for detecting methicillin-resistant *Staphylococcus aureus* in diverse specimen types in a core clinical laboratory jetting.. J. Clin. Microbiol. **44:**3794-3796.
43. **Drosten, C., L. L. Chiu, M. Panning, H. N. Leong, W. Preiser, J. S. Tam, S. Gunther, S. Kramme, P. Emmerich, W. L. Ng, H. Schmitz, and E. S. Koay.**2004 . Evaluation of advanced reverse transcription-PCR assays and an alternative PCR target region for detection of severe acute respiratory syndrome-associated coronavirus. J. Clin. Microbiol. **42:**2043-2047.
44. **Drosten, C., H. W. Doerr, W. Lim, K. Stohr, M. Niedrig.** 2004. SARS molecular detection external quality assurance. Emerg. Infect. Dis. **10:**2200-2203.
45. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin. Microbiol. Rev. **15:**631-646.
46. **Espy, M. J., J. R. Uhl, L. M. Sloan, S. P. Buckwalter, M. F. Jones, E. A. Vetter, J. D. C. Yao, N. L. Wengenack, J. E. Rosenblatt, F. R. Cockerill III, and T. F. Smith.** 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol. Rev. **19:**165-256.
47. **Espy, M. J., J. R. Uhl, L. M. Sloan, J. E. Rosenblatt, F. R. Cockerill 3rd, and T. F. Smith.** 2002. Detection of vaccinia virus, herpes simplex virus, varicella-zoster virus, and *Bacillus anthracis* DNA by LightCycler polymerase chain reaction after autoclaving: implications for biosafety of bioterrorism agents. Mayo Clin. Proc. **77:**624-628.
48.. **Evans, G. E., D. R. Murdoch, T. P. Anderson, H. C. Potter, P. M. George, and S. T. Chambers.** 2003. Contamination of Qiagen DNA extraction Kits with *Legionella* DNA. J. Clin. Microbiol. **41:**3452-3453.
49. **Fahy, E., D. Y. Kwoh, and T. R. Gingeras.** 1991. Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR. PCR Methods Applications **1:**25-33.
50. **Fang, H., and G. Hedin.** 2003. Rapid screening and identification of methicillin-resistant *Staphylococcus aureus* from clinical samples by selective-broth and real-time PCR assay.J. Clin. Microbiol. **41:**2894-2899.
51. **Fasanella, A., S. Losito, R. Adone, F. Ciuchini, T. Trotta, S. A. Altamura, D. Chiocco, and G. Ippolito.** 2003. PCR assay to detect *Bacillus anthracis* spores in heat-treated specimens. J. Clin. Microbiol. **41:**896-899.

52. **Fenollar, F, and D. Raoult.** Molecular genetic methods for the diagnosis of fastidious microorganisms. 2004. APMIS. **112:**785–807.
53. **Forman, M. S., and A. Valsamakis.** 2004. Verification of an assay for quantification of hepatitis C virus RNA by use of an analyte-specific reagent and two different extraction methods. J. Clin. Microbiol. **42:**3581-3588.
54. **Francois, P., D. Pittet, M. Bento, B. Pepey, P. Vaudaux, D. Lew, and J. Schrenzel.** 2003. Rapid detection of methicillin-resistant *Staphylococcus aureus* directly from sterile or nonsterile clinical samples by a new molecular assay. J. Clin. Microbiol. **41:**254-260.
55. **Garcia-Erce, J. A., J. M. Grasa, V. M. Solano, J. Gimeno, A. López, M. J. Hernández, M. L. Marco, J. L. Arribas, and M. Giralt.** 2002. Bacterial contamination of blood components due to *Burkholderia cepacia* contamination from chlorhexidine bottles, Vox Sang. **83:**70–71.
56. **Garcia-Quintanilla, A., J. Gonzalez-Martin, G. Tudo, M. Espasa, and M. T. Jimenez de Anta.** 2002. Simultaneous identification of *Mycobacterium* genus and *Mycobacterium tuberculosis* complex in clinical samples by 5'-exonuclease fluorogenic PCR.J. Clin. Microbiol. **40:**4646-4651.
57. **Gaydos, C. A., T. C. Quinn, D. Willis, A. Weissfeld, E. W. Hook, D. H. Martin, D. V. Ferrero, and J. Schachter.** 2003. Performance of Aptima Combo 2 assay for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in female urine and endocervical swab specimens. J. Clin. Microbiol. **41:**304-309.
58. **Gelderblom, H. C., S. Menting, and M. G. Beld.** 2006. Clinical performance of the new Roche COBAS® TaqMan HCV test and high pure system for extraction, detection and quantitation of HCV RNA in plasma and serum. Antivir. Ther. **11**:95-103.
59. **Guatelli, J. C., K. M. Whitefield, D. Y. Kwoh, K. J. Barringer, D. D. Richman, and T. R. Gingeras.** 1990. Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled retroviral replication. Proc. Natl. Acad. Sci. UAS. **87:**1874-1887.
60. **Hall, L., K. A. Doerr, S. L. Wohlfiel, and G. D. Roberts.** 2003. Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J. Clin. Microbiol. **41:**1447-1453.
61. **Harmsen, D., S. Dostal, A. Roth, S. Niemann, J. Rothgänger, M. Sammeth, J. Albert, M. Frosch, and E. Richter.** 2003. RIDOM: Comprehensive and public sequence database for identification of *Mycobacterium* species. BMC Infect. Dis. **3:**26.
62. **Harmsen, D., J. Rothganger, M. Frosch, and J. Albert.** 2002. RIDOM: Ribosomal Differentiation of Medical Micro-organisms Database. Nucleic Acids Res. **30:**416-417.
63. **Harris, K. A., and J. C. Hartley.** 2003. Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. J. Med. Microbiol. **52:**685-691
64. **Hebart, H., J. Loffler, H. Reitze, A. Engel, U. Schumacher, T. Klingebiel, P. Bader, A. Bohme, H. Martin, D. Bunjes, W. V. Kern, L. Kanz, and H. Einsele.** 2000. Prospective screening by a panfungal polymerase chain reaction assay in patients at risk for fungal infections: implications for the management of febrile neutropenia. Br. J. Haematol. **111:**635-640.
65. **Hirsch, M. S., F. Brun-Vézinet, R. T. D'Aquila, S. M. Hammer, V. A. Johnson, D. R. Kuritzkes, C. Loveday, J. W. Mellors, B. Clotet, B. Conway, L. M. Demeter, S. Vella, D. M. Jacobsen, and D. D. Richman.** 2000. Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel. JAMA **283:**2417-2426.
66. **Honest, H., S. Sharma, and K. S. Khan.** 2006. Rapid Tests for group B streptococcus colonization in laboring women: A systematic review. Pediatrics **117:**1055-1066.
67. **Huletsky, A., R. Giroux, V. Rossbach, M. Gagnon, M. Vaillancourt, M. Bernier, F. Gagnon, K. Truchon, M. Bastien, F. J. Picard, A. van Belkum, M. Ouellette, P. H. Roy, and M. G. Bergergn.** 2004. New real-time PCR assay for rapid detection of

methicillin-resistant *Staphylococcus aureus* directly from specimens ccndaining a mixture of staphylococci. J. Clin. Microbiol. **02:**1875-1884.

68. **Huletsky, A., P. Lebel, F. J. Picard, M. Bernier, M. Gagnon, N. Boucher, and M. G. Bergeron.** 2005. Identification of methicilin-resistant *Staphylococcus atreus* capriage in less then 1 hour during a hospital surveillance program. Clin. Infect. Dis. **40:** 976-981.
69. **Ieven, M., and H. Goossens.** 1997. Relevance of nucleic acid amp,ification techniques for diagnosis of respiratory tract infections in the clinical laboratory. Clin* Microbiol. Rev. **10:**242-254.
70. **Jernigan J. A., D. S. Stephens, D. A. Ashford, C. Omenaca, M. S. Topiel, M. Galbraidh, M. Tapper, T. L. Fisk, S. Zaki, T. Popovic, R. F. Meyer, C. P. Quinn, S. A. Harper, S. K. Fridkin, J. J. Sejvar, C. W. Shepard, M. McConnell, J. Guarner, W.-J. Shieh, J. M. Malecki, J. L. Gerberding, J. M. Hughes, B. A. Perkins,* and members of tha Anthrax Bioterrorism Investigation Team**. 2001. Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States. Emerg. Infect. Dis. **7:**933-944.
71. **Jespersen, D. J., K. S. Flatten, M. F. Jones, and T. F. Smith.** 2005. Prospective comparison of cell cultures and nucleic acid amplification tests for laboratory diagnosis of Chlamydia trachomatis infections. J. Clin. Microbiol. **43:**5324-5326.
72. **Jungkind, D., S. Direnzo, K. G. Beavis, and N. S. Silverman.** 1996. Evaluation of automated COBAS AMPLICOR PCR system bor detection of several infectious agents and its impact on laboratory management. J. Clin. Microbiol. **34:**2778-2783.
73. **Kalpoe, J. S., A. C. Kroes, M. D. de Jong, J. Schinkel, C. S. de Brouwer, M. F. Beersma, and E. C. Claas.** 2004. Validation of clinical application of cypomegalovirus plasma DNA load measurement and definition of treatment criteria by analysis of correlation to antig%n detection. J. Clin. Microbiol. **42:**1498-1504.
74. **Katzenstein, T. D.** 2003. Molecular biological assessment methods and understanding the course of the HIV infection. APMIS. **114:**S1–S37.
75. **Kawasaki, E. S., and A.M. Wang.** 1988. Detection of Gene Expression. In: H.A. Erlich, Editor, *PCR Technology: Principles and Applications for DNA Amplification*, Stockton Press, New York, New York p89–97.
76. **Kluytmans, J. A., H. G. Niesters, J. W. Mouton, W. G. Quint, J. A. Ijpelaar, J. H. Van Rijsoort-Vos, L. Habbema, E. Stolz, M. F. Michel, and J. H. Wagenvoort.** 1991. Performance of a nonisotopic DNA probe for detection of Chlamydia trachomatis in urogenital specimens. J Clin Microbiol. **29:**2685–2689.
77. **Kolk, A. H., A. R. Schuitema, S. Kuijper, J. van Leeuwen, P. W. Hermans, J. D. van Embden, and R. A. Hartskeerl.** 1992. Detection of *Mycobacterium tuberculosis* in clinical samples by using polymerase chain reaction and a nonradioactive detection system. J. Clin. Microbiol. **30:**2567–2575.
78. **Konnick, E. Q., S. M. Williams, E. R. Ashwood, and D. R. Hillyard.** 2005. Evaluation of the COBAS Hepatitis C Virus (HCV) TaqMan analyte-specific reagent assay and comparison to the COBAS Amplicor HCV Monitor V2.0 and Versant HCV bDNA 3.0 assays. J. Clin. Microbiol. **43:**2133-2140.
79. **Kornegay, J. R., M. Roger, P. O. Davies, A. P. Shepard, N. A. Guerrero, B. Lloveras, D. Evans, and F. Coutlee.** 2003. International proficiency study of a consensus L1 PCR assay for the detection and typing of human papillomavirus DNA: evaluation of accuracy and intralaboratory and interlaboratory agreement. J. Clin. Microbiol. **41:**1080-1086.
80. **Kreader, C. A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. **62:**1102-1106.
81. **Kwoh, D. Y., G. R. Davis, K. M. Whitefield, H. L. Chappelle, L. J. DiMichele, and T. R. Gingeras.** 1989. Transcription-based amplification systems and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc. Natl. Acad. Sci. UAS. **86:**1173-1177.
82. **Lachnik, J., B. Ackermann, A. Bohrssen, S. Maass, C. Diephaus, A. Puncken, M. Stermann, and F. C. Bange.** 2002. Rapid-cycle PCR and fluorimetry for detection of mycobacteria. J. Clin. Microbiol. **40:**3364-3373.

83. **Lemmer, K., O. Donoso Mantke, H. Bae, J. Groen, C. Drosten, and M. Niedrig.** 2004. External quality control assessment in PCR diagnostics of dengue virus infections. J. Clin. Virol. **30:**291-296.
84. **Ley, B. E., C. J. Linton, D. M. Bennett, H. Jalal, A. B. Foot, and M. R. Millar.** 1998. Detection of bacteraemia in patients with fever and neutropenia using 16S rRNA gene amplification by polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. **17:**247-253.
85. **Lina, B., B. Pozzetto, L. Andreoletti, E. Beguier, T. Bourlet, E. Dussaix, L. Grangeot- Keros, B. Gratacap-Cavallier, C. Henquell, M. C. Legrand-Quillien, A. Novillo, P. Palmer, J. Petitjean, K. Sandres, P. Dubreuil, H. Fleury, F. Freymuth, I. Leparc- Goffart, D. Hober, J. Izopet, H. Kopecka, Y. Lazizi, H. Lafeuille, P. Lebon, and M. Aymard.** 1996. Multicenter evaluating of a commercially available PCR assay for diagnosing enterovirus infection in a panel of cerebrospinal fluid specimens. J. Clin. Microbiol. **34:**3002-3006.
86. **Lindan, C., M. Mathur, S. Kumta, H. Jerajani, A. Gogate, J. Schachter, and J. Moncada.** 2005. Utility of pooled urine specimens for detection of Chlamydia trachomatis and Neisseria gonorrhoeae in men attending public sexually transmitted infection clinics in Mumbai, India, by PCR. **43:**1674-1677.
87. **Livak, K. J., S. J. A. Flood, J. Marmaro, W. Giusti, and K. Deetz.** 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. **4:**357-362.
88. **Livermore, D. M., and M. Yuan.** 1996. Antibiotic resistance and production of extended-spectrum beta-lactamases amongst *Klebsiella* spp. from intensive care units in Europe. J. Antimicrob. Chemother. **38:**409-424.
89. **Loens, K., T. Beck, D. Ursi, S. Pattyn, H. Goossens, and M. Ieven.** 2006. Two quality control exercises involving nucleic acid amplification methods for detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* and carried out 2 years apart (in 2002 and 2004). J. Clin. Microbiol. **44:**899–908.
90. **Loens, K., M. Ieven, D. Ursi, T. Beck, M. Overdijk, P. Sillekens, and H. Goossens.** 2003. Detection of *Mycoplasma pneumoniae* by real-time nucleic acid sequence-based amplification. J. Clin. Microbiol. **41:**4448-4450.
91. **Lok, A. S., E. J. Heathcote, and J. H. Hoofnagle.** 2001. Management of hepatitis B: 2000—summary of a workshop. Gastroenterology **120:**1828-1853.
92. **Longo, M. C., M. S. Berninger, and J. L Hartley.** 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reaction. Gene **93:**125-128.
93. **Luijt, D. S., P. A. J. Bos, A. A. van Zwet, P. C. van Voorst Vader, and J. Schirm.** 2005. Comparison of COBAS AMPLICOR *Neisseria gonorrhoeae* PCR, including confirmation with *N. gonorrhoeae*-specific 16S rRNA PCR, with traditional culture. J. Clin. Microbiol. **43:**1445-1447.
94. **Mackay, I. M.** 2004. Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. **10:**190-212.
95. **Malorny, B., and J. Hoorfar.** 2005. Toward standardization of diagnostic PCR testing of fecal samples: Lessons from the detection of Salmonellae in pigs. J. Clin. Microbiol. **43:**3033-3037.
96. **Malorny, B., J. Hoorfar, C. Bunge, and R. Helmuth.** 2003. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. Appl. Environ. Microbiol. **69:**290-296.
97. **Mancini, C., G. Pisanib, A. Azzic, M. L. Zerbinid, G. Gentilib, G. M. Bisso and MS participants.** 2004. Inter-laboratory comparison of qualitative and quantitative detection of hepatitis C (HCV) virus RNA in diagnostic virology: a multicentre study (MS) in Italy. J. Clin. Virol. **30:**313-319.
98. **Mignard, S., and J. P. Flandrois.** 2006. 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. J. Microbiol. Methods **67:**574-581.
99. **Mohammadi, T., H. W. Reesink, C. M. J. E. Vandenbroucke-Grauls, and P. H. M. Savelkoul.** 2005. Removal of contaminating DNA from commercial nucleic acid extraction kit reagents J. Microbiol. Methods. **61:**285-288.

100. **Mommeja-Marin, H., E. Mondou, M. R. Blum, and F. Rousseau.** 2003. Serum HBV DNA as a marker of efficacy during therapy for chronic HBV infection: analysis and review of the literature. Hepatology **37:**1309-1319.
101. **Moody, A.** 2002. Rapid diagnostic tests for malaria parasites. Clin. Microbiol. Rev. **15:**66-78.
102. **Moody, M. D., L. Burg, R. DiFransesco, D. Lovern, W. Stanick, J. Lin-Goerke, K. Mahdavi, Y. WU, and M. P. Farrell.** 1994. Evolution of host cell RNA into efficient template RNA by Q*β* replicase: The origin of RNA in untemplated reaction. Biochemistry **33:**13836-13847.
103. **Muir, P., A. Ras, P. E. Klapper, G. M. Cleator, K. Korn, C. Aepinus, A. Fomsgaard, P. Palmer, A. Samuelsson, A. Tenorio, B. Weissbrich, and A. M. van Loon.** 1999. Multicenter quality assessment of PCR methods for detection of enteroviruses. J. Clin. Microbiol. **37:**1409-1414.
104. **Mullis, K.B., and F.A. Faloona.** 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed reaction. Methods Enzymol. **155:**335-350.
105. **Muyldermans G., O. Soetens, M. Antoine, S. Bruisten, B. Vincart, K. Doucet, F. Populaire, N. K. Fry, P. Olcen, J. M. Scheftel, J. M. Senterre, A. van der Zee, M. Riffelmann, D. Pierard, and S. Lauwers.** 2005. External quality assessment for molecular detection of *Bordetella pertussis* in European laboratories. J. Clin. Microbiol. **43:**30-35.
106. **Natarajan, G., Y. R. Johnson, F. Zhang, K. Mei Chen, and M. J. Worsham.** 2006. Real-time polymerase chain reaction for the rapid detection of group B streptococcal colonization in neonates. Pediatrics. **118:**14-22.
107. **Niedrig, M., H. Meyer, M. Panning, and C. Drosten.** 2006. Follow up on diagnostic proficiency of laboratories equipped to perform orthopovirus detection and quantification by PCR: the second international external quality assurance study. J. Clin. Microbiol. **44:**1283-1287.
108. **Niedrig, M., H. Schmitz, S. Becker, S. Günther, J. Meulen, H. Meyer, H. Ellerbrok, A. Nitsche, H. R. Gelderblom, and C. Drosten.** 2004. First international quality study on the rapid detection of viral agents of bioterrorism. J. Clin. Microbiol. **42:**1753-1755.
109. **Niesters, H. G. M.** 2002. Clinical virology in real time. J. Clin. Virol. **25:**S3-S12.
110. **Niesters, H. G. M., and E. Puchhammer-Stöckl.** 2004. Standardisation and controls, why can't we overcome the hurdles? J. Clin Virol. **31:**81-83.
111. **Noordhoek, G. T., J. D. A. van Emden, and A. H. Kolk.** 1996. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: an international collaborative quality control study among 30 laboratories. J. Clin. Microbiol. **34:**2522-2525.
112. **Noordhoek, G. T., A. H. J. Kolk, G. Bjune, D. Catty, J. W. Dale, P. E. M. Fine, P. Godfrey-Faussett, S.-N. Cho, T. Shinnick, S. B. Svenson, S. Wilson, and J. D. A. Van Embden.** 1994. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. J. Clin. Microbiol. **32:**277-284.
113. **Noordhoek, G. T., S. Mulder, P. Wallace, and A. M. van Loon.** 2004. Multicentre quality control study for detection of *Mycobacterium tuberculosis* in clinical samples by nucleic acid amplification methods. Clin. Microbiol. Infect. **10:**295-301.
114. **Noordhoek, G. T., and H. Zandleven on behalf of the Dutch Working Group on Molecular Diagnostics of Infectious Diseases.** 2004. Microsoft access database on molecular diagnostics of infectious diseases, version 14-12-2004. http://www.nvmm.nl/open/vereniging/mmo_ver_main_moleculair.htm.
115. **Pawlotsky, J. M., and J. G. McHutchison.** 2004. Hepatitis C. Development of new drugs and clinical trials: promises and pitfalls. Hepatology **39:**554-567.
116. **Pellet, P. E., T. J. Spira, O. Bagasra, C. Boshoff, L. Corey, L. de Lellis, M.-L. Huang, J.-C. Lin, S. Matthews, P. Monini, P. Rimessi, C. Sosa, C. Wood, and J. A. Stewart.** 1999. Multicenter comparison of PCR assays for detection of human herpesvirus 8 DNA in semen. J. Clin. Microbiol. **37:**1298-1301.
117. **Peters, R. P. H.** 2007. Innovations in the diagnosis of bloodstream infections: A bench-bedside interaction. PhD thesis Free University of Amsterdam.

118. **Peters, R. P. H., M. A. van Agtmael, S. A. Danner, P. H. M. Savelkoul, and C. M. J. E. Vandenbroucke-Grauls.** 2004. New developments in the diagnosis of bloodstream infections. Lancet Infect. Dis. **4:**751-760.
119. **Peters, R. P. H., T. Mohammadi, C. M. J. E. Vandenbroucke-Grauls, S. A. Danner, M. A. van Agtmael, and P. H. M. Savelkoul.** 2004. Detection of bacterial DNA in blood samples from febrile patients: underestimated infection or emerging contamination? FEMS Immunol. Med. Microbiol. **42:**249–253.
120. **Peters, R. P., P. H. Savelkoul, A. M. Simoons-Smit, S. A. Danner, C. M. Vandenbroucke-Grauls, and M. A. van Agtmael.** 2006. Faster identification of pathogens in positive blood cultures by fluorescence in situ hybridization in routine practice. J. Clin. Microbiol. **44:**119-123.
121. **Piersimoni, C., and C. Scarparo.** 2003. Relevance of commercial amplification methods for direct detection of *Mycobacterium tuberculosis* complex in clinical samples. J. Clin. Microbiol. **41:**5355-5365.
122. **Pitt, T. L., and N. A. Saunders.** 2000. Molecular bacteriology: a diagnostic tool for the millennium. J. Clin. Pathol. **53:**71-75.
123. **Poon, L. L., K. H. Chan, O. K. Wong, W. C. Yam, K. Y. Yuen, Y. Guan, Y. M. Lo, and J. S. Peiris.** 2003. Early diagnosis of SARS coronavirus infection by real time RT-PCR. J. Clin. Virol. **28:**233-238.
124. **Porter, J. R.** 1976. Antony van Leeuwenhoek: Tercentenary of his discovery of bacteria. Bact. Rev. **40:**260-269.
125. **Puchhammer-Stockl, E., C. W. Mandl, J. Kletzmayr, H. Holzmann, A. Hofmann, S. W. Aberle, F. X. Heinz, B. Watschinger, and H. Hofmann.** 2000. Monitoring the virus load can predict the emergence of drug-resistant hepatitis B virus strains in renal transplantation patients during lamivudine therapy. J. Infect. Dis. **181:**2063-2066.
126. **Quint, W. G. V., R. A. Heijting, J. Schirm, W. H. Gerlich, and H. G. M. Niesters.** 1995. Reliability of methods for hepatitis B virus DNA detection. J. Clin. Microbiol. **33:**225-228.
127. **Rantakokko-Jalava, K., and J. Jalava.** 2002. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. J. Clin. Microbiol. **40:**4211-4217.
128. **Rantakokko-Jalava, K., S. Laaksonen, J. Issakainen, J. Vauras, J. Nikoskelainen, M. K. Viljanen, and J. Salonen.** 2003. Semiquantitative detection by real-time PCR of *Aspergillus fumigatus* in bronchoalveolar lavage fluids and tissue biopsy specimens from patients with invasive aspergillosis.J. Clin. Microbiol. **41:**4304-4311.
129. **Rantakokko-Jalava, K., S. Nikkari, J. Jalava, E. Eerola, M. Skurnik, O. Meurman, O. Ruuskanen, A. Alanen, E. Kotilainen, P. Toivanen, and P. Kotilainen.** 2000. Direct amplification of rRNA genes in the diagnosis of bacterial infections. J. Clin. Microbiol. **38:**32-39.
130. **Read, S. J., D. Burnett, and C. G. Fink.** 2000. Molecular techniques for clinical diagnostic virology. J. Clin. Path. **53:**502-506.
131. **Register, K. B., and G. N. Sanden.** 2006. Prevalence and Sequence Variants of IS*481* in *Bordetella bronchiseptica*: Implications for IS*481*-Based Detection of *Bordetella pertussis*. . J. Clin. Microbiol. **44:**4577-4583.
132. **Rentz, A. M., M. T. Halpern, and R. Bowden.** 1998. The impact of candidemia on length of hospital stay, outcome, and overall cost of illness. Clin. Infect. Dis. **27:**781-788.
133. **Richardson, H., and F. Smaill.** 1998. Recent advances: medical microbiology. Brit. Med. J. **317:**1060-1062.
134. **Richter, S. S.** 2002. Laboratory assays for diagnosis and management of hepatitis C virus infection. J. Clin. Microbiol. **40:**4407-4412.
135. **Rotbart, H. A.** 1991. Nucleic acid detection systems for enteroviruses. Clin. Microbiol. Rev. **4:**156-168.
136. **Rupp, J., I. Fenner, W. Solbach, and J. Gieffers.** 2006. Be aware of the possibility of false-positive results in single-locus PCR assays for methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **44:**2317.

137. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:**1350-1354.
138. **Savelkoul, P. H. M., A. Catsburg, S. Mulder, L. Oostendorp, J. Schirm, H. Wilke, A. G. M. van der Zanden, and G. T. Noordhoek.** 2006. Detection of *Mycobacterium tuberculosis* complex with Real Time PCR: Comparison of different primer-probe sets based on the IS6110 element. **66:**177-180.
139. **Schee, C. van der, A. van Belkum, L. Zwijgers, E. van der Brugge, E. L. O'neill, A. Luijendijk, T. van Rijsoort-Vos, W. I. van der Meijden, H. Verbrugh, and H. J. F. Sluiters.** 1999. Improved diagnosis of Trichomonas vaginalis infection by PCR using vaginal swabs and urine specimens compared to diagnosis by wet mount microscopy, culture, and fluorescent staining. J. Clin. Microbiol. **37:**4127-4130.
140. **Schellekens, J. F. P., L. M. Kortbeek, J. W. Dorigo-Zetsma, and L. M. Wijgergangs.** 1998. An inventory of laboratory diagnostics for public health relevant microorganisms. National Institute for Public Health and the Environment (RIVM) rapport number 216683001.
141. **Schirm, J., A. M. van Loon, E. Valentine-Thon, P. E. Klapper, J. Reid, and G. M. Cleator.** 2002. External quality assessment program for qualitative and quantitative detection of hepatitis C virus RNA in diagnostic virology. J. Clin. Microbiol. **40:**2973-2980.
142. **Schloss, L., A. M. van Loon, P. Cinque, G. Cleator, J. M. Echevarria, K. I. Falk, P. Klapper, J. Schirm, B. F. Vestergaard, H. Niesters, T. Popow-Kraupp, W. Quint, and A. Linde.** 2003. An international external quality assessment of nucleic acid amplification of herpes simplex virus. J Clin Virol. **28:**175-85.
143. **Schweitzer, B., S. Kingsmore.** 2001. Combining nucleic acid amplification and detection. Curr. Opin. Biotechnol. **12:**21–27.
144. **Seed, C. R., A. Cheng, S. L. Ismay, W. V. Bolton, P. Kiely, T. J. Cobain, A. J. Keller; Virology subcommittee of the national donor and product safety committee, Australian Red Cross blood service.** 2002. Assessing the accuracy of three viral risk models in predicting the outcome of implementing HIV and HCV NAT donor screening in Australia and the implications for future HBV NAT. Transfusion **42:**1365–1372.
145. **Sewell, D. L.** 2003. Laboratory safety practices associated with potential agents of biocrime or bioterrorism. J. Clin. Microbiol. **41:**2801-2809.
146. **Shrestha, N. K., M. J. Tuohy, G. S. Hall, U. Reischl, S. M. Gordon, and G. W. Procop.** 2003. Detection and differentiation of *Mycobacterium tuberculosis* and nontuberculous mycobacterial isolates by real-time PCR. J. Clin. Microbiol. **41:**5121-5126.
147. **Simmon, K. E., A. C. Croft, and C. A. Petti.** 2006. Application of SmartGene IDNS software to partial 16S rRNA gene sequences for a diverse group of bacteria in a clinical laboratory. J. Clin. Microbiol. **44:**4400-4406.
148. **Sing, A., K. Trebesius, A. Roggenkamp, H. Russmann, K. Tybus, F. Pfaff, J. R. Bogner, C. Emminger, and J. Heesemann.** 2000. Evaluation of diagnostic value and epidemiological implications of PCR for *Pneumocystis carinii* in different immunosuppressed and immunocompetent patient groups. J. Clin. Microbiol. **38:**1461-1467.
149. **Smalling, T. W., S. E. Sefers, H. Li, and Y.-W. Tang.** 2002. Molecular approaches to detecting herpes simplex virus and enteroviruses in the central nervous system. J. Clin. Microbiol. **40:**2317-2322.
150. **Snyder, J. W.** 2003. Role of the hospital-based microbiology laboratory in preparation for and response to a bioterrorism event. J. Clin. Microbiol. **41:**1-4.
151. **Speers, J. D.** 2006. Clinical applications of molecular biology for infectious diseases. Clin. Biochem. Rev. **27:**39-51.
152. **Stanier, R. Y., M. Doudoroff, and E. A. Adelberg.** 1963. The microbial world. 2nd edition. Prentice Hall, Inc., Englewood Cliffs, NJ.
153. **Swain, G. R., R. A. McDonald, J. R. Pfister, M. S. Gradus, G. V. Sedmak, and A. Singh.** 2004. Decision analysis: point-of-care Chlamydia testing vs. laboratory-based methods. Clin. Med. Res. **2:**29-35.

154. **Tang, P., M. Louie, S. E. Richardson, M. Smieja, A. E. Simor, F. Jamieson, M. Fearon, S. M. Poutanen, T. Mazzulli, R. Tellier, J. Mahony, M. Loeb, A. Petrich, M. Chernesky, A. McGeer, D. E. Low, E. Phillips, S. Jones, N. Bastien, Y. Li, D. Dick, A. Grolla, L. Fernando, T. F. Booth, B. Henry, A. R. Rachlis, L. M. Matukas, D. B. Rose, R. Lovinsky, S. Walmsley, W. L. Gold, and S. Krajden.** 2004. Interpretation of diagnostic laboratory tests for severe acute respiratory syndrome: the Toronto experience. Can. Med. Assoc. J. **170:**47-54.
155. **Tang, Y.-W., P. S. Mitchell, M. J. Espy, T. F. Smith, and D. H. Persing.** 1999. Molecular diagnosis of herpes simplex virus infections in the central nervous system. J. Clin. Microbiol. **37:**2127-2136.
156. **Tang, Y.-W., G. W. Procop, and D. H. Persing.** 1997. Molecular diagnostics of infectious diseases. Clin. Chem. **43:**2021-2038.
157. **Templeton, K. E..** 2005. Improved diagnosis of respiratory pathogens by real-time PCR. PhD. Thesis. University of Leiden.
158. **Templeton, K. E., C. B. Forde, A. M. Loon, E. C. J. Claas, H. G. M. Niesters, P. Wallace, and W. F. Carman.** 2006. A multi-centre pilot proficiency programme to assess the quality of molecular detection of respiratory viruses. J. Clin. Virol. **35:**51-58.
159. **The EuroGuidelines Group for HIV** Resistance**.** 2001. Clinical and laboratory guidelines for the use of HIV-1 drug resistance testing as part of treatment management: recommendations for the European setting. AIDS **15:**309-320.
160. **Turner, D., Y. Schwarz, and, I. Yust.** 2003. Induced sputum for diagnosing *Pneumocystis carinii* pneumonia in HIV patients: new data, new issues. Eur. Respir. J. **21:**204-208.
161. **Ugahary, L., W. van de Sande, J. C. van Meurs, and A. van Belkum.** 2004. An unexpected Experimental pitfall in the molecular diagnosis of bacterial endophthalmitis. J. Clin. Microbiol. **42:**5403-5405.
162. **Valentine-Thon, E.** 2002. Quality control in nucleic acid testing—where do we stand? J. Clin. Virol. **25:**S13-S21.
163. **Valentine-Thon, E., A. M. van Loon, J. Schirm, J. Reid, P. E. Klapper, and G. M. Cleator.** 2001. European proficiency program for molecular detection and quantitation of hepatitis B virus DNA. J. Clin. Microbiol. **39:**4407-4412.
164. **van Belkum, A.** 2003. Molecular diagnostics in medical microbiology: yesterday, today and tomorrow. Curr. Opin. Pharmacol. **3:**497-501.
165. **van Burik, J. A., D. Myerson, R. W. Schreckhise, and R. A. Bowden.** 1998. Panfungal PCR assay for detection of fungal infection in human blood specimens. J. Clin. Microbiol. **36:**1169-1175.
166. **van den Brule, A. J., E. C. Claas, M. du Maine, W. J. Melchers, T. Helmerhorst, W. G. Quint, J. Lindeman, C. J. Meijer, and J. M. Walboomers.** 1989. Use of anticontamination primers in the polymerase chain reaction for the detection of human papilloma virus genotypes in cervical scrapes and biopsies. J. Med. Virol. **29:**20-27.
167. **van der Zee, A., M. Peeters, C. de Jong, H. Verbakel, J. W. Crielaard, E. C. Claas, and K. E. Templeton.** 2002.Qiagen DNA extraction kits for sample preparation for *Legionella* PCR are not suitable for diagnostic purposes. J. Clin. Microbiol. **40:**1126.
168. **van Doornum, G. J., L. M. Schouls, A. Pijl, I. Cairo, M. Buimer, and S. Bruisten.** 2001. Comparison between the LCx Probe system and the COBAS AMPLICOR system for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections in patients attending a clinic for treatment of sexually transmitted diseases in Amsterdam, The Netherlands. J. Clin. Microbiol. **39:**829-835.
169. **van Esser, J. W. J.** 2003. Epstein-Barr virus lyphoproliferative disease following alogenic hematopoietic stem cell transplantation: prediction and early intervention. PhD. Thesis. Erasmus University, Rotterdam.
170. **van Hannen, E. J., P. Foppen, and B. M. de Jongh.** 2006. Molecular MRSA screening: finding negatives a.s.a.p. Clin. Microbiol. Infect. **12:**S4 P1339.
171. **van Vliet, K. E., P. Muir,.J. M. Echevarria, P. E. Klapper, G. M. Cleator, and A. M. van Loon.** 2001. Multicenter proficiency testing of nucleic acid amplification methods for the detection of enteroviruses. J. Clin. Microbiol. **39:**3390-3392.

172. **Verkooyen, R. P., G. T. Noordhoek, P. E. Klapper, J. Reid, J. Schirm, G. M. Cleator, M. Ieven, and G. Hoddevik.** 2003. Reliabilty of nucleic acid amplification methods for detection of *Chlamydia trachomatis* in urine: results of the first international collaborative quality control study among 96 laboratories. J. Clin. Microbiol. **41:**3013-3016.
173. **Versalovic, J., and J. R. Lupski.** 2002. Molecular detection and genotyping of pathogens: more accurate and rapid answers. Trends in Mircobiol. **10:**S15-S21.
174. **Verweij, J. J.** 2004. Molecular tools in the diagnosis of intestinal parasitic infections. PhD. Thesis. University of Leiden.
175. **Watson, J. D, and F. H. Crick.** 1953. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature **171:**737-738.
176. **Wheeler, D. L., T. Barrett, D. A. Benson, S. H. Bryant, K. Canese, D. M. Church, M. DiCuccio, R. Edgar, S. Federhen, W. Helmberg, D. L. Kenton, O. Khovayko, D. J. Lipman, T. L. Madden, D. R. Maglott, J. Ostell, J. U. Pontius, K. D. Pruitt, G. D. Schuler, L. M. Schriml, E. Sequeira, S. T. Sherry, K. Sirotkin, G. Starchenko, T. O. Suzek, R. Tatusov, T. A. Tatusova, L. Wagner, and E. Yaschenko.** 2005. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. **33:**D39-D45.
177. **Woo, P. C. Y., K. H. L. Ng, S. K. P. Lau, K.-T. Yip, A. M. Y. Fung, K.-W. Leung, D. M. W. Tam, T.-L. Que, and K.-Y. Yuen.** 2003. Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. J. Clin. Microbiol. **41:**1996-2001.
178. **World Health Organization.** 2000. New perspectives in malaria diagnosis. Report WHO/MAL/2000.1091. World Health Organization, Geneva, Switzerland.
179. **World Health Organization.** 2003, posting date. World Health Organization biosafety guidelines for handling of SARS specimens. [Online.] World Health Organization, Geneva, Switzerland. http://www.who.int/csr/sars/guidelines/en/.
180. **Yang, S., S. Lin, G. D. Kelen, T. C. Quinn, J. D. Dick, C. A. Gaydos, and R. E. Rothman.** 2002. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. J. Clin. Microbiol. **40:**3449-3454.
181. **Yang, S., and R.E. Rothman.** 2004. PCR-based diagnostics for infectious diseases: uses, limitations, and future application in acute-care settings. Lancet Infect. Dis. **4:**337-348.
182. **Yu, M. W., S. H. Yeh, P. J. Chen, Y. F. Liaw, C. L. Lin, C. J. Liu, W. L. Shih, J. H. Kao, D. S. Chen, and C. J. Chen.** 2005. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. J. Natl. Cancer Inst. **97:**265-272.
183. **Zaaijer, H. L., H. T. M. Cuypers, H. W. Reesink, I. N. Winkel, G. Gerken, and P. N. Lelie.** 1993. Reliability of polymerase chain reaction for detection of hepatitis C virus. Lancet **341:**722-724.

CHAPTER 2

Prospective Study of Use of PCR Amplification and Sequencing of 16S Ribosomal DNA from Cerebrospinal Fluid for Diagnosis of Bacterial Meningitis in a Clinical Setting

Tim Schuurman
Richard F. de Boer
Anna M. D. Kooistra-Smid
Anton A. van Zwet

Laboratory for Infectious Diseases, Department of Research & Development, Groningen
The Netherlands

Copyright © American Society for Microbiology,
Journal of Clinical Microbiology. 2004. **42:**734-740

ABSTRACT.

We have evaluated the use of a broad-range PCR aimed at the 16S rRNA gene in detecting bacterial meningitis in a clinical setting. To achieve a uniform DNA extraction procedure for both Gram-positive and Gram-negative organisms, a combination of physical disruption (bead beating) and a silica-guanidiniumthiocyanate procedure was used for nucleic acid preparation. To diminish the risk of contamination as much as possible, we chose to amplify almost the entire 16S rRNA gene. The analytical sensitivity of the assay was approximately 1 x 10^2 to 2 x 10^2 CFU/ml of cerebrospinal fluid (CSF) for both Gram-negative and Gram-positive bacteria. In a prospective study of 227 CSF samples, broad-range PCR proved to be superior to conventional methods in detecting bacterial meningitis when antimicrobial therapy had already started. Overall, our assay showed a sensitivity of 86%, a specificity of 97%, a positive predictive value of 80%, and a negative predictive value of 98% compared to culture. We are currently adapting the standard procedures in our laboratory for detecting bacterial meningitis; broad-range 16S ribosomal DNA PCR detection is indicated when antimicrobial therapy has already started at time of lumbar puncture or when cultures remain negative, although the suspicion of bacterial meningitis remains.

INTRODUCTION.

Bacterial meningitis is a serious disease with high morbidity and mortality. To reduce death or permanent neurological sequelae as much as possible, a fast and correct diagnosis is of the utmost importance. The current standard for the diagnosis of bacterial meningitis is microscopic examination and subsequent culture of cerebrospinal fluid (CSF). However, this approach might have some disadvantages with regard to the desired rapidity and sensitivity.

Results of culture may only be available after 24 to 48 h and sometimes, for instance, when the number of viable organisms in the CSF is low, it may take even longer. Moreover, the sensitivity of microscopic examination and culture of CSF can be debated.

First, bacterial concentration in the CSF has a profound effect on the results of microscopy. Regardless of the type of organism in the CSF, the percentage of positive microscopic results is only 25% with < 10^3 CFU/ml and 60% in the range of 10^3 to 10^5 CFU/ml [32]. Second, in an extensive study over a period of 27 years, it appeared that culture might miss the diagnosis of bacterial meningitis in at least 13% of cases [16, 55]. Acknowledged reasons for this lack in sensitivity are CSF obtained after the start of antibiotic treatment and meningitis due to fastidious or slow-growing microorganisms.

Recently, PCR-based assays have become available to provide an early and accurate diagnosis of bacterial meningitis [2, 10, 12, 14, 22, 30, 33, 35, 39, 42, 45, 46, 51, 54, 60].

Some of these assays are aimed at specific pathogens of bacterial meningitis, such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* [10, 12, 46, 51, 54], whereas others use broad-range bacterial PCR [2, 14, 22, 30, 33, 35, 39, 42, 45, 60]. The use of broad-range bacterial PCR has a great advantage in that it also detects microorganisms that are found less frequently or even unknown causative agents of bacterial origin. Even though broad-range PCR has been available since the early 1990s [5, 58], most clinical laboratories have not yet implemented this technique in their daily clinical practice because its use is hampered by two major problems.

First, this method is more vulnerable to contamination than a species-specific PCR. *Taq* DNA polymerases are frequently reported as a major

source of contaminating bacterial DNA [6, 13, 27, 40, 47]. Several approaches overcoming this problem have been reported with success in agarose gel electrophoresis detection systems, such as UV irradiation, 8-MOP treatment, DNase treatment, or restriction endonuclease treatment [9, 21, 25, 26, 28, 44, 52]. However, it has been clearly demonstrated that most decontamination procedures also affect the sensitivity of the broad-range PCR when a sensitive detection system, such as TaqMan, is used [13]. Amplicon size might have an effect on contamination, since most contaminating DNA derives from nonviable organisms. This DNA is most probably unprotected and therefore susceptible to DNA degradation. This could implicate that bigger amplicons are less sensitive for this kind of contamination than are small amplicons. In fact, for cytomegalovirus DNA it has been demonstrated that amplicon size does have a great effect on final PCR results [4]. This would implicate that amplifying the entire 16S rRNA gene would be less contamination sensitive compared to a smaller part of the gene.

Second, at this time no DNA extraction protocol is available that has the same effectiveness for both Gram-positive and Gram-negative bacteria. Most studies use enzyme treatment (e.g., proteinase K or lysozyme), boiling, phenol-chloroform extraction, ethanol precipitation, commercial kits, or some combination of these methods [12, 14, 30, 33, 42, 45]. Bead beating in a guanidinium thiocyanate buffer, followed by organic extraction, has been shown to be effective for extracting nucleic acids from clinical samples containing mycobacteria [29]. However, organic extraction procedures that use phenol and chloroform are a hazard for laboratory staff because of the toxic nature of both compounds. Most organic extraction procedures are also relatively laborious and therefore not suitable for processing samples in a clinical laboratory. Recently, it was shown that physical disruption in combination with the use of a commercial extraction kit has the same quality as standard phenol-chloroform extraction [41].

To tackle these problems, we have chosen to combine a widely used silica-guanidinium thiocyanate procedure for extracting nucleic acids [3, 7, 15, 18, 20, 23, 24, 34, 38, 43, 48, 49, 50, 53, 56, 57, 61] with bead beating in order to achieve a uniform extraction procedure. Moreover, to diminish contamination problems as much as possible, primers were chosen so that almost the entire 16S rRNA gene was amplified.

Here we describe the outcome of a prospective study of the performance of our broad-range bacterial PCR with subsequent sequencing on CSF in comparison with conventional procedures in the diagnosis of bacterial meningitis. After amplification with universal bacterial primers, positive amplicons were sequenced directly, and the obtained sequences were compared to database sequences for bacterial determination.

MATERIALS AND METHODS.

Bacterial strains.

The following bacterial strains were used in reconstruction experiments: *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Bacteria were grown on 5% sheep blood agar plates overnight at 37°C and harvested (10^9 CFU) into 1 ml of saline.

CSF samples and patients.

Between January 2002 and May 2003, 227 CSF samples from 222 patients were collected at the decentralized units of the Regional Public Health Laboratory for Groningen and Drenthe, the Laboratory for Pathology and Medical Microbiology of the Isala Clinics in Zwolle, and the Laboratory for Medical Microbiology of the Academic Hospital of Groningen. All patients included had meningitis as part of their differential diagnosis. These patients were characterized with the following features: mean age, 24.5 years (range, 0 to 87.9 years), and 125 males (56.3%) and 97 females (43.7%). Upon arrival at the laboratory, 200 to 400 μl of each CSF sample was removed under sterile conditions and stored at -20°C until further processing for DNA extraction and PCR. The remaining CSF was used for standard bacterial culture and Gram- and methylene blue staining. All included samples had sufficient volume, ensuring that both methods would produce reliable results.

Bacterial culture and direct microscopic examination.

After removal and storage of 200 to 400 μl of CSF for DNA extraction, the remaining CSF was centrifuged for 15 min at 1,500 x g. The supernatant was discarded and the sediment was resuspended in the remaining liquid by vortexing. From this suspension Gram- and methylene blue stains were made, and bacterial culture was inoculated. The bacterial culture was inoculated into 5% sheep blood and chocolate agar plates and a

thioglycolate enrichment broth with XV-factor and then incubated in a CO_2 incubator at 35°C.

DNA extraction from CSF.

DNA was extracted from CSF samples by a modification of the procedure described by Boom *et al.* [3]. Briefly, 200 µl of CSF was added to 1,000 µl of lysis buffer L6 (5.25 M guanidinium thiocyanate, 50 mM Tris-HCl [pH 6.4], 20 mM EDTA, 1.3% [wt/vol] Triton X-100) and 800 mg of acid washed 0.1-mm-diameter zirconia-silica beads (BioSpec Products, Inc., Bartlesville, Okla.). This mixture was shaken in a Mini-Beadbeater-8 (BioSpec Products, Inc.) for 1 min at 3,200 rpm. After bead beating, the sample was centrifuged for 1 min at 12,000 x g. Then, 1,000 µl of supernatant was transferred to a new screw-cap reaction tube containing 20 µl of size-fractionated silica particles prepared as described previously [3] (Silica, SiO_2, S-5631; Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). After being vortexed, the sample was left at ambient temperature for 10 min. After this 10-min binding step, the silica-DNA pellets were washed and dried as described previously [3]. DNA was eluted in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). If the DNA was not used immediately, it was stored at -20°C until further use.

PCR and sequencing primers.

All primers were obtained from Eurogentec (Eurogentec Nederland b.v., Maastricht, The Netherlands). The primer pair used for amplification consisted of 27F (5'-AGA GTT TGA TC[A/C] TGG CTC AG-3') and 1492R (5'-G[C/T]T ACC TTG TTA CGA CTT-3'). This primer pair amplifies an 1,500-bp fragment of the 16S rRNA gene between positions 8 and 1509 of the *E. coli* 16S rRNA gene and is considered to be universal for the domain Bacteria [31]. For reamplification of samples that yielded insufficient product for a successful sequencing reaction, the following primer pair was used: 357F (5'-CCT ACG GGA GGC AGC AG-3') and 1221R (5'-CAT TGT AGC ACG TGT GTA GCC-3'). Sequencing was done with the following primers: 27F (5'-AGA GTT TGA TC[A/C] TGG CTC AG-3') and 515R (5'-TAC CGC GGC TGC TGG CAC-3'). For reamplified samples, the sequencing primers used were: 357F (5'-CCT ACG GGA GGC AGC AG-3'), 797F (5'-CAA AC[A/G] GGA TTA GAT ACC C-3'), 907R (5'-CCG TCA ATT C[A/C]T TTG AGT TT-3'), and 1221R (5'-CAT TGT AGC ACG TGT GTA GCC-3').

16S ribosomal DNA (rDNA) PCR.

Amplification of the bacterial 16S rRNA gene was carried out in a GeneAmp 9600 thermocycler (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The final reaction mixture (50 µl) contained 25 µl of DNA eluate; 400 ng of each primer; 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems); 0.5 U of uracil-*N*-glycosylase (Applied Biosystems); 5 µg of bovine serum albumin (Roche Diagnostics Nederland B.V., Almere, The Netherlands); 20 µg of alpha-casein (C-6780, Sigma-Aldrich Chemie); 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 3.5 mM $MgCl_2$; dATP, dGTP, and dCTP at concentrations of 200 µM each; and 400 µM dUTP (Applied Biosystems). The thermal cycling profile used was as follows: 2 min at 50°C and 10 min at 95°C, followed by 30 cycles of 20 s at 95°C, 20 s at 50°C, and 3 min at 72°C, followed finally by 10 min at 72°C.

Amplicon detection was carried out by agarose gel electrophoresis of 12 µl of amplification product through horizontal 1% agarose (Agarose MP; Roche Diagnostics Nederland B.V.) gels containing 1 µg of ethidium bromide in 0.5x Tris-borate-EDTA buffer. After electrophoresis, amplicons were made visible by UV light irradiation.

Reamplification of samples that yielded insufficient amounts of product for direct sequencing was carried out in the same reaction mixture of the first-round PCR, except for the presence of uracil-*N*-glycosylase, which was not included in reamplification reactions. Template DNA was purified with the QIAquick PCR purification kit (Westburg, Leusden, The Netherlands) according to the manufacturer's instructions and subsequently diluted 1,000 times. The thermal cycling profile was also the same as for first-round PCR.

Precautions to prevent false-positives and false-negatives.

In order to prevent false-positive and false-negative results in the broad-range PCR, our laboratory workflow was organized in the way described by Millar *et al.* [36], with a few minor exceptions. These exceptions were as follows: (i) all reagents were molecular grade, but not UV irradiated before use, and (ii) separate pipettes were used in sample preparation, PCR setup, and post-PCR handling, but pipettes were not UV irradiated before use.

Extra measures used to prevent false positives were as follows: (i) use of the uracil-*N*-glycosylase system to prevent amplicon carry-over contamination, (ii) a negative extraction control was included for every five

clinical samples to check for cross-contamination during extraction and for quality control of extraction chemicals, and (iii) a negative PCR control was included in each run to quality control the PCR chemicals. To prevent false-negatives, all PCRs were done in duplicate—one reaction without a spike and one reaction with 40 pg of purified *E. coli* DNA as a spike to control for PCR inhibition.

Sequencing.

Amplicons were purified with the QIAquick PCR purification kit (Westburg) according to the manufacturer's instructions. The sequencing reactions were performed with the Big Dye terminator cycle sequencing kit v1.0 or v1.1 (Applied Biosystems) according to the manufacturer's instructions. Cycle reactions were purified by either manual sodium acetate-ethanol precipitation or by the use of the DyeEx 2.0 spin kit (Westburg) according to the manufacturer's instructions. The manual procedure was as follows: 20 µl of cycle sequencing product was added to 50 µl of 96% (vol/vol) ethanol and 2 µl of 3 M sodium acetate (pH 6.4), and this mixture was left at ambient temperature for 15 min. The mixture was centrifuged for 30 min at 12,000 x g, and the supernatant was discarded by pipetting. A portion (250 µl) of 70% (vol/vol) ethanol was added, and the mixture was vortexed and centrifuged for 15 min at 12,000 x g. The supernatant was again discarded by pipetting, and the pellet was centrifuged for 5 min at 12,000 x g. After the small amount of supernatant was removed by pipetting, the pellet was dried for 15 min at 60°C under vacuum conditions. This drying step is part of both the manual and the DyeEx procedures. The dried cycle sequencing products were then dissolved in 25 µl of template suppression reagent (Applied Biosystems) and analyzed on an ABI Prism 310 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions.

Data analysis.

Raw data was analyzed with the Sequence Analysis software v 3.3 and the Factura software v 2.2 (Applied Biosystems). Sequences from both strands were aligned by using the AutoAssembler software v 2.1 (Applied Biosystems) and edited, to resolve discrepancies, by evaluation of the electropherograms. The resulting consensus sequence, created from the double-stranded part of the alignment, was used for comparison with sequences stored in GenBank, EMBL, and DDJB by using the basic local

alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) and the similarity matrix tool from the Ribosomal Database Project II (RDP-II; http://rdp.cme.msu.edu/html/analyses.html) [11]. For both BLAST and RDP-II analyses, the highest-scoring species was reported. Sequences identified in the present study are deposited in GenBank under accession numbers AY325737 to AY325764, AY332613 to AY332615, and AY429260.

RESULTS.

Lower limit of detection of the procedure.

To assess the lower limit of detection, the procedure was performed on 10-fold serial dilutions of *E. coli* and *S. aureus* ranging from 10^7 to 10^0 CFU/ml of pooled CSF, which were previously proven to be both culture and broad-range 16S rDNA PCR negative. Bacteria used for spiking were quantified by agar dilution. Agarose gel electrophoresis of the amplified products showed a lower limit of detection of 100 and 200 CFU/ml of CSF for *E. coli* and *S. aureus*, respectively. Amplicons obtained from the dilutions that contained 100 and 1,000 CFU/ml needed reamplification for a successful sequencing identification. All amplicons yielded the correct identification of the spiked organism after sequencing (data not shown).

Laboratory findings for CSF samples from conventional procedures.

A total of 227 CSF samples were analyzed with both conventional and broad-range 16S rDNA PCR and the results are summarized in Table 2.1. Microbial growth in bacterial culture was detected for 37 CSF samples. In 21 samples the direct microscopic examination was confirmed by culture, but for the remaining 16 culture-positive samples no bacteria were seen. None of the culture-negative samples had any evidence of bacteria in the direct microscopic examination, but in four samples yeasts were seen. Bacterial species isolated during culture were the following: *N. meningitidis* (14 isolates), coagulase-negative staphylococci (CNS) (8 isolates), *S. pneumoniae* (8 isolates), *Haemophilus influenzae* (2 isolates), *E. coli* (2 isolates), viridans group streptococci (1 isolate), *Listeria monocytogenes* (1 isolate), and a *Pantoea* species. (1 isolate).

Laboratory findings from broad-range 16S rDNA PCR.

Broad-range 16S rDNA PCR resulted in a total of 30 positive and 197 negative specimens. Sequencing of these positive amplicons resulted in the

following identifications: *S. pneumoniae* (11 times), *N. meningitidis* (10 times), *H. influenzae* (2 times), *E. coli* (2 times), *Streptococcus salivarius* (1 time), *Streptococcus agalactiae* (1 time), *Prevotella bivia* (1 time), and *Fusobacterium nucleatum* (1 time). One sample resulted in a mixed sequence.

TABLE 2.1. Overall results obtained by 16S rDNA PCR compared to culture.

		Culture		
		Positive	Negative	Total number of specimens
16S rDNA PCR	Positive	24	6	30
	Negative	13	184	197
	Total	37	190	227

Concordant PCR-positive and culture-positive samples.

Table 2.2 shows the results for 22 CSF samples for which conventional and molecular methods were in complete concordance. Pathogens identified by both methods included most of the common causative agents of bacterial meningitis. *N. meningitidis* was identified most often, with a total of nine cases, followed by *S. pneumoniae* (eight cases), *E. coli* and *H. influenzae* (two cases each), and finally *S. salivarius* (viridans group streptococci in culture) with one episode. In this group the broad-range 16S rDNA PCR showed supplementary value in one sample. This sample yielded a strain of *S. pneumoniae* with very poor growth characteristics, making biochemical identification not possible, even after 8 days. Identification of the isolated strain, resulting in *S. pneumoniae*, was only possible by 16S rRNA gene sequencing. Broad-range 16S rDNA PCR, followed by sequencing of the original CSF sample, resulted in *S. pneumoniae* within 48 h.

Concordant culture-negative and PCR-negative samples.

Bacteria were not detectable in the vast majority, i.e., 81.1% (184 of 227 specimens), of analyzed CSF samples by both conventional and molecular methods, as illustrated in Table 2.1. Retrospective analysis of these 184 CSF samples revealed a virus or yeast by culture, microscopy, or specific viral PCR in 7 samples: enterovirus RNA and herpes simplex virus DNA, respectively, were detected in 1 sample each. In the other five samples yeasts were detected by microscopy and/or culture. The low number of viral agents that were finally detected is due to the fact that only a minority of the samples was analyzed for the presence of viruses.

TABLE 2.2. Results of concordant PCR positive and culture positive specimens.

Lab	Broad-range 16S rDNA method			Conventional methods	
number	PCR[a]	BLAST	RDP-II	Culture result[b]	Microscopy[c]
1489	+	*Escherichia coli* (100%)	*E. coli* (1.000)	*E. coli* (+)	(-)
1495	+++	*S. salivarius* (99.9%)	*S. salivarius* (1.000)	viridans-group streptococci (+++)	GPC (++++)
1530	+++	*S. pneumoniae* (100%)	*S. pneumoniae* (1.000)	*S. pneumoniae* (+++)	GPC (++++)
1532	+++	*N. meningitidis* (100%)	*N. meningitidis* (0.998)	*N. meningitidis* (+++)	GNC (++++)
1563	+++	*S. pneumoniae* (100%)	*S. pneumoniae* (0.998)	*S. pneumoniae* (+++)	GPC (++++)
1577	++	*N. meningitidis* (100%)	*N. meningitidis* (1.000)	*N. meningitidis* (++)	GNC (+++)
1682	+++	*N. meningitidis* (99.8%)	*N. meningitidis* (0.995)	*N. meningitidis* (+++)	GNC (+++)
1713	++	*N. meningitidis* (100%)	*N. meningitidis* (0.998)	*N. meningitidis* (++)	GNC (++)
1826	+++	*N. meningitidis* (99.8%)	*N. meningitidis* (0.993)	*N. meningitidis* (+)	GNC (+)
1835	+++	*S. pneumoniae* (99.8%)	*S. pneumoniae* (0.998)	*S. pneumoniae* (++)	GPC (++++)
1836	+	*S. pneumoniae* (99.9%)	*S. pneumoniae* (0.999)	*S. pneumoniae* (+)	GPC (+)
1863	+	*E. coli* (99.5%)	*E. coli* (0.998)	*E. coli* (+++)	GNR (++)
1898	+++	*N. meningitidis* (99.8%)	*N. meningitidis* (0.993)	*N. meningitidis* (++)	GNC (++++)
1908	+++	*N. meningitidis* (99.8%)	*N. meningitidis* (0.993)	*N. meningitidis* (+++)	GNC (++)
1963	+	*H. influenzae* (99.7%)	*H. influenzae* (0.999)	*H. influenzae* (++)	(-)
2055	+++	*S. pneumoniae* (99.6%)	*S. pneumoniae* (0.994)	*S. pneumoniae* (+)[d]	(-)
2108	++	*H. influenzae* (99.7%)	*H. influenzae* (0.997)	*H. influenzae* (+++)	GNR (++++)
2109	++	*S. pneumoniae* (99.8%)	*S. pneumoniae* (0.998)	*S. pneumoniae* (+++)	GPC (++++)
2302	++	*N. meningitidis* (100%)	*N. meningitidis* (0.999)	*N. meningitidis* (++)	GNC (+)
2452	++	*S. pneumoniae* (100%)	*S. pneumoniae* (0.998)	*S. pneumoniae* (+)	GPC (++)
2457	+++	*S. pneumoniae* (99.8%)	*S. pneumoniae* (0.998)	*S. pneumoniae* (+++)	GPC (++++)
2471	++	*N. meningitidis* (100%)	*N. meningitidis* (1.000)	*N. meningitidis* (+++)	GNC (++)

[a] PCR result is reported as follows: +, weakly positive; ++, moderately positive; and +++, strongly positive. Numbers in parentheses are identity percentages.

[b] Symbols in parentheses are relative number of colonies on primary plate: + few or enrichment broth positive, ++ moderate and +++ many.

[c] GPC, Gram-positive cocci, GNC, Gram-negative cocci, and GNR, Gram-negative rods. Symbols in parentheses are relative number of bacterial cells seen in direct microscopy: - none, + sporadic, ++ few, +++ moderate, ++++ many.

[d] Enrichment broth yielded positive result, however sub-culturing of the strain did not yield enough growth for identification procedures. Strain was identified by 16S rDNA sequencing.

PCR-negative and culture-positive discrepant samples.

For 13 CSF samples the positive culture result obtained could not be confirmed by the broad-range 16S rDNA PCR. Eight of these CSF samples resulted in the isolation of CNS, as is shown in Table 2.3. From the remaining five samples, *N. meningitidis* was isolated three times, and *L. monocytogenes* and a *Pantoea* sp. were both isolated once each. The CNS and *Pantoea* sp. culture results were regarded as contamination of bacterial culture and therefore as false positives. In the other four patients the diagnosis of bacterial meningitis was confirmed clinically.

PCR-positive and culture-negative discrepant samples.

For six culture-negative CSF samples the broad-range 16S rDNA PCR did yield a positive result. In three of these samples *S. pneumoniae* was identified, and in the other three *N. meningitidis*, *S. agalactiae*, and *P. bivia* were detected once each (Table 2.3). For five of these CSF samples antimicrobial therapy had already started at the time of lumbar puncture.

PCR-positive and culture-positive discrepant samples.

For two samples sequence data did not match the organism isolated by culture (Table 2.3). The first sample, from which *N. meningitidis* was isolated in culture, showed clear signs of a mixed sequence in its electropherograms obtained after broad-range 16S rDNA PCR. When this sequence was analyzed by using the BLAST algorithm, the sequence was most related to a *Lactococcus* species. However, when the sequence was analyzed with the similarity matrix algorithm from RDP-II it resulted in a *Neisseria* species. Another sample, from which also a *N. meningitidis* strain was isolated in culture, resulted in a sequence identification of *F. nucleatum*.

Overall performance of the broad-range PCR assay compared to culture.

For calculation of the overall performance of the assay compared to bacterial culture, the data from Table 2.1 were used, with the exception that the nine false-positive culture results were excluded from the analysis. This results in an overall sensitivity of 86%, a specificity of 97%, a positive predictive value (PPV) of 80%, and a negative predictive value (NPV) of 98%.

Table 2.3 . Results of discordant PCR and culture specimens.

Lab number	Broad-range 16S rDNA methods[a]			Conventional methods		Remarks[g]
	PCR	BLAST	RDP-II	Microscopy[b]	Culture[c]	
PCR negative and culture positive discrepancies						
1572	-	N.D.[f]	N.D.	(-)	CNS(+)	Contamination*
1705	-	N.D.	N.D.	(-)	CNS(+)	Contamination*
1751	-	N.D.	N.D.	(-)	CNS(+)	Contamination*
1818	-	N.D.	N.D.	(-)	*N. meningitidis* (+)	Bacterial meningitis*
1824	-	N.D.	N.D.	(-)	CNS(+)	Contamination*
1961	-	N.D.	N.D.	(-)	*N. meningitidis* (+)	Bacterial meningitis*
2061	-	N.D.	N.D.	(-)	*L. monocytogenes* (+)	Bacterial meningitis*
2106	-	N.D.	N.D.	(-)	CNS(+)	Contamination*
2225	-	N.D.	N.D.	(-)	CNS(+)	Contamination*
2228	-	N.D.	N.D.	(-)	CNS(+)	Contamination*
2251	-	N.D.	N.D.	(-)	*Pantoea species* (+)	Contamination*
2263	-	N.D.	N.D.	(-)	CNS(+)	Contamination*
2266	-	N.D.	N.D.	GNC (+)	*N. meningitidis* (+)	Bacterial meningitis*
PCR positive and culture negative discrepancies						
1750	+	*S. pneumoniae* (99.9%)	*S. pneumoniae* (0.999)	(-)	(-)	Antibiotic treatment†
2125	+++	*S. agalactiae* (99.7%)	*S. agalactiae* (0.997)	(-)	(-)	Antibiotic treatment†
2145	+++	*P. bivia* (98.5%)	*P. bivia* (0.998)	(-)	(-)	Anaerobic culture not performed†
2264	++	*N. meningitidis* (99.8%)	*N. meningitidis* (1.000)	(-)	(-)	Antibiotic treatment†
2304	+++	*S. pneumoniae* (100%)	*S. pneumoniae* (0.999)	(-)	(-)	Antibiotic treatment†
2403	+++	*S. pneumoniae* (100%)	*S. pneumoniae* (0.999)	(-)	(-)	Antibiotic treatment†
PCR and culture identification discrepancies						
1936[d]	+	*Lactococcus sp.* (87,8%)	*Neisseria sp.* (0.925)	(-)	*N. meningitidis* (+)	SSEARCH result *N. meningitidis*
2253[e]	+++	*F. nucleatum* (99%)	*F. nucleatum* (0.998)	GNC (+)	*N. meningitidis* (+)	PCR contamination

[a] PCR result is reported as follows: +, weakly positive; ++, moderately positive; and +++, strongly positive. Numbers in parentheses are identity percentages.
[b] GNC, gram-negative cocci. Symbols in parentheses reflect relative numbers of bacterial cells seen in direct microscopy: -, none; and +, sporadic.
[c] Symbols in parentheses reflect relative numbers of colonies on the primary plate: +, few or enrichment broth positive; -, none.
[d] Electropherogram showed clear signs of a mixed sequence and resulted in discrepant sequence results between BLAST and RDP-II. A third analysis with the SSEARCH algorithm identified N. meningitidis.
[e] The sequencing result of F. nucleatum did not match the clinical presentation of the patient, although all controls were valid. Reanalysis of the original CSF and the original DNA isolate identified both N. meningitidis. The first result was therefore reported as contamination of the PCR reaction.
[f] ND, not done.
[g] *, Clinical interpretation; †, possible reason for negative culture.

DISCUSSION.

In the present study we evaluated broad-range 16S rDNA PCR and subsequent sequencing in diagnosis of bacterial meningitis in a clinical setting. Our results show that the molecular findings were in good agreement with those obtained from direct microscopy and bacterial culture. The results were in complete concordance for 206 CSF specimens (91%), including 184 culture-negative and PCR-negative samples and 22 culture-positive and PCR-positive samples.

Our broad-range 16S rDNA PCR proved to be extremely valuable in detecting bacterial meningitis when antibiotics had been started prior to lumbar puncture. For five CSF samples obtained after the start of antimicrobial therapy, a significant bacterial pathogen (*S. pneumoniae* [three times], *S. agalactiae* [one time], and *N. meningitidis* [one time]) was identified by broad-range 16S rDNA PCR, whereas the culture results remained negative. These data confirm the findings of earlier studies [10, 30, 42, 45].

Our broad-range 16S rDNA PCR also proved of value in the case of a 2.5-month-old boy with clinical symptoms of sepsis and meningitis. *P. bivia* was found in the CSF of this child, while conventional methods remained negative, since anaerobic culture is not part of the standard operation procedure for detecting bacterial meningitis in most microbiological laboratories. The boy was treated empirically with ampicillin and ceftazidime and recovered completely. Recently, isolation of *P. bivia* from CSF in a critical ill young patient has been described [8].

In two samples, both PCR and culture results were positive, but the microorganism found was different for each method. The first discrepant identification involved a CSF sample from which *N. meningitidis* was isolated. The sequence obtained after broad-range bacterial PCR showed clear signs of a mixed sequence in its electropherograms. This sequence was analyzed with the algorithms from the both BLAST and RDP-II databases. The BLAST search resulted in a *Lactococcus* species, most closely related to *L. lactis* (87.8%), with *N. meningitidis* (86.5%) as the next possible match, whereas the RDP-II search resulted in a *Neisseria* species (percent similarity values, 0.925 to 0.923). Upon manual comparison, > 99% of the sequences from both *L. lactis* and *N. meningitidis* could be retrieved

within the electropherogram. Finally, to confirm the previous findings, the original sequence was also subjected to a third search algorithm, SSEARCH from the Software and Tools for Genome Analysis website (http://www-btls.jst.go.jp/), resulting in *N. meningitidis*. Although lactococci have recently been associated with human disease [1, 17, 19, 37, 59], we concluded that, based upon the clinical evaluation, the lactococcal DNA was most likely a contaminant introduced at the time of lumbar puncture. The second discrepant result was the detection of *F. nucleatum* in the CSF of a 13-year-old girl presenting with mild symptoms of bacterial meningitis. Culture of the CSF yielded *N. meningitidis*, and the child was treated accordantly. The detection of *F. nucleatum* did not match the clinical presentation of the patient, even though all controls included were valid. Therefore, the original CSF sample, as well as the original DNA isolate, was retested, resulting in the identification of *N. meningitidis* for both samples. Resequencing the original PCR product again yielded *F. nucleatum* as in the first analysis, confirming that the first PCR had to be contaminated. This case emphasizes that all laboratory findings should always be placed in perspective with the clinical symptoms, even when all controls to prevent false-positive results make a run valid.

One of the shortcomings of our approach seems to be the sensitivity in the lower range of bacterial loads encountered in bacterial meningitis. A total of 13 culture-positive CSF samples remained negative by broad-range 16S rDNA PCR. In four of these samples it was evident that a bacterial pathogen was present, whereas the others were considered to be contaminants of bacterial culture on clinical grounds. All of these samples, which had low numbers of colonies on primary plates, were estimated to have bacterial loads below our limit of detection. However, it should be emphasized that the sensitivity of our assay (100 CFU/ml of CSF) should be sufficient for detecting the majority of bacterial meningitis cases, since La Scolea *et al.* showed that only 16% of culture-positive CSF samples in bacterial meningitis have bacterial loads of < 1,000 CFU/ml [32].

The overall performance of our assay, compared to bacterial culture, is in line with other published broad-range PCR methods [30, 33, 42], with an overall sensitivity of 86%, a specificity of 97%, a PPV of 80%, and an NPV of 98%. It should be emphasized that the relatively low PPV is at least partly

explained by the inadequacy of bacterial culture: in six PCR-positive, culture-negative CSF samples a significant bacterial pathogen was identified in a patient with all of the signs of bacterial meningitis (Table 2.3).

The molecular method validated here is not only suitable for the detection of bacteria in CSF but can also be used for the detection of bacteria in many other clinical samples from anatomic sites that are considered to be sterile in healthy subjects. We have demonstrated that we can detect clinically significant bacteria in other clinical samples, such as blood culture fluids, a brain abscess, a liver abscess, pus from a vertebra, synovial fluids, and pericardial fluid, for which conventional methods could not (data not shown).

In conclusion, our broad-range 16S rDNA PCR with subsequent sequencing has proven to be a valuable supplementary test in daily clinical practice. Bacterial culture of CSF remains the cornerstone in the diagnosis of bacterial meningitis. However, especially when antimicrobial therapy has already started at the time of lumbar puncture, conventional methods should always be accompanied by molecular detection, since the sensitivity of direct microscopic examination and bacterial culture drops substantially when therapy has started. We are currently adapting the standard procedures in our laboratory for the detection of bacterial meningitis; broad-range 16S rDNA detection is indicated when antimicrobial therapy has already been started at the time of lumbar puncture or when cultures remain negative, although the suspicion of bacterial meningitis remains.

ACKNOWLEDGMENTS.

We thank all of the members of the decentralized units of the Regional Public Health Laboratory for Groningen and Drenthe, the members of the Laboratory for Pathology and Medical Microbiology of the Isala Clinics in Zwolle, and the members of the Laboratory for Medical Microbiology of the Academic Hospital of Groningen for their contributions to this study.

REFERENCES.

1. **Akhaddar, A., B. El Mostarchid, M. Gazzaz, and M. Boucetta.** 2002. Cerebellar abscess due to *Lactococcus lactis*: a new pathogen. Acta Neurochir. **144:**305-306.
2. **Bäckman, A., P. G. Lantz, P. Rädström, and P Olcén.** 1999. Evaluation of an extended diagnostic PCR assay for detection and verification of the common causes of bacterial meningitis in CSF and other biological samples. Mol. Cel. Probes **13:**49-60.
3. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495-503.
4. **Boom, R., C. J. A. Sol, T. Schuurman, A. van Breda, J. F. L. Weel, M. Beld, I. J. M. ten Berge, P. M. E. Wertheim-van Dillen, and M. D. de Jong.** 2002. Human cytomegalovirus DNA in plasma and serum specimens of renal transplant recipients is highly fragmented. J. Clin. Microbiol. **40:**4105-4113.
5. **Böttger, E. C.** 1989. Rapid determination of bacterial rRNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol. Lett. **53:**171-176.
6. **Böttger, E. C.** 1990. Frequent contamination of *Taq* polymerase with DNA. Clin. Chem. **36:**1258-1259.
7. **Brisson-Noël, A., C. Aznar, C Chureau, S. Nguyen, C. Pierre, M. Bartoli, R. Bonete, G. Pialoux, B. Gicquel, and G. Garrique.** 1991. Diagnosis of tuberculosis by DNA amplification in clinical practice evaluation. Lancet **338:**364-366.
8. **Busch, N., P. R. Mertens, T. Schonfelder, H. Nguyen, H. U. Marschall, H. Kierdorf, G. Haase, R. Urhahn, H. G. Sieberth, and S. Matern.** 1996. Lemierre's post-tonsillitis sepsis with meningitis and intravascular consumption coagulopathy as complication of infectious mononucleosis with pansinusitis. Dtsch. Med. Wochenschr. **121:**94-98.
9. **Carroll, N. M., P. Adamson, and N. Okhravi.** 1999. Elimination of bacterial DNA from *Taq* DNA polymerases by restriction endonuclease digestion. J. Clin. Microbiol. **37:**3402-3404.
10. **Cherian, T., M. K. Lalitha, A. Manoharan, K. Thomas, R. H. Yolken, and M. C. Steinhoff.** 1998. PCR-enzyme immunoassay for detection of *Streptococcus pneumoniae* DNA in cerebrospinal fluid samples from patients with culture-negative meningitis. J. Clin. Microbiol. **36:**3605-3608.
11. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxynomy. Nucleic Acids Res. **31:**442-443.
12. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, A. J. Fox, and E. B. Kaczmarski.** 2001. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J. Clin. Microbiol. **39:**1553-1558.
13. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, E. B. Kaczmarski, and A. J. Fox.** 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J. Clin. Microbiol. **38:**1747-1752.
14. **Dicuonzo, G., G. Lorino, D. Lilli, D. Rivanera, P. Guarino, S. Angeletti, G. Gherardi, and F. Filadoro.** 1999. Polymerase chain reaction, with sequencing, as a diagnostic tool in culture-negative bacterial meningitis. Clin. Microbiol. Infect. **5:**92-96.
15. **Dorigo-Zetsma, J. W., R. P. Verkooyen, H. P. van Helden, H. van der Nat, and J. M. van den Bosch.** 2001. Molecular detection of *Mycoplasma pneumoniae* in adults with community-acquired pneumonia requiring hospitalization. J. Clin. Microbiol. **39:**1184-1186.
16. **Durand, M. L., S. B. Calderwood, D. J. Weber, S. I. Miller, F. S. Southwick, V. S. Caviness, Jr., and M. N. Schwartz.** 1993. Acute bacterial meningitis in adults: a review of 493 episodes. N. Engl. J. Med. **328:**21-28.

17. **Durand, J. M., M. C. Rousseau, J. M. Gandois, G. Kaplanski, M. N. Mallet, and J. Soubeyrand.** 1995. *Streptococcus lactis* septicemia in a patient with chronic lymphocytic leukemia. Am. J. Hematol. **50:**64-65.
18. **Dyer, J. R., B. L. Gilliam, J. J. Eron, Jr., L. Grosso, M. S. Cohen, and S. A. Fiscus.** 1996. Quantitation of human immunodeficiency virus type 1 RNA in cell free seminal plasma: comparison of NASBA™ with Amplicor™ reverse transcription-PCR amplification and correlation with quantitative culture. J. Virol. Methods **60:**161-170.
19. **Facklam, R., and J. A. Elliott.** 1995. Identification, classification and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. Clin. Microbiol. Rev. **8:**479-495.
20. **Fidler, H. M., G. A. Rook, N. M. Johnson, and J. McFadden.** 1993. *Mycobacterium tuberculosis* DNA in tissue affected by sarcoidosis. BMJ **306:**546-549.
21. **Frothingham, R., R. B. Blitchington, D. H. Lee, R. C. Greene, and K. H. Wilson.** 1992. UV absorption complicates PCR decontamination. BioTechniques **13:**208-210.
22. **Hall, L. M. C., B. Duke, and G. Urwin.** 1995. An approach to the identification of the pathogens of bacterial meningitis by the polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. **14:**1090-1094.
23. **Hale, A. D., J. Green, and D. W. G. Brown.** 1996. Comparison of four RNA extraction methods for the detection of small round viruses in faecal specimens. J. Virol. Methods **57:**195-201.
24. **Hand, O., M. Höss, M. Krings, and S. Pääbo.** 1994. Ancient DNA: methodological challenges. Experientia **50:**524-529.
25. **Heininger, A., M. Binder, A. Ellinger, K. Botzenhart, K. Unertl, and G. Döring.** 2003. DNase pretreatment of master mix reagents improves the validity of universal 16S rRNA gene PCR results. J. Clin. Microbiol. **41:**1763-1765.
26. **Hilali, F., P. Saulnier, E. Chachaty, and A. Andremont.** 1997. Decontamination of polymerase chain reaction reagents for detection of low concentration of 16S rRNA genes. Mol. Biotechnol. **7:**207-216.
27. **Hughes, M. S., L. A. Beck, and R. A. Skuce.** 1994. Identification and elimination of DNA sequences in *Taq* DNA polymerase. J. Clin. Microbiol. **32:**2007-2008.
28. **Jinno, Y., K. Yoshiura, and N. Niikawa.** 1990. Use of psoralen as extinguisher of contaminating DNA in PCR. Nucleic Acids Res. **18:**6739.
29. **Kirschner, P., J. Rosenau, B. Springer, K. Teschner, K. Feldmann, and E. C. Böttger.** 1996. Diagnosis of mycobacterial infections by nucleic acid amplification: 18-month prospective study. J. Clin. Microbiol. **34:**304-312.
30. **Kotilainen, P., J. Jalava, O. Meurman, O. Lehtonen, E. Rintala, O. Seppälä, E. Eerola, and S. Nikkari.** 1998. Diagnosis of meningococcal meningitis by broad-range bacterial PCR with cerebrospinal fluid. J. Clin. Microbiol. **36:**2205-2209.
31. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley & Sons, Chichester, United Kingdom.
32. **La Scolea, L. J., Jr., and D. Dryja.** 1984. Quantitation of bacteria in cerebrospinal fluid and blood of children with meningitis and its diagnostic significance. J. Clin. Microbiol. **19:**187-190.
33. **Lu, J.-J., C.-L. Perng, S.-Y. Lee, and C.-C. Wan.** 2000. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. J. Clin. Microbiol. **38:**2076-2080.
34. **Mahony, J. B., X. Song, S. Chong, M. Faught, T. Salonga, and J. Kapala.** 2001. Evaluation of the NucliSENS basic kit for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genital tract specimens using nucleic acid sequence-based amplification of 16S rRNA. J. Clin. Microbiol. **39:**1429-1435.
35. **Margall Coscojuela, N., M. Majó Moreno, C. Latorre Otín, D. Fontanals Aymerich, À. Domínquez García, and G. Prats Pastor.** 2002. Use of universal PCR on cerebrospinal fluid to diagnose bacterial meningitis in culture-negative patients. Eur. J. Clin. Microbiol. Infect. Dis. **21:**67-69.
36. **Millar, B. C., J. Xu, and J. E. Moore.** 2002. Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. J. Clin. Microbiol. **40:**1575-1580.

37. **Nakarai, T., K. Morita, Y. Nojiri, J. Nei, and Y. Kawamori.** 2000. Liver abscess due to *Lactococcus lactis cremoris.* Pediatr. Int. **42:**699-701.
38. **Nijhuis, M., N. van Maarseveen, R. Schuurman, S. Verkuijlen, M. de Vos, K. Hendriksen, and A. M. van Loon.** 2002. Rapid and sensitive routine detection of all members of the genus *Enterovirus* in different clinical specimens by real-time PCR. J. Clin. Microbiol. **40:**3666-3670.
39. **Nikkari, S., F. A. Lopez, P. W. Lepp, P. R. Cieslak, S. Ladd-Wilson, D. Passaro, R. Danila, and D. A. Relman.** 2002. Broad-range bacterial detection and the analysis of unexplained death and critical illness. Emerg. Infect. Dis. **8:**188-193.
40. **Rand, K. H., and H. Houck.** 1990. *Taq* polymerase contains bacterial DNA of unknown origin. Mol. Cell. Probes **4:**445-450.
41. **Rantakokko-Jalava, K., and J. Jalava.** 2002. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. J. Clin. Microbiol. **40:**4211-4217.
42. **Rantakokko-Jalava, K., S. Nikkari, J. Jalava, E. Eerola, M. Skurnik, O. Meurman, O. Ruuskanen, A. Alanen, E. Kotilainen, P. Toivanen, and P. Kotilainen.** 2000. Direct amplification of rRNA genes in diagnosis of bacterial infections. J. Clin. Microbiol. **38:**32-39.
43. **Revello, M. G., F. Baldanti, A. Sarasini, M. Zavattoni, M. Torsellini, and G. Gerna.** 1997. Prenatal diagnosis of rubella virus infection by direct detection and semiquantitation of viral RNA in clinical samples by reverse transcriptase-PCR. J. Clin. Microbiol. **35:**708-713.
44. **Rochelle, P. A., A. J. Weightman, and J. C. Fry.** 1992. DNase I treatment of *Taq* DNA polymerase for complete PCR decontamination. BioTechniques **13:**520.
45. **Saravolatz, L. D., O. Manzor, N. VanderVelde, J. Pawlak, and B. Belian.** 2003. Broad-range bacterial polymerase chain reaction for early detection of bacterial meningitis. Clin. Infect. Dis. **36:**40-45.
46. **Saunders, N. B., D. R. Shoemaker, B. L. Brandt, and W. D. Zollinger.** 1997. Confirmation of suspicious cases of meningococcal meningitis by PCR and enzyme-linked immunosorbent assay. J. Clin. Microbiol. **35:**3215-3219.
47. **Schmidt, T. M., B. Pace, and N. R. Pace.** 1991. Detection of DNA contamination in *Taq* polymerase. BioTechniques **11:**176-177.
48. **Schee, C. van der, A. van Belkum, L. Zwijgers, E. van der Brugge, E. L. O'neill, A. Luijendijk, T. van Rijsoort-Vos, W. I. van der Meijden, H. Verbrugh, and H. J. F. Sluiters.** 1999. Improved diagnosis of *Trichomonas vaginalis* infection by PCR using vaginal swabs and urine specimens compared to diagnosis by wet mount microscopy, culture, and fluorescent staining. J. Clin. Microbiol. **37:**4127-4130.
49. **Schoone, G. J., L. Oskam, N. C. M. Kroon, H. D. F. H. Schallig, and S. A. Omar.** 2000. Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. J. Clin. Microbiol. **38:**4040-4072.
50. **Secchiero, P., D. R. Carrigan, Y. Asano, L. Benedetti, R. W. Crowley, A. L. Komaroff, R. C. Gallo, and P. Lusso.** 1995. Detection of human herpesvirus 6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction. J. Infect. Dis. **171:**273-280.
51. **Seward, R. J., and K. J. Towner.** 2000. Use of an automated DNA analysis system (DARAS®) for sequence-specific recognition of *Neisseria meningitidis* DNA. Clin. Microbiol. Infect. **6:**29-33.
52. **Sharma, J. K., V. Gopalkrishna, and B. C. Das.** 1992. A simple method for elimination of unspecific amplifications in polymerase chain reaction. Nucleic Acids Res. **20:**6117-6118.
53. **Solmone, M., E. Girardi, F. Costa, L. Pucillo, G. Ippolito, and M. R. Capobianchi.** 2002. Simple and reliable method for detection and genotyping of hepatitis C virus RNA in dried blood spots stored at room temperature. J. Clin. Microbiol. **40:**3512-3514.
54. **Taha, M.-K.** 2000. Simultaneous approach for nonculture PCR-based identification and serogroup prediction of *Neisseria meningitidis.* J. Clin. Microbiol. **38:**855-857.

55. **Tunkel, A. R., and W. M. Scheld.** 1995. Acute bacterial meningitis. Lancet **346:**1675-1680.
56. **Vernazza, P. L., J. R. Dyer, S. A. Fiscus, J. J. Eron, and M. S. Cohen.** 1997. HIV-1 viral load in blood, semen and saliva. AIDS **11:**1058-1059.
57. **Wall, S., Z. M. Kunze, S. Saboor, J. Soufleri, P. Seechurn, R. Chiodini, and J. J. McFadden.** 1993. Identification of sheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction. J. Clin. Microbiol. **31:**1241-1245.
58. **Wilson, K., R. Blitchington, and R. Greene.** 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. **28:**1942-1946.
59. **Wood, H. F., K. Jacobs, and M. McCarthy.** 1985. *Streptococcus lactis* isolated from a patient with subacute bacterial endocarditis. Am. J. Med. **18:**345-347.
60. **Xu, J., B. C. Millar, J. E. Moore, K. Murphy, H. Webb, A. J. Fox, M. Cafferkey, and M. J. Crowe.** 2003. Employment of broad-range 16S rRNA PCR to detect etiological agents of infection from clinical specimens in patients with acute meningitis: rapid separation of 16S rRNA PCR amplicons without the need for cloning. J. Appl. Microbiol. **94:**197-206.
61. **Zipeto, D., F. Baldanti, D. Zella, M. Furione, A. Cavicchini, G. Milanesi, and G. Gerna.** 1993. Quantification of human cytomegalovirus DNA in peripheral blood polymorphonuclear leukocytes of immunocompromised patients by the polymerase chain reaction. J. Virol. Methods **44:**45-46.

CHAPTER 3

Reduced PCR Sensitivity Due to Impaired DNA Recovery with the MagNA Pure LC Total Nucleic Acid Isolation Kit

Tim Schuurman [1]
Alex van Breda [2]
Richard de Boer [1]
Mirjam Kooistra-Smid [1]
Marcel Beld [2]
Paul Savelkoul [3]
René Boom [1]

[1] Laboratory for Infectious Diseases, Department of Research & Development, Groningen
[2] Academic Medical Center, Department of Medical Microbiology, Section of Clinical Virology, Amsterdam
[3] VU Medical Center, Department of Medical Microbiology and Infection Control, Amsterdam
The Netherlands

Copyright © American Society for Microbiology,
Journal of Clinical Microbiology. 2005. **43:**4616-4622

ABSTRACT.

The increasing demand for molecular diagnostics in clinical microbiology laboratories necessitates automated sample processing. In the present study, we evaluated the performance of the MagNA Pure LC total nucleic acid isolation kit (M extraction) in comparison with the manual method (Si extraction) according to Boom *et al.* (R. Boom, C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa, J. Clin. Microbiol. 28:495-503, 1990) for the detection of viral DNA by competitive quantitative PCR. Reconstruction experiments with *Hin*dIII-digested phage lambda DNA and *Hae*III-digested ΦX174 DNA showed that the recovery of DNA from phosphate-buffered saline, cerebrospinal fluid, EDTA-anticoagulated plasma, and EDTA-anticoagulated whole blood by M extraction is, on average, 6.6-fold lower compared to Si extraction. PCR signals of spiked PCR control DNAs for Epstein-Barr virus and varicella-zoster virus were also between 1.9- and 14.2-fold lower after M extraction compared to Si extraction, also suggesting impaired DNA recovery. M extraction of spiked cytomegalovirus strain AD 169 in whole blood showed a 5- to 10-fold reduction in PCR sensitivity compared to Si extraction. This reduction of PCR sensitivity was also observed when clinical whole blood samples were processed by M extraction. Before implementing M extraction, the clinical consequences of the reduced recovery should first be considered, especially when maximal sensitivity is required.

INTRODUCTION.

In recent years, molecular diagnostic testing has become an essential part of the routine work flow in clinical microbiology laboratories. To comply with the demands for sensitivity and reliability, highly qualitative methods for extraction, amplification, and detection of nucleic acids (NA) are required. Most amplification-detection systems today, like PCR-hybridization and real-time PCR, have the analytical power to detect and identify a single target molecule [5, 7, 8, 9, 22]. When combined with the extraction procedure developed by Boom *et al.* (Si extraction) [3], acknowledged for its potency in removing inhibitors from clinical samples and widely used for the purification of NA from a variety of clinical samples [2, 6, 24], optimal clinical sensitivity and reliability are achieved. However, with the increasing number of molecular assays becoming available to the clinical laboratory, the total throughput of samples also increases and makes automation of the extraction procedure mandatory. The use of an automated commercial NA extraction method also has other potential benefits, like a high degree of standardization and transferring part of the quality control from the clinical microbiology laboratory to the manufacturer.

Several robotic platforms have recently become available for sample preparation in molecular diagnostics. For low-throughput settings, the BioRobot EZ1 (QIAGEN) and MagNA Pure Compact (Roche) systems are suitable platforms, whereas for medium- to high-throughput settings the MagNA Pure LC instrument (Roche) and the BioRobot M48/9604 system (QIAGEN) are available. For most clinical microbiology laboratories performing several DNA- and RNA-based assays on a wide range of clinical specimens, medium- to high-throughput extractors combined with generic extraction chemistry will provide an efficient solution for sample preparation, although for smaller laboratories low-throughput extractors can also be sufficient.

The MagNA Pure LC total NA isolation kit (M extraction) on the MagNA Pure LC instrument provides generic extraction chemistry on a medium- to high-throughput extraction platform. Several reports have described the application of M extraction in the sample preparation of cytomegalovirus (CMV), enterovirus, hepatitis B virus, hepatitis C virus (HCV), herpes simplex virus type 1 and 2, human immunodeficiency virus type 1, and broad-range

bacterial rRNA genes [10, 11, 13, 14, 15, 16, 18, 20, 21]. Most of these reported assays perform with satisfactory analytical and clinical sensitivities compared to reference procedures. However, there is some evidence for reduced extraction efficiency with M extraction. For instance, HCV RNA recovery seems to be less efficient with M extraction and analysis by the (COBAS) AMPLICOR HCV 2.0 test [10, 11, 14] compared to other manual (Roche) and automated (NucliSENS) extraction procedures [1, 19]. Furthermore, for enterovirus RNA, *Knepp et al.* [16] have shown that M extraction was less sensitive than the automated BioRobot viral RNA M48 and manual QiaAmp viral RNA isolation kits. Also, data presented by Mohammadi *et al.* suggest less efficient recovery of the *Escherichia coli* 16S rRNA gene by M extraction in comparison with Si extraction (manual NucliSENS extraction) [21]. Finally, Burghoorn-Maas *et al.* (C. Burghoorn-Maas, P. van Deursen, M. Jacobs, and H. G. M. Niesters, Abstr., 2nd Eur. Congr. Virol., p. 7-10, 2004) have shown that M extraction was 0.5 to 1.0 $\log_{10}$ less sensitive compared to the NucliSENS miniMAG platform.

Because the lower recovery of M extraction is currently supported by a limited number of small-scale experiments, it has to date not been recognized as a possible issue of concern. Furthermore, in most reports the extraction methods used are difficult to compare directly, due to differences in input/output ratios and therefore the equivalent of sample volume tested by the amplification-detection system. In this respect, a more extensive study of the extraction efficiency in a direct comparison, with equivalent sample volumes, could provide more insights into the possible drawbacks of M extraction. Here we report the evaluation of M extraction in comparison with the Si extraction reported by Boom *et al.* [3], which is currently widely used throughout the field of molecular diagnostic testing [2, 6].

MATERIALS AND METHODS.

Clinical specimens.

EDTA-anticoagulated whole blood (whole blood) was obtained from healthy volunteers. EDTA-anticoagulated plasma (plasma) was obtained by separation from the whole blood after centrifugation for 10 min at 1,750 x g. Clinical whole blood specimens were obtained from patients for whom CMV

and/or EBV PCR was requested. Cerebrospinal fluid (CSF), stored at –20°C, was obtained from a previous study (24).

Human CMV.

Sucrose density gradient-purified human CMV strain AD 169 (lot no. 80-165-1; 5.38 x 10^9 viral particles/ml of virus dilution buffer [10 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5], as determined electron microscopically by direct particle counting, which discriminates between full and empty particles) was obtained from Advanced Biotechnologies Inc. (Colombia, Md.). The virus titer was corrected to 1.61 x 10^{10} viral particles/ml as described previously [5].

DNA purification.

DNA was purified from 200 μl of phosphate-buffered saline (PBS), plasma, or CSF or 50 μl of whole blood by manual Si extraction or automated M extraction. Specimens were spiked with either *Hin*dIII-digested phage lambda (λ-*Hin*dIII) DNA (Gibco BRL, Breda, The Netherlands), *Hae*III-digested ΦX174 DNA (molecular weight marker IX; Roche Diagnostics Nederland BV, Almere, The Netherlands), or internal control (IC) DNA before extraction.

Manual Si extraction was carried out as described previously [3], with 20 μl of size-fractionated silica particles (SC). Elution was in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]).

Automated M extraction on the MagNA Pure LC instrument (Roche Diagnostics Nederland BV) was carried out with the MagNA Pure LC total NA isolation kit (Roche Diagnostics Nederland BV) according to the manufacturer's instructions, using the protocol total NA external lysis, with the elution volume set at 100 μl.

Recovery of lost DNA.

DNA potentially lost during washing steps of the purification procedures was retrieved by re-extraction of the discarded washing solutions. For M extraction, 200 μl of washing solution was processed by Si extraction and eluted in 50 μl of TE buffer. For Si extraction, the discarded lysis buffer L6 was added to 20 μl of fresh SC and subsequently extracted by Si extraction with elution in 50 μl of TE buffer. For the washing stages (L2, 70% ethanol and acetone), 200 μl of the discarded washing solution was re-extracted by Si extraction and eluted in 50 μl of TE buffer. Noneluted DNA from the silica

matrices of both methods (magnetic glass particles or SC) was recovered by elution in 50 µl of TE buffer for 1 h at 56°C.

Agarose gel electrophoresis.

DNA was electrophoresed through 1% agarose (Agarose MP; Roche Diagnostics Nederland BV) gels containing 1 µg ethidium bromide per ml of 0.5 x Tris-borate-EDTA buffer. Appropriate recovery markers were included in all gels for estimation of the DNA recovery after image capturing under UV illumination. The Gel Doc 1000 system (Bio-Rad, Veenendaal, The Netherlands) was used for image capturing, and the graphic files were exported as 8-bit TIFF images. DNA band intensities were calculated from the peak surface areas using Scion Image Release Beta 4.0.2 (Scion Corporation, Frederick, Md.). Recoveries were calculated in comparison to the appropriate recovery markers.

Primers and probes.

The primers and probes used (high-performance liquid chromatography purified; Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) are listed in Table 3.1.

TABLE 3.1. Primers and probes.

Name	Function	Sequence[a]	Ref
CMV-531	Forward primer	5'-ACA AGG TGC TCA CGC ACA TTG ATC-3'	[5]
bio-CMV-1107	Reverse primer	5'-biotin-CAC TGG CTC AGA CTT GAC AGA CAC-3'	[5]
TBR-CMV-1	CMV-probe	5'-TBR-TGA AGG TTG CCC AGT ACA TTC T-3'	[5]
TBR-CMV-2	CMV-IC-probe	5'-TBR-CCC TTT ACA TCT TTC TGA AGT AGG G-3'	[5]
VZV-3	Forward primer	5'-TCT TTC ACG GAG GCA AAC AC-3'	[8]
bio-VZV-4	Reverse primer	5'-biotin-TCC AAG GCG GGT GCA TAT CT-3'	[8]
TBR-VZV-1	VZV-probe	5'-TBR-AAC GGT TTG GGT TTT CAC GCT GCC-3'	[8]
TBR-VZV-2	VZV-IC-probe	5'-TBR-ACC TGT CGG ACT CGT AGT TGC TGT-3'	[8]
bio-EBV-1	Forward primer	5'-biotin-TGG GTC GCC GGT GTG TTC GTA TA-3'	-
EBV-2	Reverse primer	5'-CTA AAC GGA GGG ACC AAA GGT GG-3'	-
TBR-EBV-1	EBV-probe	5'-TBR-GGC CAT TCC AAA GGG GAG ACG-3'	-
TBR-EBV-2	EBV-IC-probe	5'-TBR-GAG CAG TCA GGA TCC GAG AGC-3'	-

[a] TBR; Tris-(2,2'-bipyridine)-ruthenium (II) chelate.

IC DNA.

Construction of the IC DNAs for CMV and VZV has been described previously [5, 8]. Construction of the IC DNA for EBV will be described elsewhere (V. Bekker *et al.*, submitted for publication). The design of the EBV-IC DNA is essentially the same as for CMV and VZV.

CMV, VZV, and EBV PCRs.

CMV and VZV PCRs were carried out as described previously [5, 8]. EBV PCR was carried out in the same format as the CMV and VZV PCRs and in general has performance characteristics similar to both the CMV and VZV PCRs. The validation of the EBV PCR assay will be described elsewhere (Bekker et al., submitted). Briefly, the reaction mixtures (50 µl) consisted of 10 mM Tris HCl (pH 8.3); 50 mM KCl; 3 (CMV), 4 (VZV), or 4.5 (EBV) mM $MgCl_2$; dATP, dGTP, and dCTP at a concentration of 200 µM each; 400 µM dUTP; 2.5 U of AmpliTaq DNA polymerase (CMV and VZV) or AmpliTaq Gold DNA polymerase (EBV); 0.5 U of Amperase (uracil-*N*-glycosylase); 200 ng each of the forward and reverse primers (Table 3.1); 20 µg of alpha-casein (C 6780; Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands); 5 µg of bovine serum albumin (Roche Diagnostics Nederland BV); and 25 µl of DNA. Amplifications were done in a PE 9600 thermocycler (Applied Biosystems) under the following conditions: 2 min at 50°C, 5 min (CMV and VZV) or 10 min (EBV) at 95°C, followed by 35 cycles each consisting of 20 s at 95°C, 20 s at 63°C, and 1 min at 72°C, followed by 5 min at 72°C.

Removal of excess primers, hybridization, and electrochemiluminescence measurement.

Excess primers were removed as described previously [5] by protocol Delta Y-A. Hybridization and electrochemiluminescence detection were also performed as described previously [4, 5, 8].

Algorithm for quantitation.

For quantitation of CMV and EBV, the following algorithms were used. After correcting for the background, the ratio of the virus DNA-specific signal to the IC DNA-specific signal (*R*) was determined and the number of copies of viral DNA per milliliter of whole blood was calculated by multiplying *R* by factor *F*. Factor *F* was derived by multiplying the factors $IC_{extraction}$ x *D*. The factor $IC_{extraction}$ represents the number of IC DNA molecules present in the DNA extraction, and factor *D* is required to reach the copy number per milliliter, which is set at 20 for whole blood.

RESULTS.

Recovery of *Hin*dIII-digested phage λ DNA.

Four micrograms of spiked *Hin*dIII-digested phage lambda DNA was extracted from PBS, CSF, and plasma with four to eight replicates each by both M extraction and Si extraction. Table 3.2 shows the summarized data for all specimen types tested.

TABLE 3.2. Recovery of DNA[a].

Method and specimen type[b]	DNA type[c]	Mean % DNA recovery[d]				% of DNA which was not recovered
		By method	From binding step	From washing steps	From silica matrix	
M extraction						
PBS	HMW	10	10	5	15	60
	LMW	5	15	20	10	50
CSF	HMW	15	20	0	5	60
	LMW	15	25	0	5	55
Plasma	HMW	25	25	0	5	45
	LMW	25	25	0	5	45
Whole blood	HMW[f]	20	20	0	60	NA[g]
	LMW	25	40	0	10	25
Si extraction						
PBS	HMW	100	0	ND[e]	ND	0
	LMW	100	0	ND	ND	0
CSF	HMW	80	5	ND	5	10
	LMW	80	5	ND	5	10
Plasma	HMW	90	5	ND	5	0
	LMW	70	5	ND	5	20
Whole blood	HMW[f]	70	5	0	25	NA
	LMW	65	15	0	5	15

[a] For PBS, CSF, and plasma, *Hin*dIII-digested phage λ DNA was used. For whole blood, *Hae*III-digested ΦX174 DNA was used.

[b] Plasma, EDTA-anticoagulated plasma; whole blood, EDTA-anticoagulated whole blood.

[c] HMW DNA, 23.1 kb, 9.4 kb, 6.6 kb, and 4.4 kb (λ-*Hin*dIII) and chromosomal DNA (marker IX); LMW DNA, 2.3 kb and 2.0 kb (λ-*Hin*dIII) and 1.4 kb, 1.1 kb, 0.9 kb, and 0.6 kb (marker IX).

[d] Calculated percentages were rounded to the nearest multiple of 5% for simplified representation.

[e] ND, not determined.

[f] Due to the fact that the exact input of chromosomal DNA was unknown, the percentages reflect the relative amounts of DNA that could be detected.

[g] NA, not applicable (could not be determined because the exact input was unknown).

Table 3.2 clearly shows that M extraction had a substantially lower recovery compared to Si extraction when λ-*Hin*dIII DNA was extracted from PBS, CSF, and plasma, with, on average, 7.8-fold (range, 2.8-fold to 20-fold)

lower recoveries. To identify purification steps where DNA was possibly lost in the procedure, DNA was extracted from the fluids in which binding to the silica matrix takes place for both procedures. Approximately 20% of the missing DNA is not bound by the magnetic glass particles in M extraction, whereas approximately 3% of the λ-*Hin*dIII DNA remains unbound in Si extraction. In a second elution of the silica matrix, about 7.5% and 5% of the missing DNA could be retrieved for M and Si extractions, respectively. Since a large portion (on average, 53%) of the λ-*Hin*dIII DNA was still missing with M extraction, the other washing solutions were also investigated for potentially lost DNA. However, within the detection limit of agarose gel electrophoresis, no DNA could be retrieved from discarded wash buffers I, II, and III, thus leaving about 53% of the input of λ-*Hin*dIII DNA irretrievable.

Recovery of DNA from whole blood.

For whole blood, *Hae*III-digested ΦX174 DNA was used to monitor low-molecular-weight (LMW) DNA recovery. For recovery of high-molecular-weight (HMW) DNA, the human chromosomal DNA present in the blood was used, but no estimation of irretrievable DNA was possible for HMW DNA, since the exact amount of chromosomal DNA per milliliter was unknown.

With whole blood, a similar trend in recovery was observed with M extraction, with, on average, 3.1-fold lower recovery compared to Si extraction (Table 3.2). Again part of the missing DNA could be retrieved from the binding step (30% with M extraction and 10% with Si extraction), and no DNA was lost during the washing procedures. For both extraction procedures, more HMW DNA could be retrieved from the silica matrix compared to PBS, CSF, and plasma, whereas the amount of LMW DNA retrieved by a second elution was the same as for the other specimen types.

Influence of DNA recovery on PCR.

To assess the influence of the impaired DNA recovery of M extraction on PCR assays, we spiked PCR-negative whole blood samples with low (50 copies [LPC]) and high (250 copies [HPC]) positive control DNAs for EBV or VZV and the appropriate IC DNA (200 copies of EBV-IC or 50 copies of VZV-IC). Both M and Si extractions were performed in five replicates for each sample. After correcting for the background signal level, the signals obtained by M extraction were between 7.6- and 14.2-fold lower for both EBV wild-type and IC DNAs compared to the signals obtained after Si

extraction, as can be seen in Table 3.3. For VZV, similar results were obtained with signals between 1.9- and 7.9-fold lower after M extraction (Table 3.3).

TABLE 3.3. Summarized data for EBV and VZV LPC and HPC control DNAs, CMV strain AD 169, and EBV and CMV clinical samples after Si extraction and M extraction from EDTA-treated whole blood.

		Average signal ratio Si-extraction/M-extraction[c]	
Specimen type[a]	Quantity[b]	Virus	IC
EBV			
LPC	3	9.0 (2.8 to 20.3; n = 4)	14.2 (4.4 to 37.7; n = 5)
HPC	3.7	7.6 (4.1 to 13.1; n = 5)	7.7 (3.9 to 10.2; n = 5)
VZV			
LPC	3	7.9 (3.5 to 12.8; n = 4)	5.1 (2.4 to 8.2; n = 5)
HPC	3.7	2.1 (0.7 to 3.3; n = 4)	1.9 (0.2 to 3.5; n = 3)
CMV			
strain AD169	1.5	NA[d]	2.2 (0.1 to 7.2; n = 8)
	2.5	1.8 (0.8 to 2.9; n = 7)	3.5 (2.5 to 7.3; n = 8)
	3.5	1.1 (0.7 to 1.6; n = 8)	2.2 (1.1 to 3.2; n = 8)
	4.5	1.1 (0.9 to 1.4; n = 8)	3.2 (2.3 to 4.2; n = 8)
EBV clinical	unknown	9.4 (1.0 to 33.5; n = 16)	7.2 (0.9 to 26.9; n = 23)
CMV clinical	unknown	4.8 (2.2 to 6.2; n = 3)	3.6 (1.7 to 8.8; n = 16)

[a] LPC, 1,000 copies/ml, 50 copies/extraction; HPC, 5,000 copies/ml, 250 copies/extraction.
[b] Log_{10} number of viral DNA copies/ml.
[c] Ratio calculated from background-corrected luminosity signals. Values in parentheses reflect the range of the measurements and the number of samples.
[d] NA, not applicable.

Extraction and PCR of human CMV strain AD 169 from whole blood.

In order to assess the influence of the impaired DNA recovery of M extraction with complete virus, another reconstruction experiment was performed with human CMV strain AD 169. A PCR-negative whole blood sample was spiked with 10-fold serial dilutions of CMV ranging from 1.5 to 4.5 log_{10} copies/ml, and each dilution was extracted in eight replicates by both procedures in the presence of 140 copies of CMV-IC DNA. Figure 3.1A shows the quantitative results of both procedures after CMV PCR. At 1.5 log_{10} copies/ml, all eight replicates isolated by M extraction were PCR negative, whereas only 50% (4/8) of the replicates from Si extraction were negative. All replicates at subsequent dilutions for both extraction procedures were positive, except 1/8 replicates (13%) for M extraction at 2.5 log_{10} copies/ml. Also the luminosity signals were lower after M extraction compared to Si extraction, especially for CMV-IC. For CMV and for CMV-IC,

the signals were between 1.1- to 1.8-fold and 2.2- to 3.5-fold lower, respectively, as can be seen in Table 3.3. The correlation between quantitative results was in excellent accordance for both extraction procedures, as illustrated in Figure 3.1B, with r = 0.9807, a slope of 1.1033, and an intercept of – 0.0219.

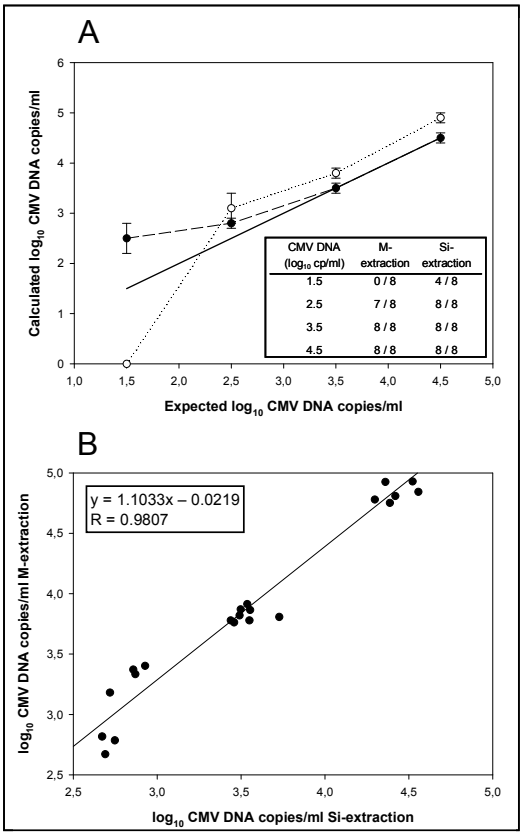


Figure 3.1. Extraction of CMV AD 169 from whole blood.

(A) Quantitative results obtained for human CMV strain AD 169 by CMV PCR after extraction from EDTA-antico-agulated whole blood by Si extraction (closed symbols) and M extraction (open symbols). Error bars indicate the standard deviation of the log-transformed quantitative results. The table shows the number of positives for the eight replicates tested with the different dilutions of CMV.
(B) Correlation between the quantitative results obtained by Si extraction and M extraction.

At 2.5, 3.5, and 4.5 $\log_{10}$ CMV copies/ml, the mean difference from the expected value, 0.1 (range, –0.2 to 0.4) $\log_{10}$ copies/ml, was well within the accepted range (± 0.5 $\log_{10}$ copies/ml) for Si extraction. For M extraction, the mean difference was greater, with approximately 0.4 (range, 0.2 to 0.9) $\log_{10}$ copies/ml, but still acceptable. At the lowest input of 1.5 $\log_{10}$ copies/ml, Si extraction overestimated the expected CMV DNA load by 1.0 $\log_{10}$ copies/ml, which is significantly outside the acceptable range of ± 0.5 $\log_{10}$

copies/ml. This overestimation is most likely caused by the Poisson distribution of CMV near the limit of detection of the assay, resulting in inconsistent positive and negative reactions and therefore overestimation of the CMV DNA load in the portion of positive reaction mixtures.

Effect of impaired DNA recovery in clinical whole blood samples.

To assess the effect of impaired DNA recovery of M extraction in clinical samples, whole blood samples were analyzed by PCR in parallel for EBV (n = 23) and CMV (n = 16) after M extraction and Si extraction in the presence of 200 EBV-IC or 70 CMV-IC molecules. PCR and quantitation results are shown in Figure 3.2. Figure 3.2A shows that for 17 (Fig. 3.2A, samples 1, 3 to 8, 10 to 15, and 18 to 21) of the 23 samples (74%), both EBV and EBV-IC signals were lower after M extraction compared to Si extraction. Another five samples (Fig. 3.2A, samples 2, 9, 16, 17, and 23) (22%) had comparable EBV signals but lower EBV-IC signals. Only one sample (Fig. 3.2A, sample 22) (4%) showed comparable results in both EBV and EBV-IC signals with both extraction methods. For CMV, 8 of the 16 samples (Fig. 3.2B, samples 1, 5, and 11 to 16) (50%) showed lower signals for both CMV and CMV-IC after M extraction (Fig. 3.2B). Another seven samples (Fig. 3.2B, samples 2, 4, and 6 to 10) (44%) showed comparable signals for CMV but lower signals for CMV-IC. One sample (Fig. 3.2B, sample 3) (6%) showed a comparable CMV-IC signal but yielded a lower CMV signal after M extraction. In addition, M extraction yielded four and six false-negative results for EBV- and CMV-positive specimens with viral DNA loads ranging from 2.4 to 3.4 $\log_{10}$ copies/ml, respectively (Fig. 3.2C, samples 6, 11, 13, and 15, and D, samples 5, 8, and 11 to 14). The EBV and CMV status of these samples had been confirmed by a reference PCR that was conducted a day earlier (data not shown). One weakly CMV-positive whole blood sample with a viral DNA load of approximately 2.0 $\log_{10}$ copies/ml, as judged by the reference PCR, yielded negative results with both methods (Fig. 3.2D, sample 4). This specimen's viral DNA load was near the limit of detection for the assay (1.9 $\log_{10}$ copies/ml). For the EBV- and CMV-positive specimens, the luminosity signals obtained after M extraction were, on average, 9.4-fold (range, 1.0-fold to 33.5-fold) and 4.8-fold (range, 2.2-fold to 6.2-fold) lower compared to the signals obtained after Si extraction (Table 3.3). For EBV-IC and CMV-IC, respectively, the luminosity signals were 7.2-fold (range, 0.9-fold to 26.9-

fold) and 3.6-fold (range, 1.7-fold to 8.8-fold) lower after M extraction compared to Si extraction (Table 3.3). Although M extraction resulted in lower luminosity signals, this did not seem to significantly influence the viral load calculation for EBV, resulting in a high correlation between the quantitation results for EBV by both methods. This is shown in Figure 3.2E with an overall correlation of 0.8116, a slope of 1.1958, and an intercept of – 0.7087. The agreement between viral load values for the specimens processed by both methods was measured by determining the differences in log10 DNA loads for each sample (i.e., the loads obtained by Si extraction minus the loads obtained by M extraction) and calculating the mean and standard deviation of the differences. The values were in good agreement, with a mean $\log_{10}$ difference of 0.01 and a standard deviation of 0.463. The 95% confidence interval (± 2 standard deviations) for the mean difference was + 0.94 and – 0.92 $\log_{10}$. A plot of the difference versus the average $\log_{10}$ viral load for each sample is shown in Figure 3.2F. Furthermore, for the majority (75%) of the EBV-positive specimens, the difference between the two methods was within the ± 0.5 $\log_{10}$ copies/ml range (Fig. 3.2F). For CMV, only three samples were available for comparison of the quantitation results between the two extraction procedures. These three samples (Fig. 3.2D, samples 1, 3, and 15) showed differences of + 0.60, + 0.75, and – 0.43 $\log_{10}$ copies/ml, respectively.

DISCUSSION.

We have evaluated the performance of M extraction as an automated NA extraction system, aiming to replace the manual Si extraction procedure that we currently use for several applications. DNA recovery experiments with *Hin*dIII-digested phage lambda DNA and *Hae*III-digested ΦX174 DNA showed substantially lower DNA yields for M extraction compared to Si extraction, with, on average, 6.6-fold lower recoveries for M extraction. A major problem identified with M extraction was the retrieval of DNA from the magnetic glass particles, where up to 60% of the DNA could not be retrieved. PCR signals of spiked PCR control DNAs for EBV and VZV were also between 1.9- and 14.2-fold lower after M extraction compared to Si extraction, also suggesting impaired DNA recovery. This resulted in a 5 - 10-

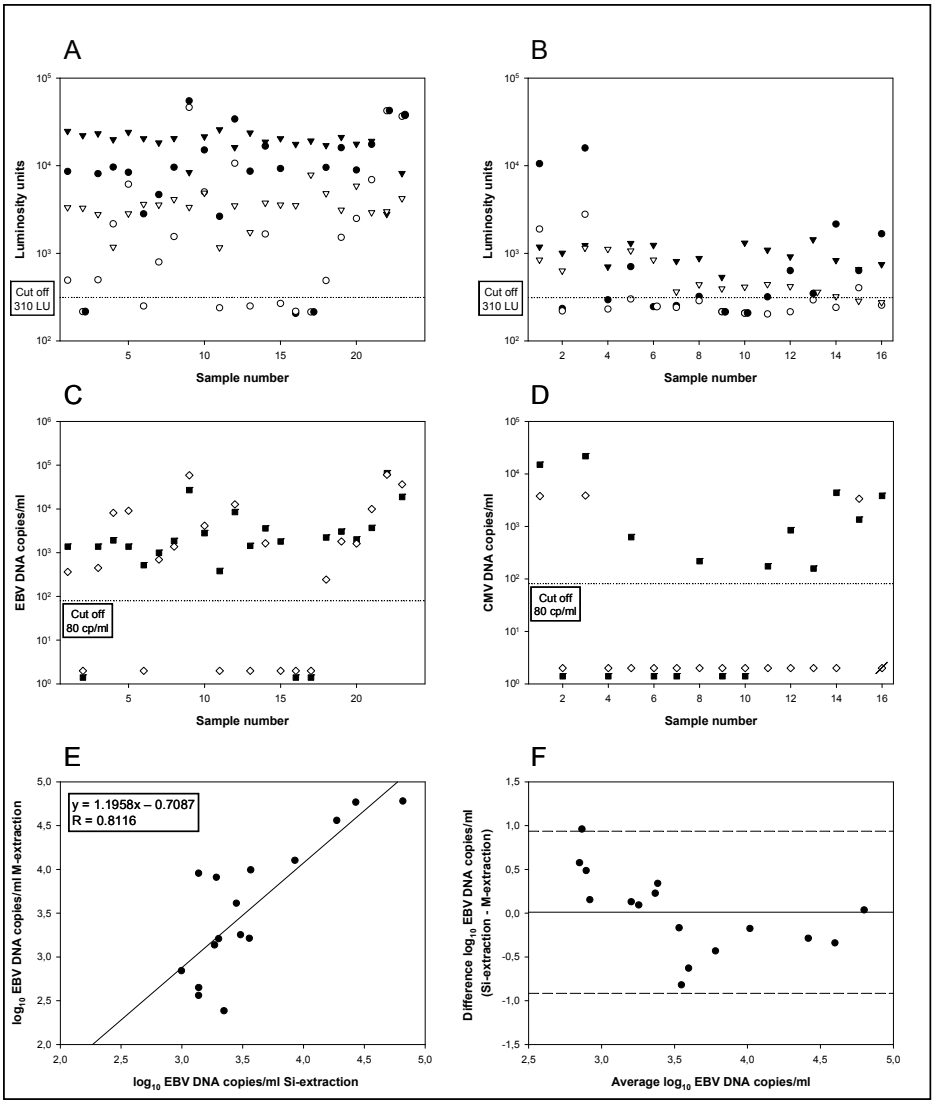


Figure 3.2. PCR signals in luminosity units obtained for wild-type (circles) and IC (triangles) DNAs after processing with Si extraction (closed symbols) and M extraction (open symbols) for EBV

(A) and CMV (B). The cutoff value of 310 luminosity units (LU) is indicated by the horizontal dotted line. Quantitative results for EBV (C) and CMV (D) after Si extraction (closed squares) or M extraction (open diamonds). A diagonal rod through the diamond symbol shows one invalid result for M extraction. The correlation (E) and mean difference (F) between quantitative results for EBV after Si extraction and M extraction are also shown. The solid line (F) represents the mean difference (0.01 $\log_{10}$ copies of EBV DNA/ml) between the quantitative results for Si extraction and Mextraction. The dashed lines (F) represent the upper and lower 95% agreement lines (mean 2 standard deviations [0.94 and -0.92 $\log_{10}$ copies of EBV DNA/ml]).

fold reduction in PCR sensitivity when spiked CMV strain AD 169 was extracted from whole blood by M extraction. Furthermore, it also resulted in false-negative results with 10 (4 EBV, 6 CMV) of the 39 clinical whole blood specimens tested. For manual Si extraction, high yields were observed with minimal loss of DNA during the procedure, which is in accordance with previous studies [2, 3, 5, 6, 8].Although the experiments presented here do not exclude the contribution of PCR inhibition to the lower signals obtained after M extraction, no evidence for PCR inhibition was found in several experiments where eluates extracted from whole blood and TE buffer with M extraction were spiked with EBV-IC DNA and analyzed by EBV PCR. The signals obtained with the spiked eluates were in excellent accordance with the signals obtained from Si-extracted samples with the corresponding amount of EBV-IC DNA, showing that PCR inhibition did not contribute to the lower signals after M extraction (data not shown).

Several reports have described less efficient recovery of small RNA transcripts used as IC or quantification standard (QS) samples for (COBAS) AMPLICOR HCV 2.0 and HIV-1 MONITOR v1.5 tests [10, 11, 14, 20]. Therefore, the amount of these transcripts per specimen needed to be increased when applying M extraction to allow reliable quantification. Our data suggest that relatively small DNA molecules, like the ICs (3- to 4-kb plasmids) used in this study are not susceptible to the lower extraction efficiency that has been described for the small RNA transcripts. If the ICs used in this study had been extracted with lower efficiency compared to the larger viral chromosomal DNAs (125 to 230 kb) by M extraction, overestimation of the viral DNA loads would have occurred. However, quantification did not seem to be significantly influenced by the lower recovery of DNA by M extraction, as was shown by high correlations between quantitative results from the two methods. Furthermore, the differences in quantitative results for the majority of specimens were within the acceptable range of ± 0.5 $\log_{10}$ copies/ml. This is important when monitoring disease progression in solid organ and stem cell transplant recipients, where both primary and reactivated CMV infections need to be detected and quantified quickly and reliably in order to start pre-emptive therapy [15, 18]. For managing EBV-associated posttransplantation lymphoproliferative disease, it has also been suggested that EBV load

measurements can contribute to pre-emptive therapy and therefore reliable quantification is necessary [17].

We consider that the problems we have identified in our study are structural for M extraction and not the result of a malfunctioning instrument or one specific lot of M extraction reagents. This conclusion is based on the observation that we obtained results similar to those presented here with multiple lots of M extraction reagents on two different MagNA Pure LC instruments at two different locations over a 4-year time period (data not shown). Furthermore, the impaired recovery of DNA seems to be a specific M extraction problem, since we did not observe substantially lower recoveries in preliminary experiments with other DNA extraction kits available for the MagNA Pure LC instrument (DNA I Isolation Kit, DNA III Isolation Kit Bacteria Fungi, Microbiology Kit MGRADE). However, the use of DNA- and RNA-specific isolation kits is a disadvantage when both DNA- and RNA-targeted assays have to be performed and limited material is available.

The clinical impact of the lower efficiency of M extraction does not seem to be widespread, as is shown by other investigators who were satisfied with the performance of M extraction [10, 11, 13, 14, 15, 16, 18, 20, 21]. However, there are some applications were the reduced recovery might have clinical implications. For instance, molecular diagnosis of herpes simplex virus (HSV) encephalitis, with its severe morbidity and high mortality, requires optimal methods for demonstrating the presence of HSV DNA near the lower range of the observed concentrations (200 to 4 x 10^7 copies/ml) of HSV DNA in primary CSF specimens from HSV encephalitis patients [23]. High sensitivity is also required for detecting bacterial meningitis, where bacterial counts of less than 1,000 CFU/ml have been observed in 15% of the cases in children [17]. Furthermore, Schuurman *et al.* have also shown that a very sensitive assay (100 to 200 CFU/ml) utilizing Si extraction for sample preparation did not detect 14% of the culture-proven cases of bacterial meningitis due to the small numbers of bacteria present in these samples [24]. Finally, bacterial loads have been reported as low as 1 to 10 CFU/ml in bacteremia [12] and therefore requiring maximal sensitivity when molecular detection methods are used.

To what extent the reduced recovery of M extraction may prove detrimental to an application is largely dependent upon the extraction

method that it needs to replace. For instance, the manual extraction method provided with the (COBAS) AMPLICOR HCV, and HIV tests can readily be replaced with M extraction [10, 11, 14, 20], due to the fact that the NA are concentrated by M extraction compared to the manual procedure. This concentrating of the NA counteracts the effects of the reduced recovery and results in a sensitivity similar to the manual extraction method. However, compared with Si extraction, this advantage is largely redundant because the sample input/output ratios are identical for the two methods.

In conclusion, M extraction seems to have a structurally impaired DNA recovery, resulting in a loss of sensitivity in molecular diagnostic testing. Whether this reduced recovery is detrimental to the application in which M extraction is used is largely dependent on the method that is intended to be replaced by M extraction and the clinical application. We therefore recommend that the clinical consequences of the reduced recovery, especially when maximal sensitivity is required, should be considered before implementing M extraction in the routine work flow.

REFERENCES.

1. **Beld, M., M. R. Habibuw, S. P. H. Rebers, R. Boom, and H. W. Reesink.** 2000. Evaluation of automated RNA extraction technology and a qualitative HCV assay for sensitivity and detection of HCV RNA in pool-screening systems. Transfusion **40:**575-579.
2. **Boom, R., C. Sol, M. Beld, J. Weel, J. Goudsmit, and P. Wertheim-van Dillen.** 1999. Improved silica-guanidiniumthiocyanate DNA isolation procedure based on selective binding of bovine alpha-casein to silica particles. J. Clin. Microbiol. **37:**615-619.
3. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495-503.
4. **Boom, R., C. J. A. Sol, T. Schuurman, A. van Breda, J. F. L. Weel, M. Beld, I. J. M. ten Berge, P. M. E. Wertheim-van Dillen, and M. D. de Jong.** 2002. Human cytomegalovirus DNA in plasma and serum specimens of renal transplant recipients is highly fragmented. J. Clin. Microbiol. **40:**4105-4113.
5. **Boom, R., C. Sol, J. Weel, Y. Gerrits, M de Boer, and P. Wertheim-van Dillen.** 1999. A highly sensitive assay for the detection and quantitation of human cytomegalovirus DNA in serum and plasma by PCR and electrochemiluminescence. J. Clin. Microbiol. **37:**1489-1497.
6. **Boom, R., C. Sol, J. Weel, K. Lettinga, Y. Gerrits, A. van Breda, and P. Wertheim-Van Dillen.** 2000. Detection and quantitation of human cytomegalovirus DNA in faeces. J. Virol. Methods **84:**1-14.
7. **Chen, W., G. Martinez, and A. Mulchandani.** 2000. Molecular beacons: a real-time polymerase chain reaction assay for detecting *Salmonella*. Anal. Biochem. **280:**166-172.
8. **de Jong, M. D., J. F. Weel, T. Schuurman, P. M. Wertheim-van Dillen, and R. Boom.** 2000. Quantitation of varicella-zoster virus DNA in whole blood, plasma, and serum by PCR and electrochemiluminescence. J. Clin. Microbiol. **38:**2568-2573.
9. **Dhar, A. K., M. M. Roux, and K. R. Klimpel.** 2001. Detection and quantification of infectious hypodermal and hematopoietic necrosis virus and white spot virus in shrimp using real-time quantitative PCR and SYBR Green chemistry. J. Clin. Microbiol. **39:**2835-2845.
10. **Fiebelkorn, K. R., B. G. Lee, C. E. Hill, A. M. Caliendo, and F. S. Nolte.** 2002. Clinical evaluation of an automated nucleic isolation system. Clin. Chem. **48:**1613-1615.
11. **Germer, J. J., M. M. Lins, M. E. Jensen, W. S. Harmsen, D. M. Ilstrup, P. S. Mitchell, F. R. Cockerill III, and R. Patel.** 2003. Evaluation of the MagNA Pure LC instrument for extraction of hepatitis C virus RNA for the COBAS AMPLICOR hepatitis C virus test, version 2.0. J. Clin. Microbiol. **41:**3503-3508.
12. **Henry, N. L., C. A. McLimans, A. J. Wright, R. L. Thompson, W. R. Wilson, and J. A. Washington II.** 1983. Microbiological and clinical evaluation of the isolator lysis-centrifugation blood culture tube. J. Clin. Microbiol. **17:**864-869.
13. **Kalpoe, J. S., A. C. M. Kroes, M. D. de Jong, J. Schinkel, C. S. de Brouwer, M. F. C. Beersma, and E. C. J. Claas.** 2004. Validation of clinical application of cytomegalovirus plasma DNA load measurement and definition of treatment criteria by analysis of correlation to antigen detection. J. Clin. Microbiol. **42:**1498-1504.
14. **Kessler, H. H., A. M. K. Clarici, E. Stelzl, G. Mühlbauer, E. Daghofer, B. I. Santner, E. Marth, and R. E. Stauber.** 2002. Fully automated detection of hepatitis C virus RNA in serum and whole-blood samples. Clin. Diagn. Lab. Immunol. **9:**1385-1388.
15. **Kessler, H. H., G. Mühlbauer, E. Stelzl, E. Daghofer, B. I. Santner, and E. Marth.** 2001. Fully automated nucleic acid extraction: MagNA Pure LC. Clin. Chem. **47:**1124-1126.

16. **Knepp, J. H., M. A. Geahr, M. S. Forman, and A. Valsmakis.** 2003. Comparison of automated and manual nucleic acid extraction methods for detection of enterovirus RNA. J. Clin. Microbiol. **41:**3532-3536.
17. **La Scolea, L. J., Jr., and D. Dryja.** 1984. Quantitation of bacteria in cerebrospinal fluid and blood of children with meningitis and its diagnostic significance. J. Clin. Microbiol. **19:**187-190.
18. **Leb, V., M. Stöcher, E. Valentine-Thon, G. Hölzl, H. Kessler, H. Stekel, and J. Berg.** 2004. Fully automated internally controlled quantification of hepatitis B virus DNA by real-time PCR by use of the MagNA Pure LC and LightCycler instruments. J. Clin. Microbiol. **42:**585-590.
19. **Lee, S. C., A. Antony, N. Lee, J. Leibow, J. Q. Yang, S. Soviero, K. Guteknust, and M. Rosenstraus.** 2000. Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. J. Clin. Microbiol. **38:**4171-4179.
20. **Lee, B. G., K. R. Fiebelkorn, A. M. Caliendo, and F. S. Nolte.** 2003. Development and verification of an automated sample processing protocol for quantification of human immunodeficiency virus type 1 RNA in plasma. J. Clin. Microbiol. **41:**2062-2067.
21. **Mohammadi, T., H. W. Reesink, C.M.J.E. Vandenbroucke-Grauls, and P. H. M. Savelkoul.** 2003. Optimization of real-time PCR assay for rapid and sensitive detection of eubacterial 16S ribosomal DNA in platelet concentrates. J. Clin. Microbiol. **41:**4796-4798.
22. **Patel, S., M. Zuckerman, and M. Smith.** 2003. Real-time quantitative PCR of Epstein-Barr virus BZLF1 DNA using the LightCycler. J. Virol. Methods **109:**227-233.
23. **Schloss, L., A. M. van Loon, P. Cinque, G. Cleator, J.-M. Echevarria, K. l. Falk, P. Klapper, J. Schirm, B. F. Vestergaard, H. Niesters, T. Popow-Kraupp, W. Quint, and A. Linde.** 2003. An international external quality assessment of nucleic acid amplification of herpes simplex virus. J. Clin. Virol. **28:**175-185.
24. **Schuurman, T., R. F. de Boer, A. M. D. Kooistra-Smid, and A. A. van Zwet.** 2004. Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for the diagnosis of bacterial meningitis in a clinical setting. J. Clin. Microbiol. **42:**734-740.

CHAPTER 4

Comparative Evaluation of In-House Manual and Commercial Semi-Automated and Automated DNA Extraction Platforms in the Sample Preparation of Human Stool Specimens for a S*almonella enterica* 5'-Nuclease Assay

Tim Schuurman
Richard de Boer
Rachèl Patty
Mirjam Kooistra-Smid
Anton van Zwet

Laboratory for Infectious Diseases, Department of Research & Development, Groningen
The Netherlands

Journal of Microbiological Methods. 2007. **71:**238-245

ABSTRACT.

In the present study, three methods (NucliSENS miniMAG [bioMérieux], MagNA Pure DNA Isolation Kit III Bacteria/Fungi [Roche], and a silica-guanidiniumthiocyanate {Si-GuSCN-F} procedure) for extracting DNA from stool specimens were compared with regard to analytical performance (relative DNA recovery and down stream real-time PCR amplification of *Salmonella enterica* DNA), stability of the extracted DNA, hands-on time (HOT), total processing time (TPT), and costs. The Si-GuSCN-F procedure showed the highest analytical performance (relative recovery of 99%, *S. enterica* real-time PCR sensitivity of 91%) at the lowest associated costs per extraction (€ 4.28). However, this method did require the longest HOT (144 min) and subsequent TPT (176 min) when processing 24 extractions. Both miniMAG and MagNA Pure extraction showed similar performances at first (relative recoveries of 57% and 52%, *S. enterica* real-time PCR sensitivity of 85%). However, when difference in the observed Ct-values after real-time PCR were taken into account, MagNA Pure resulted in a significant increase in Ct-value compared to both miniMAG and Si-GuSCN-F (with on average + 1.26 and + 1.43 cycles). With regard to inhibition all methods showed relatively low inhibition rates (< 4%), with miniMAG providing the lowest rate (0.7%). Extracted DNA was stable for at least 1 year for all methods. HOT was lowest for MagNA Pure (60 min) and TPT was shortest for miniMAG (121 min). Costs, finally, were € 4.28 for Si-GuSCN, € 6.69 for MagNA Pure and € 9.57 for miniMAG.

INTRODUCTION.

In recent years, molecular diagnostics have become an integral part of the routine workflow in clinical microbiology laboratories. With the introduction of molecular detection of pathogens, the numbers of specimens processed also increased rapidly. This makes automation of the nucleic acid (NA) extraction procedure attractive. Several (semi-)automated NA extraction systems have recently become available to the clinical microbiology laboratory. Examples of such systems are the BioRobot series (EZ1/M48/M96/MDx/9604 [QIAGEN]), MagNA Pure series (Compact/LC [Roche]), m1000 (Abbott), and the NucliSENS series (miniMAG/easyMAG/Extractor [bioMérieux]). Nearly all extraction chemistries used by these systems are based on the silica-guanidinium thiocyanate (Si-GuSCN) procedure developed by Boom *et al.* [5], also referred to as the Boom chemistry. This chemistry has been acknowledged for its overall performance and especially its efficient removal of inhibitory substances [6, 7, 21]. Therefore, it is regarded by many in the field of molecular diagnostics as the “gold standard” in NA extraction technology.

The choice of a NA extraction method is dependent on several factors, including assay targets (RNA/DNA-based), specimen type, sample throughput, laboratory workflow, costs, and, foremost, the performance of the extraction system. Recent studies have shown that the performance of the automated systems is satisfactory [8, 10, 11, 14, 15, 17, 20, 23]. However, the number of specimen types studied in many of these reports remains limited and extrapolation to other specimen types is difficult and should at least be done with care [23]. It is, therefore, still necessary to conduct comparative performance studies for sample types that have not been thoroughly evaluated.

Fecal specimens are probably the most difficult specimen type processed by the clinical microbiology laboratory. This originates from the complex nature of the fecal sample matrix, which is renowned for the presence of very potent inhibitors of NA amplification in the form of complex polysaccharides, bilirubin and bile salts [9, 18, 24]. These inhibitory effects can be minimized by the addition of amplification facilitators, such as bovine serum albumin (BSA) or T4 gene 32 protein [2, 16] to the PCR, the use of thermostable DNA polymerases that are more resistant to PCR inhibition [1],

or efficient purification of NA from the stools. The Si-GuSCN extraction chemistry has proven its applicability with stools [3, 4, 7], but the impaired DNA recovery in 9% of the stools as a result of competition for binding to the silica matrix between the NA and (an) unknown substance(s) present in some fecal sample matrices remains problematic. Therefore, the standard protocol had to be modified for use with stools in order to reach a satisfactory performance. These issues highlight the importance of performing a comparative performance study when a NA extraction system for processing stool specimens needs to be selected.

In the present study we assessed the performance of 3 Si-GuSCN-based DNA extraction procedures with human stool specimens as the sample matrix. The 3 extraction procedures studied were an in-house manual Si-GuSCN procedure optimized for stools (Si-GuSCN-F), the semi-automated NucliSENS miniMAG (miniMAG), and the MagNA Pure LC DNA Isolation Kit III Bacteria/Fungi (MPLC DNA III). Performance was studied in recovery experiments and by a real-time PCR assay for detecting *Salmonella enterica* [13]. Furthermore, the stability of the extracted DNA, the hands-on time (HOT), the total processing time (TPT), and costs were assessed.

MATERIALS AND METHODS.

Fecal specimens.

Sixty-nine stools positive and 67 stools negative for *S. enterica* were selected at random from archived specimens stored at −70°C. Identification of these stools was carried out previously by routine culturing methods using selenite enrichment, selective culturing on *Salmonella*/*Shigella* agar and hektoen enteric agar, biochemical identification (urea, lysine decarboxylase, tryptone water, triple sugar iron agar, and *o*-nitrophenyl-β-D-galactopyranoside), and group-specific *Salmonella* (long/short polyvalent, A, B, b, C1, C2, D, d, Vi, E, F, and G) serological identification [19].

Efforts to minimize variation introduced by other factors than the extraction procedures or the specimen.

To limit the effects of methodological inconsistencies, all extractions were performed in parallel on the same fecal suspension. Input/output volumes were identical for all 3 methods. Storage conditions for DNA eluates

were 2–8°C for 48 hours and subsequently at −20°C. All specimens were processed in parallel in batches of 22 fecal specimens and 2 phosphate buffered saline (PBS) specimens. Nucleic acid extracts from a single specimen were analyzed in the same real-time PCR run and on the same agarose gel to minimize the variation introduced by the analysis systems.

DNA extraction procedures.

i) Manual Si-GuSCN-F procedure.

Manual Si-GuSCN-F was performed as follows. Briefly, 100 µl of fecal suspension (33–50% wt/vol) was added to a mixture of 50 µl silica particles (SC-F, prepared as described by Beld et al. [3]) (Silica, SiO_2, S-5631, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands), 900 µl of lysis buffer L6, and 2 µg of *Hin*dIII-digested phage lambda DNA (λ-*Hin*dIII DNA) (Invitrogen, Paisley, UK), mixed and left at ambient temperature for 10 minutes. After centrifugation, the silica-NA complexes were washed as described previously [5]. The nucleic acids were eluted in 100 µl of 1 x TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]).

ii) Semi-automated NucliSENS miniMAG.

Semi-automated NucliSENS miniMAG (bioMérieux, Boxtel, The Netherlands) extraction was carried out with the NucliSENS Magnetic Extraction Reagents (bioMérieux) on the NucliSENS miniMAG instrument (bioMérieux) according to the manufacturer's instructions. Briefly, 100 µl of fecal suspension was added to a lysis tube containing 2 ml of lysis buffer. The mixture was incubated at room temperature for 10 min., after which 2 µg of λ-*Hin*dIII DNA (Invitrogen) and 50 µl of magnetic silica particles were added. The mixture was incubated for 10 min. at room temperature, after which the magnetic silica-NA complexes were washed according to the manufacturer's instructions. Elution was in 100 µl of elution buffer.

iii) MagNA Pure LC DNA III Bacteria/Fungi Kit.

Automated DNA extraction on the MagNA Pure LC instrument (Roche Diagnostics Nederland B.V., Almere, The Netherlands) was performed with the MagNA Pure LC DNA Isolation Kit III Bacteria/Fungi (Roche Diagnostics), according to the manufacturer's instructions. Briefly, 100 µl of fecal suspension was added to 130 µl of Bacteria Lysis Buffer and 20 µl of Proteinase K solution, and the mixture was incubated at 65°C for 10 min. Subsequently, the mixture was incubated at 95°C for 10 min to kill any non-

lysed microorganisms. After a short spin, the mixture was transferred to the Sample Cartridge and 2 µg of λ-*Hin*dIII DNA (Invitrogen) was added. Further processing on the MagNA Pure LC instrument was performed with the protocol “DNA III Bacteria”. Elution was in 100 µl of elution buffer.

Agarose gel electrophoresis.

DNA was electrophoresed through 1% agarose gels (Agarose MP, Roche Diagnostics Nederland BV) containing 1 µg ethidium bromide per ml of 0.5 × Tris-borate-EDTA (TBE) buffer. The Gel Doc 1000 system (Bio-Rad, Veenendaal, The Netherlands) was used for image capturing under UV-illumination and the graphic files were exported as 8-bit TIFF images. Relative DNA recoveries were calculated from the peak surface areas using Scion Image Release Beta 4.0.2 (Scion Corporation, Frederick, Maryland, USA). For each specimen, the extraction method with the largest peak surface area was set at a relative recovery of 100%. A relative recovery ≤ 33% was interpreted as a low recovery.

Real-time PCR for *S. enterica*.

Real-Time amplification was carried out on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) using the TaqMan Universal PCR Master Mix (Applied Biosystems). For *S. enterica*, the assay targeting the *invA* gene described by Hoorfar *et al.* [13] was used with minor modifications. Briefly, primers and probe were purchased from Applied biosystems, and the reaction mixture (25 µl) consisted of 1 × TaqMan Universal PCR Mastermix (Applied Biosystems), 2.5 µg bovine serum albumin (Roche Diagnostics), 5 µl of template DNA, and the primers at 300 nM each, and the FAM-TAMRA labeled TaqMan probe at 400 nM. Reactions were run under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 50°C for 15 s, and 60°C for 1 min. PCR inhibition was monitored by running a duplicate reaction with a spike of 15 pg *S. enterica* serovar Virchow DNA, resulting in a Ct of approximately 29–30 with non-inhibited fecal specimens. Amplification data were analyzed with the Sequence Detector Software v1.9.1 (Applied Biosystems).

Costs calculation.

To calculate the costs per extraction, full extraction runs of 24 specimens were assumed. The list prices of chemicals and consumables

supplied by the manufacturer were used, which results in a maximum price per extraction. All consumables not supplied by the manufacturer were also included, as were the costs of the technician performing the extractions. Not included were the costs of generally available laboratory equipment and instruments. The mean costs per hour for a technician in our laboratory in The Netherlands at the time of the study were € 25.

RESULTS.

Relative DNA recovery in stool and PBS.

The DNA recoveries of all 3 methods were assessed using co-extraction of *Hin*dIII-digested phage lambda DNA as a model system. Recoveries were assigned relative to the method with the highest band intensity (100%).

Table 4.1 shows the overall results for the relative recovery from both stool specimens and PBS. For stool specimens, Si-GuSCN-F showed the best relative recovery performance, based both on the number of specimens with the highest recovery (n = 126), as well as on the average relative recovery (99%). Both miniMAG and MPLC DNA III performed nearly identical, although miniMAG showed slightly higher recoveries as compared to MPLC DNA III (average relative recovery 57% vs. 52%).

With PBS, Si-GuSCN-F again showed the best relative recovery performance (97%), shortly followed by miniMAG (84%), whereas MPLC DNA III showed the lowest efficiency (64%).

TABLE 4.1. Relative recovery of DNA from feces and PBS[a].

	Extraction method		
Specimen type and relative recovery score	Si-GuSCN-F	miniMAG	MPLC DNA III
Stool			
100%	126	6	4
> 67-99%	9	40	37
> 33-67%	1	68	62
0-33% (Low)	0	22	33
Average relative recovery	99 ± 4%	57 ± 24%	52 ± 25%
PBS			
100%	11	1	0
> 67-99%	0	11	5
> 33-67%	1	0	7
0-33% (Low)	0	0	0
Average relative recovery	97 ± 10%	84 ± 9%	64 ± 14%

[a] Presented are the numbers of specimens with in a relative recovery range and the average relative recovery for all specimens ± the standard deviation.

Detection of *S. enterica* DNA by real-time amplification.

Table 4.2 shows the results obtained with the *S. enterica* real-time PCR on the DNA extracted from the panel of stools. Of the 69 stool specimens positive for *S. enterica* by culture, 63 were detected after Si-GuSCN-F extraction, whereas miniMAG and MPLC DNA III both detected *S. enterica* DNA in 59 of the culture positive stools. None of the culture negative stools resulted in a positive signal in the real-time PCR after Si-GuSCN-F and MPLC DNA III extraction. However, a single culture negative stool produced a weak positive real-time PCR result after miniMAG processing.

TABLE 4.2. *S. enterica* real-time PCR results.

		S. enterica culture result						
		positive			negative			Total
Extraction method	PCR result[a]	Recovery[b]			Recovery[b]			number of specimens
		Normal	Low	*Total*	Normal	Low	*Total*	
Si-GuSCN-F	Pos	63[c]	0	**63**	0	0	**0**	63
	Neg	6	0	**6**	63	0	**63**	69
	Unresolved	0	0	**0**	4[d]	0	**4**	4
	Total	69	0	**69**	67	0	**67**	136
miniMAG	Pos	49	10	**59**	1	0	**1**	60
	Neg	6	4	**10**	58	7	**65**	75
	Unresolved	0	0	**0**	1[e]	0	**1**	1
	Total	55	14	**69**	60	7	**67**	136
MPLC DNA III	Pos	47	12	**59**	0	0	**0**	59
	Neg	5	3	**8**	50	15	**65**	73
	Unresolved	2[f]	0	**2**	1[g]	1[g]	**2**	4
	Total	54	15	**69**	51	16	**67**	136

[a] Pos, positive; Neg, negative; Unresolved, unresolved due to PCR inhibition.

[b] Low DNA recovery is defined as relative recovery ≤ 33%.

[c] One specimen showed a positive PCR result for *S. enterica* although it was severely inhibited.

[d] Three specimens were severely inhibited, 1 specimen was mildly inhibited.

[e] This specimen showed mild PCR inhibition.

[f] One specimen was mildly inhibited, whereas the other specimen was severely inhibited.

[g] Specimen with low recovery was mildly inhibited, whereas the specimen with normal recovery was severely inhibited.

A real-time PCR was considered to be inhibited when the Ct-value of the spiked reaction ($Ct_{[spike]}$) was outside the range of the mean Ct-value + 2 standard deviations (determined for the culture negative specimens that were clearly not inhibited) and the cut-off $Ct_{[spike]}$ value was determined to be 33.51 cycles. Mild inhibition of the real-time PCR was observed with DNA extracted by miniMAG in a single culture negative specimen ($Ct_{[spike]}$ of 33.80), making this PCR result unresolved. For Si-GuSCN-F, 1 specimen showed mild inhibition ($Ct_{[spike]}$ of 34.38), whereas severe inhibition was detected for 4 stools ($Ct_{[spike]}$'s of 39.14, 38.21, > 45.00, and 39.35).

Therefore 4 of the 5 inhibited stools (1 mildly and 3 severely inhibited) were considered unresolved. The last stool did produce a positive real-time PCR signal in the unspiked reaction (Ct of 44.50), despite the detection of severe PCR inhibition in the spiked reaction (Ct $_{[spike]}$ of 39.14). After MPLC DNA III extraction, 4 stools were inhibitory to real-time PCR. Two stools showed mild inhibition (Ct $_{[spike]}$'s of 34.80 and 33.60) and the other 2 stools severely inhibited the real-time PCR (Ct $_{[spike]}$'s of > 45.00 and 39.52).

Figure 4.1 shows the direct comparison between the 3 methods for all culture positive specimens that did not show evidence of PCR inhibition. Si-GuSCN-F and miniMAG showed the most similar performance with a slope close to the ideal line (0.9433) and an r^2-value of 0.8919. Overall, the distribution of the paired concordant specimens was not statistically significant (two-sided Student's T-test, $p > 0.5$) and resulted in an average difference in Ct-value of + 0.17 ± 2.01 (mean ± standard deviation) cycles for miniMAG compared to Si-GuSCN-F. MPLC DNA III showed a slightly lower performance compared to both Si-GuSCN-F and miniMAG (Fig. 4.1B and C), with slopes of 0.8864 and 0.8622, although correlation was comparable with r^2-values of 0.8605 and 0.9079 respectively. For MPLC DNA III, the distribution of paired concordant specimens was significantly different from both Si-GuSCN-F and miniMAG (two-sided Student's T-test, *p*-values < 0.00005) and resulted in average differences in Ct-values of + 1.43 ± 1.47 and + 1.26 ± 2.06 cycles when compared to Si-GuSCN-F and miniMAG, respectively.

Compared to culture, all three methods were significantly less sensitive (McNemar's Test, $p < 0.04$ [Si-GuSCN-F], $p < 0.02$ [miniMAG], $p < 0.008$ [MPLC DNA III]). When the three extraction methods were compared to one another, no significant differences in sensitivity were found (McNemar's Test, $p > 0.5$ [Si-GuSCN-F vs. miniMAG], $p > 0.2$ [Si-GuSCN-F vs. MPLC DNA III], $p > 0.9$ [miniMAG vs. MPLC DNA III]).

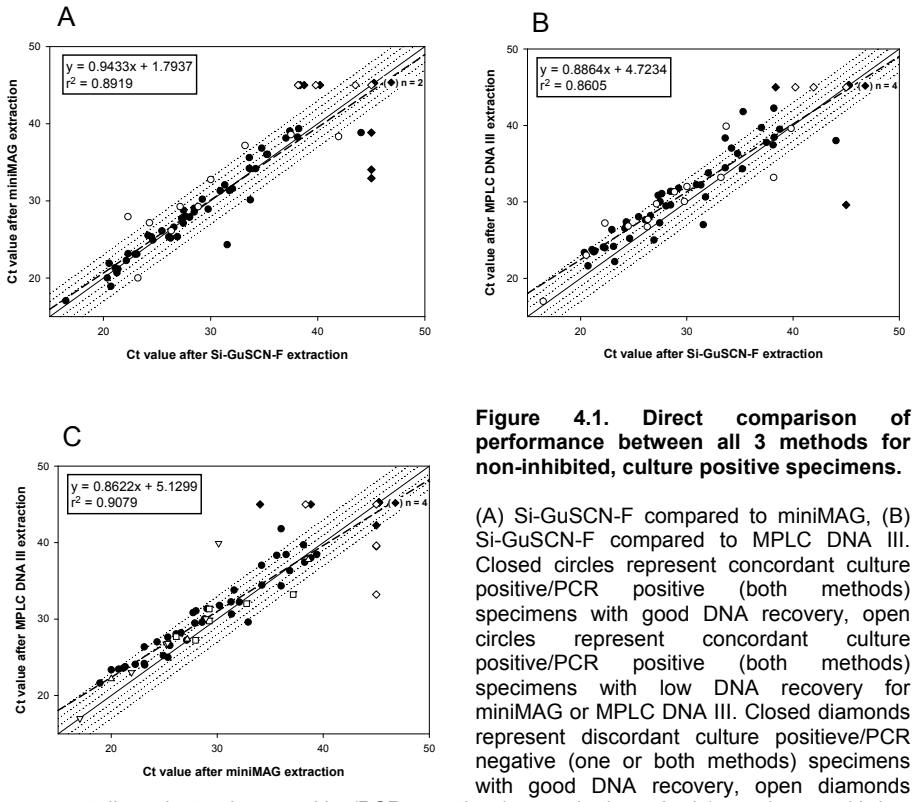


Figure 4.1. Direct comparison of performance between all 3 methods for non-inhibited, culture positive specimens.

(A) Si-GuSCN-F compared to miniMAG, (B) Si-GuSCN-F compared to MPLC DNA III. Closed circles represent concordant culture positive/PCR positive (both methods) specimens with good DNA recovery, open circles represent concordant culture positive/PCR positive (both methods) specimens with low DNA recovery for miniMAG or MPLC DNA III. Closed diamonds represent discordant culture positieve/PCR negative (one or both methods) specimens with good DNA recovery, open diamonds represent discordant culture positive/PCR negative (one or both methods) specimens with low DNA recovery for miniMAG or MPLC DNA III. (C) miniMAG compared to MPLC DNA III. Closed circles represent concordant culture positive/PCR positive (both methods) specimens, open upward pointing triangles represent concordant culture positive/PCR positive (both methods) specimens with low recovery for miniMAG, open downward pointing triangles represent concordant culture positive/PCR positive (both methods) specimens with low recovery for MPLC DNA III, open squares represent concordant culture positive/PCR positive (both methods) specimens with low recovery for both miniMAG and MPLC DNA III, closed diamonds discordant culture positive/PCR negative (one or both methods) specimens with good DNA recovery, the open dotted diamond represents a discordant culture positive/PCR negative (one or both methods) specimen with low DNA recovery for miniMAG, open diamonds represent discordant culture positive/PCR negative (one or both methods) specimens with low DNA recovery for both miniMAG and MPLC DNA III. The solid line (A, B, C) represents the hypothetical identical performance between 2 methods, the dotted lines represent differences in Ct-value of ± 1, 2, or 3 cycles, and the dashed line shows the actual correlation between 2 methods.

Influence of impaired DNA recovery on real-time PCR detection.

To assess if the low recovery for 16.2% and 24.3% of the stools with miniMAG and MPLC DNA III influenced the detection of *S. enterica* DNA by real-time PCR, the real-time PCR and culture results were compared to the recovery score (normal or low), as is shown in Table 4.2. Although a trend

was observed indicating low recovery may have influenced the detection of *S. enterica* DNA by miniMAG, this did not reach statistical significance (p > 0.1, Fisher's exact test). For the MPLC DNA III this relation was also not statistically significant (p > 0.3, Fisher's exact test).

Performance of the extraction methods with specimens containing *S. enterica* DNA quantities near the limit of detection.

To asses the performance of the extraction methods near the limit of detection, culture positive specimens with discordant PCR results (n = 15) between one or more extraction methods were subjected to a second extraction and PCR. With these low positive specimens, Si-GuSCN-F showed the highest detection rate in the original analysis, detecting 9 out of 15 (60%) specimens, followed by both miniMAG and MPLC DNA III with a detection rate of only 33%. Upon retesting these specimens by a new extraction and PCR, the detection rate improved slightly to 47% for MPLC DNA III, whereas for Si-GuSCN-F and miniMAG the detection rates remained identical at 60% and 33% respectively. Overall, the detection rates for the original analysis and the reanalysis were 42% and 47% respectively, indicating that, based on the Poisson distribution, these samples were near the lower limit of molecular detection.

Stability of the extracted DNA stored at −20°C for 1 year.

The stability of the DNA extracted from the fecal specimens, and stored at −20°C for a year was assessed with DNA extracted from the 35 stools whose original real-time PCR results for the 3 extraction methods resulted in an average Ct-value of ≤ 30, and which showed no PCR inhibition. Stools resulting in higher average Ct-values were excluded, as inconsistent detection in these specimens is expected by the Poisson distribution of *S. enterica* DNA near the limit of detection of the assay. The Ct-values of the real-time PCR results obtained after storage of the DNA at −20°C for 1 year, were compared to the original Ct-values. None of the methods showed evidence of significant DNA degradation, in fact, for all 3 methods Ct-values obtained after storage for 1 year were generally lower compared to the original Ct-values (miniMAG, − 1.08 ± 0.73, range + 0.37 to − 2.25; Si-GuSCN-F, − 1.15 ± 1.44, range + 1.44 to − 7.35; MPLC DNA III, − 1.41 ± 0.65, range + 0.10 to − 2.69 [mean ± standard deviation, range]). Furthermore, high r^2 values of 0.9636, 0.9543, and 0.8241 and slopes of

1.0070, 0.9742, and 0.9108 were established for MPLC DNA III, miniMAG, and Si-GuSCN-F respectively. The somewhat lower r^2 value and slope for Si-GuSCN-F might be the result of PCR inhibition for one specimen, which was missed by the PCR inhibition algorithm. This specimen showed a Ct-value of 31.55 with a Ct $_{[spike]}$ of 31.09 after Si-GuSCN-F extraction, whereas after miniMAG and MPLC DNA III the Ct-values were 24.32 (Ct $_{[spike]}$ 24.33) and 27.02 (Ct $_{[spike]}$ 27.28). When this sample was excluded from the comparison for the Si-GuSCN-F method, the r^2 value improved to 0.9195 and the slope increased to 1.0232.

TABLE 4.3. Hands-on time, total turn-around time, and costs[a].

	Extraction method		
	Si-GuSCN-F	miniMAG	MPLC DNA III
HOT (minutes)	143.8 ± 18.1	96.4 ± 6.0	59.8 ± 3.4
TPT (minutes)	175.8 ± 18.1	121.4 ± 6.0	134.8 ± 3.4
Chemicals and/or disposables/specimen (€)	1.46	6.90[b]	5.24[b]
Additional disposables/ specimen (€)	0.29	0.95[c]	0.39[c]
Additional disposables /run (€)	0.03	0.05[c]	0.02[c]
Technician/specimen (€)	2.50	1.67	1.04
Total costs/specimen (€)	4.28	9.57	6.69

[a] To compare all methods, run sizes of 24 specimens (22 stools + 2 PBS) were used. All prices are including tax rate of 19%. MPLC DNA III can process 32 specimens per run, resulting in a higher HOT (+ 10-15 min) and TPT (+ 25-30 min), lower chemicals and disposables/specimen costs (€ 4.97), identical additional disposables/specimen costs (€ 0.39), lower additional disposables/run costs (€ 0.01), lower technician time/specimen cost (€ 0.95). The total costs for processing 32 specimens with MPLC DNA III are therefore estimated to be € 6.32.

[b] Based on the 2005 list prices supplied by the manufacturer (€ 5.80 for miniMAG and € 4.40 for MPLC DNA III) plus 19% taxes. This includes the chemicals and disposables used on the instruments.

[c] This includes additional disposables used for transferring specimens, preparing reagents, and pre- and post-extraction storage of specimens.

Hands-on time, total processing time, and costs.

HOT, and TPT were determined for all the extraction runs performed (22 stools and 2 PBS specimens per run) and the mean values are shown in Table 4.3. The distribution of the mean HOT values was as expected in correlation with the level of automation of the extraction method, resulting in the manual Si-GuSCN-F method being the most labor intensive, followed by the semi-automated miniMAG, and finally the fully automated MPLC DNA III being the least labor intensive. The Si-GuSCN-F method required the longest time to complete the NA extraction of 24 specimens, followed by the MPLC DNA III, and with the miniMAG requiring the least time.

The total costs per extraction are shown in Table 4.3. The manual Si-GuSCN method proved to be the least expensive, followed by the MPLC DNA III, and the miniMAG.

DISCUSSION.

Excellent recovery of *Hin*dIII-digested phage lambda DNA was demonstrated from stool specimens by the Si-GuSCN-F procedure with the highest relative recovery in 92.6% of the specimens tested. Furthermore, none of the specimens resulted in a low recovery of λ-*Hin*dIII DNA compared to miniMAG and MPLC DNA III, whereas for miniMAG and for MPLC DNA III, 16.2% and 24.3% of the stools showed low recovery. The proposed mechanism for this reduced recovery is competition, between the DNA and (an) unknown competitor(s) present in some of the fecal specimens, for the silica matrix in the binding stage of the NA extraction [7]. Additional testing with 2 low recovery specimens by miniMAG, supported this hypothesis, as the recovery could partially be restored by multiple additions of magnetic silica to the fecal lysate, which has also been described for the standard Si-GuSCN extraction [7].

The manual Si-GuSCN-F procedure also showed the best performance with the subsequent real-time PCR detection of *S. enterica* DNA, obtaining a sensitivity of 91%, whereas miniMAG and MPLC DNA III both correctly detected *S. entercia* DNA in 85.5% of the culture positive stools. Although these differences were not statistically significant between the three methods, they were shown to be significantly less sensitive compared to culture detection. To increase sensitivity, culture enrichment is still mandatory for the detection of *S. enterica* from stool specimens by PCR.

Although miniMAG and MPLC DNA III obtained similar sensitivities when compared to culture, miniMAG more closely resembled the analytical performance of the Si-GuSCN-F procedure than did MPLC DNA III. Correlation between Ct-values for miniMAG/Si-GuSCN-F (r^2 = 0.8919) was slightly higher than for MPLC DNA III/Si-GuSCN-F (r^2 = 0.8605). Furthermore, also the observed Ct-values after MPLC DNA III extraction proved to be significantly different compared to both Si-GuSCN and miniMAG, showing a shift of on average + 1.43 and + 1.26 cycles compared to Si-GuSCN-F and miniMAG, respectively. Finally, miniMAG outscored

MPLC DNA III with regard to relative recovery, and also showed fewer specimens designated with low DNA recovery.

With regard to PCR inhibition, miniMAG showed the best performance, with only mild inhibition of the real-time PCR in a single specimen (0.7%), whereas MPLC DNA III and Si-GuSCN-F showed inhibition in 2.9% (4/136) and 3.7% (5/136) of the specimens, which resulted in an unresolved PCR result for 2.9% (4/136) of the stools tested for both methods. Although miniMAG obtained the lowest inhibition rate, the difference did not reach statistical significance compared to both Si-GuSCN-F and MPLC DNA III (Fisher's exact test, $p > 0.2$ and $p > 0.3$), which probably relates to the sample size of our study. However, the inhibition rates reported in this work are still regarded as excellent with regard to the difficult specimen type tested, especially in perspective of the relatively high equivalent of fecal material (1.67 mg [or µl]/PCR) that eventually ends up in the amplification reaction. Others have reported inhibitions rates of 8 to 15% at similar input levels [4, 7, 12], and 1 to 63% at lower input levels [12, 18].

All extracted DNA remained stable for at least a year when stored at −20°C. The stability of stored DNA at −20°C was studied after we experienced stability problems with some applications when MPLC extracted DNA was stored at −20°C (unpublished data). For all methods no evidence for DNA degradation was observed. In fact, for most specimens, Ct-values obtained after storage were even lower when compared to the original analysis. This may have resulted from the instability of trace amounts of PCR inhibitory substances in the DNA eluate due to freeze–thawing of the specimen. We have for instance seen that overnight storage of extracted DNA at −20°C can relieve PCR inhibition for several specimen types, including stool (unpublished data).

For a single specimen, contamination was suspected when extracted by miniMAG, although it can not be ruled out that *S. enterca* (DNA) was present, since this specimen originated from a patient with diarrhea. However, this seems unlikely, as both culture and Si-GuSCN-F/PCR were shown to be more sensitive compared to miniMAG/PCR.

Although the analytical performance is the primary criterion in the choice for a NA extraction method, other aspects may also influence the final decision. These include, amongst others, costs, HOT, TPT, flexibility, user-

friendliness, and laboratory workflow. In this respect, Si-GuSCN-F provides excellent performance at low costs. In addition, it is highly flexible, as it has been shown effective for both DNA and RNA [3, 7]. However, this is opposed by the labor intensive nature and the long TPT associated with this method. MPLC DNA III, provides full automation and therefore has a low associated HOT. The material and labor costs per specimen are about 50% higher compared to Si-GuSCN-F, but this does not include the high start-up costs associated with the purchase of the instrument (€ 80.000–100.000). Furthermore, with regard to flexibility, the use of separate kits for DNA and RNA extractions may prove to be a limiting factor. Although a kit is available for total nucleic acid isolation, it was recently shown to have a lower recovery compared to standard Si-GuSCN extraction resulting in reduced sensitivity [22]. With regard to TPT, miniMAG is the fastest method, although due to the semi-automated format it does require an additional 35 min of HOT compared to MPLC DNA III. Furthermore, miniMAG is flexible with its generic extraction chemistry, although the cost per specimen is the highest of all three methods studied. The instrument on the other hand is relatively cheap (€ 5,000).

In conclusion, of all three tested methods, Si-GuSCN-F provided the best performance for the extraction of DNA from stool specimens at the lowest costs. However, due to its laborious nature, this method does not seem feasible in today's molecular microbiology laboratory. Both miniMAG and MPLC DNA III provide sufficient performance with stool specimens. With regard to performance, miniMAG may be preferred over MPLC DNA III, as it shows more similar results compared to Si-GuSCN-F, and, more importantly, proved to remove inhibitory substances more efficiently. MPLC DNA III on the other hand is a walk-away system, and also proved to be cheaper compared to miniMAG, although it does have higher start-up costs.

ACKNOWLEDGMENTS.

The authors would like to acknowledge Alex van Belkum for the critical reading of the manuscript and the helpful discussions and suggestions.

This work was supported in part by bioMérieux by providing the miniMAG instrument and reagents used during the study.

REFERENCES.

1. **Abu Al-Soud, W., and P. Rådström.** 1998. Capacity of nine thermostable DNA polymerases to mediate amplification in the presence of PCR-inhibiting samples. Appl. Environ. Microbiol. **64:**3748–3753.
2. **Abu Al-Soud, W., and P. Rådström.** 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. J. Clin. Microbiol. **38:**4463–4470.
3. **Beld, M., R. Sentjes, S. Rebers, J. Weel, P. Wertheim-Van Dillen, C. Sol, and R. Boom.** 2000. Detection and quantification of Hepatitis C Virus RNA in feces of chronically infected individuals. J. Clin. Microbiol. **38:**3442–3444.
4. **Beld, M., R. Minnaar, J. Weel, C. Sol, M. Damen, H. van der Avoort, P. Wertheim-van Dillen, A. van Breda, and R. Boom.** 2004. Highly sensitive assay for detection of enterovirus in clinical specimens by reverse-transcription PCR with an armoured RNA internal control. J. Clin. Microbiol. **42:**3059–3064.
5. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
6. **Boom, R., C. Sol, M. Beld, J. Weel, J. Goudsmit, and P. Wertheim-van Dillen**. 1999. Improved silica-guanidiniumthiocyanate DNA isolation procedure based on selective binding of bovine alpha-casein to silica particles. J. Clin. Microbiol. **37:**615–619.
7. **Boom, R., C. Sol, J. Weel, K. Lettinga, Y. Gerrits, A. van Breda, and P. Wertheim-Van Dillen.** 2000. Detection and quantitation of human cytomegalovirus DNA in faeces. J. Virol. Methods **84:**1–14.
8. **Beuselinck, K., M. van Ranst and J. van Eldere.** 2005. Automated extraction of viral-pathogen RNA and DNA for high-throughput quantitative real-time PCR. J. Clin. Microbiol. **43:**5541–5546.
9. **Chiu, C.-H., and J.T. Ou.** 1996. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *invC*, by an enrichment broth culture-multiplex PCR combination assay. J. Clin. Microbiol. **34:**2619–2622.
10. **Espy, M. J., P. N. Rys, A. D. Wold, J. R. Uhl, L. M. Sloan, G. D. Jenkins, D. M. Ilstrup, F. R. Cockerill III, R. Patel, J. E. Rosenblatt, and T.F. Smith.** 2001. Detection of herpes simplex virus DNA in genital and dermal specimens by LightCycler PCR after extraction using the IsoQuick, MagNA Pure, and BioRobot 9604 methods. J. Clin. Microbiol. **39:**2233–2236.
11. **Gobers, E., T. A. M. Oosterlaken, M. J. A. W. M. van Bussel, R. Melsert, A. C. M. Kroes, and E. C. J. Claas.** 2001. Efficient extraction of virus DNA by NucliSENS extractor allows sensitive detection of hepatitis B virus by PCR. *J.* Clin. Microbiol. **39:**4339–4343.
12. **Holland, J. J., L. Louie, A. E. Simor, and M. Louie.** 2000. PCR detection of *Escherichia coli* O157:H7 directly from stools: Evaluation of commercial extraction methods for purifying fecal DNA. J. Clin. Microbiol. **38:**4108–4113.
13. **Hoorfar, J., P. Ahrens, and P. Rådström.** 2000. Automated 5'nuclease PCR assay for identification of *Salmonella enterica*. J. Clin. Microbiol. **38:**3429–3435.
14. Knepp, J. H., M. A. Geahr, M. S. Forman, and A. Valsamakis. 2003. Comparison of automated and manual nucleic acid extraction methods for detection of enterovirus RNA. J. Clin. Microbiol. **41:**3532–3536.
15. **Konnick, E. Q., S. M. Williams, E. R. Ashwood, and D. R. Hillyard.** 2005. Evaluation of the COBAS hepatitis C virus (HCV) taqman analyte-specific reagent assay and comparison to the COBAS amplicor HCV monitor v2.0 and versant HCV bDNA 3.0 assays. J. Clin. Microbiol. **43:**2133–2140.
16. **Kreader, C. A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. **62:**1102–1106.

17. **Leb, V. , M. Stöcher, E. Valentine-Thon, G. Hölzl, H. Kessler, H. Stekel, and J. Berg.** 2004. Fully automated internally controlled quantification of hepatitis B virus DNA by Real-time PCR by use of the MagNA Pure LC and LightCycler instruments. J. Clin. Microbiol. **42:**585–590.
18. **Monteiro, L., D. Bonnemaison, A. Vekris, K. G. Petry, J. Bonnet, R. Vidal, J. Cabrita, and F. Megraud.** 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J. Clin. Microbiol. **35:**995–998.
19. **Murray, P. R., E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken.** 1999. Manual for Clinical Microbiology (7th edition), American Society for Microbiology, Washington, DC (1999).
20. **Schrag, S. J., J. T. Brooks, C. van Beneden, U. D. Parashar, P. M. Griffin, L. J. Anderson, W. J. Bellini, R. F. Benson, D. D. Erdman, A. Klimov, T. G. Ksiazek, T. C. T. Peret, D. F. Talkington, W. L. Thacker, M. L. Tondella, J. S. Sampson, A. W. Hightower, D. F. Nordenberg, B. D. Plikaytis, A. S. Khan, N. E. Rosenstein, T. A. Treadwell, C. G. Whitney, A. E. Fiore, T. M. Durant, J. F. Perz, A. Wasley, D. Feikin, J. L. Herndon, W. A. Bower, B. W. Kilbourn, D. A. Levy, V. G. Coronado, J. Buffington, C. A. Dykewicz, R. F. Khabbaz, and M. E. Chamberland.** 2004. SARS surveillance during emergency public health response, United States, March–July 2003. Emerg. Infect. Dis. **10:**185–194.
21. **Schuurman, T., R. F. de Boer, A. M. D. Kooistra-Smid, and A. A. van Zwet.** 2004. Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for the diagnosis of bacterial meningitis in a clinical setting. J. Clin. Microbiol. **42:**734–740.
22. **Schuurman, T., A. van Breda, R. de Boer, M. Kooistra-Smid, M. Beld, P. Savelkoul, and R. Boom.** 2005. Reduced PCR sensitivity due to impaired DNA recovery with the MagNa Pure LC total nucleic acid isolation kit. J. Clin. Microbiol. **43:**4616–4622.
23. **Tang, Y.-W., S. E. Sefers, H. Li, D. J. Kohn, and G.W. Procop.** 2005. Comparative evaluation of three commercial systems for nucleic acid extraction from urine specimens. J. Clin. Microbiol. **43:**4830–4833.
24. **Widjojoatmodjo, M. N., A. C. Fluit, R. Torensma, G. P. H. Verdonk, and J. Verhoef.** 1992. The magnetic immuno polymerase chain reaction assay for direct detection of *Salmonellae* in fecal samples. J. Clin. Microbiol. **30:**3195–3199.

CHAPTER 5

Feasibility of a Molecular Screening Method for Detection of *Salmonella enterica* and *Campylobacter jejuni* in a Routine Community-Based Clinical Microbiology Laboratory

T. Schuurman [1]
R. F. de Boer [1]
E. van Zanten [1]
K. R. van Slochteren [1]
H. R. Scheper [2]
B. G. Dijk-Alberts [2]
A. V. M. Möller [2]
A. M. D. Kooistra-Smid [1]

Laboratory for Infectious Diseases, Departments of [1] Research & Development and [2] Bacteriology, Groningen
The Netherlands

Copyright © American Society for Microbiology,
Journal of Clinical Microbiology. 2007. **45:**3692-3700

ABSTRACT.

Conventional diagnostic methods for the detection of *Salmonella enterica* and *Campylobacter jejuni* are laborious and time-consuming procedures, resulting in final results, for the majority of specimens, only after 3 to 4 days. Molecular detection can improve the time to reporting of the final results from several days to the next day. However, molecular assays for the detection of gastrointestinal pathogens directly from stool specimens have not made it into the routine clinical microbiology laboratory. In this study we have assessed the feasibility of a real-time PCR-based molecular screening method (MSM), aimed at *S. enterica* and *C. jejuni*, in the daily practice of a routine clinical microbiology laboratory. We have prospectively analyzed 2,067 stool specimens submitted for routine detection of gastrointestinal bacterial pathogens over a 7-month period. The MSM showed 98 to 100% sensitivity but routine culture showed only 77.8 to 86.8% sensitivity when an extended "gold standard" that included all culture-positive and all MSM-positive specimens, as confirmed by an independent secondary PCR of a different target gene, was used. An overall improvement in the rate of detection of both pathogens of 15 to 18% was observed. Both approaches performed nearly identically with regard to the specificity, positive predictive value, and negative predictive value, with the values for MSM being 99.7%, 93.1 to 96.6%, and 99.8 to 100%, respectively, and those for routine culture being 100%, 100%, and 97.6 to 99.5%, respectively. Finally, the final results were reported between 3 and 4 days earlier for negative specimens compared to the time of reporting of the results of routine culture. Positive specimens, on the other hand, required an additional 2 days to obtain a final result compared to the time required for routine culture, although preliminary MSM PCR-positive results were reported, on average, 2.9 to 3.8 days before the final routine culture results were reported. In conclusion, MSM can be incorporated into the daily practice of a routine clinical microbiology laboratory with ease. Furthermore, it provides an improvement in the screening for *S. enterica* and *C. jejuni* and substantially improves the time to the reporting of negative results.

INTRODUCTION.

Infectious gastroenteritis is still a major public health burden in developed countries, although the related mortality is low [14]. In The Netherlands, the total number of gastroenteritis cases in the population was estimated to be 4.5 million in 1999 [36], with an estimated annual cost of € 345 million to Dutch society [34]. *Salmonella enterica* and *Campylobacter jejuni* are the most prevalent enteric bacterial pathogens responsible for infectious gastroenteritis in The Netherlands, with reported incidences of 24/100,000 population and 36 to 37.3/100,000 population for *S. enterica* and *C. jejuni*, respectively [9, 10, 35, 36]. *C. jejuni* seems to be more prevalent in older children (ages 5 to 14 years) and young adults (ages 15 to 29 years), whereas *S. enterica* tends to infect young children more often [10]. Most infections with *S. enterica* and *C. jejuni* usually result in mild, self-limiting gastrointestinal disease. However, both organisms can give rise to more severe disease forms and complications, like typhoid fever, which is caused by *S. enterica* serovars Typhi and Paratyphi, or Guillain-Barré syndrome, which is a neurodegenerative disorder triggered by molecular mimicry of *C. jejuni* lipo-oligosacchrides and neural gangliosides [33, 38]. About 1 in 1,000 *C. jejuni* infections develops into Guillain-Barré syndrome [3].

Routine diagnostic procedures for the screening of fecal samples for *S. enterica* and *C. jejuni* consist of selective culture methods, preceded by enrichment for *S. enterica*, and followed by biochemical identification, partial serotyping (*S. enterica*), and resistance profiling. These procedures are laborious and time-consuming, and it can take up to 4 days or more to obtain a final result. Furthermore, several diagnostic problems are encountered when stool specimens are screened for these pathogens. For instance, *S. enterica* grows with a suspicious colony morphology on salmonella-shigella medium (SS) and Hektoen enteric agar (HEA) plates, but bacteria present in the normal human gastrointestinal flora also show the same colony type [11, 31]. These false-positive findings result in increases in hands-on times and the delay of the reporting of the final result for these negative specimens. Another type of problem may be caused by the viable but nonculturable state of *C. jejuni* [29, 30]. This may compromise the sensitivity of culture approaches. Furthermore, the majority of stool specimens received by the clinical laboratory for examination for bacterial gastrointestinal pathogens do

not yield a positive result. For instance, in The Netherlands, the average percentage of positive findings for bacterial gastrointestinal pathogens (i.e., *Salmonella* spp., *Campylobacter* spp., and *Shigella* spp.) was only 6.2% [36]. The total workload involved in stool screening is high, as, for instance, our laboratory receives approximately 10,000 stool specimens annually, resulting in an average of about 40 stool specimens per day. Furthermore, the throughput of these specimens shows seasonal variance, with peaks at the end of winter and during the summer and early autumn [36]. All of this makes screening of stool specimens for enteric pathogens a laborious procedure, especially during the seasonal peaks. Therefore, culture-independent methods may facilitate routine screening, especially when the bulk of the specimens, which are negative, can be eliminated quickly.

Real-time PCR provides a means for the accurate and fast detection of pathogens; however, only a limited number of reports describe the detection of *S. enterica* and/or *C. jejuni* directly from human stool specimens [13, 17, 23, 25]. Furthermore, all of these reports performed the analysis with a (relatively) small number of fecal specimens (i.e., 25 to 145 specimens).

Therefore, to date, the applicability of (real-time) PCR for the diagnosis of gastroenteritis has remained limited. The major issue here is the inhibition caused by fecal constituents [7, 28, 37]. However, this can be overcome by the use of amplification facilitators, like bovine serum albumin [2, 21] or thermostable DNA polymerases that are more resistant to inhibition [1]. Furthermore, with the advances in real-time PCR technology, PCR diagnostics are no longer restricted to highly specialized (research and academic) laboratories but are increasingly becoming available to community laboratories as well.

The aim of the present study was to assess the feasibility of a molecular screening method (MSM) for *S. enterica* and *C. jejuni* in the routine practice of a community-based clinical microbiology laboratory. MSM-positive specimens were subsequently cultured for antibiotic resistance profiling and epidemiology. We prospectively analyzed 2,067 stool specimens sent to the laboratory for the detection of *S. enterica* and/or *C. jejuni* by both the standard approach and MSM.

MATERIALS AND METHODS.

Bacterial and fungal strains.

A panel of 33 *S. enterica* strains, 6 *C. jejuni* strains, and a total of 36 bacterial and fungal negative control strains representing other gastrointestinal pathogens and normal gastrointestinal flora was used in this study. The 33 *S. enterica* strains included the following serotypes; Agona, Albany, Anatum, Berta, Blockley, Bochum, Bovis Morbificans, Corvallis, Derby, Dublin, Enterica, Enteritidis (ATCC 13076), Goldcoast, Hadar, Infantis, London, Montevideo, Munster, Panama, Paratyphi A (two strains), Paratyphi B, Paratyphi var. Java, Poona, Rubislaw, Salamea, Saint Paul, Senftenberg, Stanley, Takoradi, Typhi, Typhimurium (ATCC 14028), and Virchow. The six *C. jejuni* strains comprised the following: ATCC 29428, CCUG 10935 (Penner serotype 1), CCUG 10936 (Penner serotype 2), CCUG 10937 (Penner serotype 3), CCUG 10938 (Penner serotype 4), and NTCC 11168 (Penner serotype 2). The 36 bacterial and fungal strains other than *S. enterica* and *C. jejuni* have been described previously [32].

All strains were from the strain database of the Laboratory for Infectious Diseases (LvI), unless stated otherwise (ATCC), and were grown on the appropriate media and under appropriate conditions for 16 to 48 h. After harvesting of the strains (approximately 10^9 CFU) into 1 ml of physiological saline, chromosomal DNA was extracted from the strains by heat lysis for 10 min at 95°C.

Study design and fecal specimens.

All stool specimens used in this study were sent to the Department of Bacteriology of LvI for routine examination of enteric pathogens during the period from 27 June 2005 to 25 January 2006. A total of 2,067 stools specimens with sufficient sample volume to ensure reliable results for both routine culture and MSM were enrolled in the study. Of these 2,067 specimens, 2,055 stool specimens were eligible for use in the evaluation of the procedures for *S. enterica* (no *S. enterica* culture was performed for the remaining 12 specimens) and 2,009 stool specimens were eligible for use in the evaluation of the procedures for *C. jejuni* (no *C. jejuni* culture was performed for the remaining 58 specimens) (Table 5.1). All experiments were performed fully blinded, and the results of both routine culture and MSM were linked only when both approaches were completed.

Bacterial culture for *S. enterica*.

Routine culture for enteric pathogens was carried out by the Department of Bacteriology of LvI and for *S. enterica* consisted of selenite enrichment and selective culturing on SS medium and HEA at 35°C, biochemical identification (urea, lysine decarboxylase, tryptone water, triple sugar iron agar, and *o*-nitrophenyl-ß-D-galactopyranoside), and group-specific *Salmonella* (long/short polyvalent, A, B, b, C1, C2, D, d, Vi, E, F, and G) serological identification. All culture and identification media were from Mediaproducts BV (Mediaproducts BV, Groningen, The Netherlands), whereas the *Salmonella* agglutination sera were from Remel (Remel Europe Ltd., Dartford, United Kingdom). Resistance profiling was performed with the VITEK 2 system (bioMérieux, Boxtel, The Netherlands). All strains isolated were stored at –75°C so that they were available for further testing, if necessary.

Bacterial culture for *Campylobacter* species.

For *Campylobacter* species, routine culture consisted of selective culture on *Campylobacter* selective agar (48 h at 42°C) and charcoal cefoperazone desoxycholate agar (72 h at 35°C) under microaerophilic conditions (6.0% O_2, 7.1% CO_2, 3.6% H_2, and 83.3% N_2) (Anoxomat system; Mart Microbiology BV, Lichtenvoorde, The Netherlands). Identification was carried out by Gram-staining, oxidase testing, determination of hippurate hydrolysis, determination of the absence of aerobic growth at 42°C, and resistance profiling (disk diffusion method with ciprofloxacin, erythromycin, nalidixic acid, norfloxacin, tetracycline, and trimethoprim [Neo-Sensitabs 73412, 74012, 75812, 76212, 78417, and 79012, respectively; Rosco, Taastrup, Denmark] and cephalothin [kf30; Oxoid Limited, Basingstoke, United Kingdom]). Culture and identification media were from Mediaproducts BV. All strains isolated were stored at –75°C so that they were available for further testing, if necessary.

Molecular screening method for *S. enterica* and *C. jejuni*.

(i) Specimen collection.

Stool specimens, stored at 2 to 8°C after inoculation of routine bacterial cultures for enteric pathogens, were collected at the end of the day and brought to the Research and Development Department. A fecal suspension (33 to 50% [wt/vol]) was prepared and stored at –20°C until DNA extraction

on the next day. A selenite enrichment broth from the same stool specimen was also inoculated and incubated for 16 h at 35°C, whereas the remaining portion of the stool specimen was stored at 2 to 8°C until further culture, depending on the real-time PCR result.

(ii) DNA extraction.

DNA was extracted from the fecal suspension and selenite enrichment broth as described previously [32] by using NucliSENS magnetic extraction reagents (bioMérieux) and a NucliSENS miniMAG instrument (bioMérieux), according to the manufacturer's instructions. Approximately 6,000 copies of phocine herpesvirus 1 (PhHV) were added to the extraction mixture to serve as an internal control to monitor DNA extraction and PCR inhibition. An aliquot (1 ml) from the selenite enrichment broth was stored at –20°C, in case of inhibition of the PCR, whereas the remaining selenite enrichment broth was stored at room temperature until further culture, depending on the real-time PCR result.

(iii) Real-time PCR.

Real-time amplification was carried out on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) by using the TaqMan Universal PCR master mix (Applied Biosystems). The primers and probes have been described previously for *S. enterica* [16], *C. jejuni* [5], and PhHV [32] and were purchased from Applied Biosystems. Probes were labeled (at the 5' end) with 6-carboxyfluorescein (*S. enterica*), VIC (*C. jejuni*), or 2'-chloro-5'-fluoro-7',8'-fused phenyl-1,4-dichloro-6-carboxyfluorescein (NED) (PhHV). Quenching of the 3' end was conducted with either 6-carboxytetramethylrhodamine or a nonfluorescent quencher combined with a minor groove binder group. The reactions were set up as single PCRs for each individual target, with the reaction mixtures (25 μl) consisting of 1 x TaqMan Universal PCR master mix (Applied Biosystems), 2.5 μg bovine serum albumin (Roche Diagnostics Nederland BV, Almere, The Netherlands), 5 μl of template DNA, the primers at 300 nM, and the probe at 100 nM (except for *S. enterica*, for which the probe was used at 400 nM). The reactions were run in a single reaction plate under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 50°C for 15 s, and 60°C for 1 min. Amplification data

were acquired and analyzed with Sequence Detector software (version 1.9.1; Applied Biosystems).

(iv) Real-time PCR interpretation and MSM-guided culture for *S. enterica* and *C. jejuni*. A real-time PCR was considered positive when a threshold cycle (Ct) of less than 40 cycles for *S. enterica* and/or *C. jejuni* was recorded. A real-time PCR was considered inhibited when the Ct-value for the PhHV real-time PCR exceeded 35.40 cycles (i.e., the mean Ct-value for uninhibited specimens + 2 standard deviations). When a real-time PCR was inhibited, the specimen was retested in the next run by using a new DNA extract from the stored fecal suspension and selenite enrichment broth. For all real-time PCR-positive and inhibited specimens, cultures for *S. enterica* and/or *C. jejuni* were immediately started from the stored (2 to 8°C) fecal specimens by the same procedures described above for the routine culture of enteric pathogens. When an inhibited specimen showed a valid negative result upon the second real-time PCR analysis, the culture for that specimen was discontinued. Culture was not performed for all other PCR-negative specimens. All isolated strains were stored at –75°C so that they were available for further testing, if necessary.

Confirmatory real-time PCR assays.

For confirmation of the results for the real-time PCR-positive and culture-negative (both routine and real-time PCR-guided) specimens, DNA was extracted from the stored fecal suspension (–20°C) and selenite enrichment broth (–20°C) and subjected to a real-time PCR targeting a different region of the designated organism's genome.

For *S. enterica*, the real-time PCR primer/probe set targeting the *ttrBCA* region described by Malorny *et al.* [27] was used in the same reaction mixture set up as described above for the *invA*, *mapA*, and PhHV assays, with the primers (Applied Biosystems) at 300 nM and the probe (5'-6-carboxyfluorescein, 3'-eclipse dark quencher; Eurogentec Nederland BV, Maastricht, The Netherlands) at 100 nM.

For *C. jejuni* confirmation, a primer set targeting a *Campylobacter* sp.-specific region of the 16S rRNA gene was used. The reaction mixture consisted of 1 x SYBR green PCR master mix (Applied Biosystems), primers 5'-AGC GCA ACC CAC GTA-3' and C1228R [25] at 300 nM (kindly donated by H. Wilke, Laboratory for Medical Microbiology Twente Achterhoek,

Enschede, The Netherlands), 2.5 µg bovine serum albumin (Roche Diagnostics Nederland BV), and 5 µl of template DNA.

The reactions for confirmation of the results for both *S. enterica* and *C. jejuni* were run on a ABI 7500 real-time PCR system (Applied Biosystems) by applying the following thermoprofile: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The specific amplification of *C. jejuni* DNA was confirmed by melting curve analysis in comparison with the results for a positive control specimen (*C. jejuni* ATCC 29428).

Both assays showed identical sensitivities compared to the results of the real-time PCR assays targeting *invA* and *mapA*, which were used as the screening assays (data not shown).

For confirmation of *C. coli* detection by routine culture, a *C. coli*-specific real-time PCR targeting the *glyA* gene described by Jensen *et al.* [18] was used, with the reaction set up identically to the reaction setups described above for the real-time PCR assays targeting *invA*, *mapA*, and the *ttrBCA* region. The reactions were run on an ABI 7500 real-time PCR system with the same thermoprofile used for the *S. enterica* and *C. jejuni* confirmation assays.

RESULTS.

Selectivities of primer/probe sets for *S. enterica* and *C. jejuni*.

The primer/probe sets for the *S. enterica invA* and *C. jejuni mapA* genes were tested for their selectivity, consisting of inclusivity and exclusivity, with DNA preparations from pure bacterial strains. The *S. enterica invA*-specific primer/probe set detected all 33 *S. enterica* strains, and no cross-reactivity with any of the non-*S. enterica* strains was observed (data not shown).

The *C. jejuni mapA* primer/probe set correctly identified all available strains of *C. jejuni* and showed no cross-reactivity with any of the non-*C. jejuni* strains (data not shown).

Although only a limited number of *S. enterica* and *Campylobacter* spp. were tested, no further testing was attempted since both tests have already been tested with 210 *Salmonella* strains and over 6,000 clinical *Campylobacter* isolates [5, 16].

LODs.

The analytical sensitivities of the *S. enterica* and *C. jejuni* real-time PCR assays were assessed with spiked fecal specimens. For *S. enterica*, two fecal specimens (negative for *S. enterica* by culture and real-time PCR) were inoculated into selenite enrichment broth and spiked with 10-fold serial dilutions of *S. enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028, resulting in a concentration range of 1.8×10^9 to 0 CFU/g of feces for each fecal specimen. An unspiked fecal suspension (33 to 50% [wt/vol]) was also made and was stored at –20°C. DNA was extracted from the fecal suspensions and the enrichment broths as described above for the clinical stool specimens, and real-time PCR was subsequently performed with the isolated nucleic acids. Culture for *S. enterica* was also inoculated from the selenite enrichment broths for the lower 5 dilutions of the concentration range. Both real-time PCR and culture showed identical lower limits of detection (LODs) of 18 CFU/g of feces, although for one of the two stool specimens culture did not detect *Salmonella* serovar Typhimurium from the SS medium plate inoculated with 1.8×10^1 CFU/g (data not shown).

For *C. jejuni*, two fecal suspensions (33 to 50% [wt/vol]), prepared from two individual specimens (negative for *C. jejuni* by culture and real-time PCR), were spiked with serial dilutions of *C. jejuni* strain ATCC 29428, resulting in a range of from 2.4×10^7 to 2.4×10^1 CFU/ml of suspension. Depending on the consistency of the suspended stool specimen, this corresponds to a range of 4.8×10^6 to 7.2×10^6 to 4.8 to 7.2 CFU/g of feces. After real-time PCR analysis, the LODs for the *C. jejuni* real-time PCR were shown to be 4.8×10^2 to 7.2×10^2 CFU/g of feces (data not shown).

Detection of *S. enterica* and *C. jejuni* by real-time PCR and routine culture.

A total of 2,067 clinical stool specimens were screened for *S. enterica* and *C. jejuni* by MSM, and for 2,055 and 2,009 of these specimens, routine culture for *S. enterica* and *C. jejuni*, respectively, was performed in parallel. Both sample and specimen receipt dates were available for 1,349 specimens and resulted in a mean transportation time of 1.7 days (range, 0 to 18 days; median, 1 day). For *S. enterica*, the routine culture result was regarded as positive only if a strain of *Salmonella* was isolated and not if other enteropathogens such as *Shigella* species were recovered from the

SS medium and HEA plates. Routine culture for *Campylobacter* species was regarded as positive only when the isolated strain was typed as *C. jejuni*. This was mandatory due to the fact that the MSM was aimed only at *C. jejuni* and not at the other thermophilic *Campylobacter* species. Table 5.1 shows all the individual results of MSM, subsequent and routine culture, confirmatory PCR testing, and PCR inhibition for the 2,067 specimens tested.

Performance of MSM and routine culture compared to that of an extended "gold standard."

To independently compare the performance of the MSM and routine culture, an extended gold standard was defined to establish the status of the clinical stool specimens. True-positive specimens were defined as all specimens with a positive culture result for the targeted organism by either routine culture or MSM-guided culture and all additional specimens positive by both MSM and confirmatory real-time PCRs. True-negative specimens were defined as all specimens not regarded as true positive. Table 5.2 shows the performance of the MSM and routine culture for both *S. enterica* and *C. jejuni* in regard to this extended gold standard.

For both *S. enterica* and *C. jejuni*, the MSM showed the highest sensitivity, with sensitivities of 100% and 98.0%, respectively, whereas routine culture showed sensitivities of only 86.8% and 77.8%, respectively. With regard to specificity, both the MSM and routine culture showed excellent performance, with 99.7% specificity for both bacteria with the MSM and 100% specificity for both bacteria by routine culture. The positive predictive value (PPV) of the MSM was slightly lower, with PPVs of 93.1% and 96.9% for *S. enterica* and *C. jejuni*, respectively, compared to the results of routine culture (PPVs of 100% for both bacteria). With regard to the negative predictive value (NPV), both the MSM and routine culture showed excellent performance, with NPVs for *S. enterica* and *C. jejuni* of 100% and 99.8%, respectively, by MSM, and 99.5% and 97.6%, respectively, by routine culture. Finally, the diagnostic accuracies for *S. enterica* and *C. jejuni* were established to be 99.8% and 99.5%, respectively, for MSM and 99.6% and 97.8%, respectively, for routine culture.

TABLE 5.1. Overall MSM, MSM-guided culture, routine culture, confirmatory, and inhibition results for all 2,067 tested stool specimens[a].

Specimens	Number of	MSM result		MSM-guided culture result		Routine culture result		Confirmatory PCR results		PCR inhibition	
tested for	specimens	*S. enterica*	*C. jejuni*	*S. enterica*	*C. jejuni*	*S. enterica*	*C. jejuni*	*S. enterica*	*C. jejuni*	Primary	Secondary
S. enterica	48	Pos	Neg	Pos	ND	Pos	Neg	ND	ND	Non Inh.	NA
+ *C. jejuni*	1	Pos	Neg	Pos	ND	Pos	Neg	ND	ND	Inh.	Non Inh.
	2	Pos	Neg	Pos	ND	Pos	Neg	ND	ND	Inh.	Inh.
	2	Pos	Neg	Pos	ND	Neg	Neg	ND	ND	Non Inh.	NA
	6	Pos	Neg	Neg	ND	Neg	Neg	Pos	ND	Non Inh.	NA
	4	Pos	Neg	Neg	ND	Neg	Neg	Neg	ND	Non Inh.	NA
	119	Neg	Pos	ND	Pos	Neg	Pos	ND	ND	Non Inh.	Non Inh.
	1	Neg	Pos	ND	Pos	Neg	Pos	ND	ND	Inh.	Non Inh.
	2	Neg	Pos	ND	Pos	Neg	Pos	ND	ND	Inh.	Inh.
	16	Neg	Pos	ND	Pos	Neg	Neg	ND	ND	Non Inh.	Non Inh.
	20	Neg	Pos	ND	Neg	Neg	Pos	ND	ND	Non Inh.	Non Inh.
	2	Neg	Pos	ND	Neg	Neg	Pos	ND	ND	Inh.	Non Inh.
	27	Neg	Pos	ND	Neg	Neg	Neg	ND	Pos	Non Inh.	Non Inh.
	6	Neg	Pos	ND	Neg	Neg	Neg	ND	Neg	Non Inh.	Non Inh.
	1	Pos	Pos	Neg	Pos	Neg	Pos	Neg	ND	Non Inh.	Non Inh.
	1	Pos	Pos	Pos	Pos	Pos	Pos	ND	ND	Non Inh.	Non Inh.
Subtotal	*258*										
	1,669	Neg	Neg	ND	ND	Neg	Neg	ND	ND	Non Inh.	Non Inh.
	34	Neg	Neg	ND	ND	Neg	Neg	ND	ND	Inh.	Non Inh.
	31	Neg	Neg	ND	ND	Neg	Neg	ND	ND	Inh.	Inh.
	1	Neg	Neg	ND	ND	Neg	Neg	ND	ND	Inh.	NP
	3	Neg	Neg	ND	ND	Neg	Pos	ND	ND	Non Inh.	Non Inh.
	1	Neg	Neg	ND	ND	Neg	Pos	ND	ND	Inh.	Inh.
Subtotal	*1,739*										

TABLE 5.1. Continued.

Specimens tested for	Number of specimens	MSM result		MSM-guided culture result		Routine culture result		Confirmatory PCR results		PCR inhibition	
		S. enterica	*C. jejuni*	*S. enterica*	*C. jejuni*	*S. enterica*	*C. jejuni*	*S. enterica*	*C. jejuni*	Primary	Secondary
S. enterica only											
	5	Pos	NA	Pos	NA	Pos	ND	ND	NA	Non Inh.	Non Inh.
	1	Pos	NA	Pos	NA	Pos	ND	ND	NA	Inh.	Non Inh.
	1	Pos	NA	Pos	NA	Neg	ND	ND	NA	Non Inh.	Non Inh.
	1	Pos	NA	Neg	NA	Pos	ND	ND	NA	Non Inh.	Non Inh.
	50	Neg	NA	ND	NA	Neg	ND	ND	NA	Non Inh.	Non Inh.
Subtotal	*58*										
C. jejune only											
	3	NA	Pos	NA	Pos	ND	Pos	NA	ND	Non Inh.	Non Inh.
	1	NA	Pos	NA	Neg	ND	Neg	NA	Pos	Non Inh.	Non Inh.
	7	NA	Neg	NA	ND	ND	Neg	NA	ND	Non Inh.	Non Inh.
	1	NA	Neg	NA	ND	ND	Pos	NA	ND	Non Inh.	Non Inh.
Subtotal	*12*										
Total	2,067										

[a] Pos, Positive; Neg, Negative; ND, Not done; Non Inh., Not inhibited; NA, Not applicable; Inh., Inhibited; NP, Not possible due to insufficient specimen volume.

To assess if the observed difference in sensitivity between routine culture and both the MSMs reached statistical significance, the data from Table 5.1, excluding those for the inhibited and nonconfirmed PCR-positive specimens, were analyzed by McNemar's test. This revealed that both MSMs were significantly more sensitive than routine culture ($p < 0.005$).

TABLE 5.2. Test performance of MSM and routine culture compared to that of the extended gold standard.

Application and result	Extended gold standard[a]		Total number of specimens
	Positive	Negative	
S. enterica, MSM			
Positive	68	5	73
Negative	0	1,947	1,947
Unresolved	0	35	35
Total	68	1,987	2,055
S. enterica, routine culture			
Positive	59	0	59
Negative	9	1,987	1,996
Total	68	1,987	2,055
C. jejuni, MSM			
Positive	193	6	199
Negative	4	1,771	1,775
Unresolved	1	34	35
Total	198	1,811	2,009
C. jejuni, routine culture			
Positive	154	0	154
Negative	44	1,811	1,855
Total	198	1,811	2,009

[a] The extended gold standard result was defined as positive when the target organism was isolated by either routine culture or MSM-guided culture upon an MSM-positive result or when an MSM-positive result was confirmed by the species-specific confirmation PCR.

TATs for final results with MSM and routine culture.

To assess the influence of the MSM on the turnaround time (TAT), the time to the final result was recorded for the specimens positive by both routine culture and MSM-guided culture for *S. enterica* (n = 58) and *C. jejuni* (n = 127) (concordant positive specimens), as well as for the concordant negative specimens (n = 1,792). The TAT for positive specimens was, on average, 6.9 ± 2.0 days (mean ± standard deviation; median, 7 days) for *S. enterica* by the MSM, with preliminary MSM PCR-positive results reported after, on average, 1.4 ± 0.8 days (median, 1 day), whereas routine culture needed 5.2 ± 1.8 days (median, 5 days). For *C. jejuni* the TAT was, on average, 6.3 ± 1.6 days (median, 6 days) for the MSM, with MSM preliminary PCR-positive results reported after 1.5 ± 0.9 days (median, 1 day) and

routine culture results reported after 4.4 ± 1.3 days (median, 4 days). The results for the concordant negative specimens were reported by MSM after 1.5 ± 1.1 days (median, 1 day), whereas routine culture needed 3.9 ± 1.3 days (median, 4 days).

TABLE 5.3. Effect of delayed inoculation of MSM-guided culture compared to that of routine culture.

Target and delay in days between routine and MSM-guided culture	Culture results for routine culture/MSM-guided culture[a]		*p*-value[b] for pair-wise comparisons between different delays (in days)				
	pos/pos	pos/neg	2	3	4	≥ 2	≥ 3
S. enterica							
1	27	1	0.99	0.99	1.0	0.47	0.99
2	16	0	NA	1.0	1.0	NA	1.0
≤ 2	43	1	NA	1.0	1.0	NA	0.99
3	5	0	NA	NA	1.0	NA	NA
≤ 3	48	1	NA	NA	0.99	NA	NA
4	10	0	NA	NA	NA	NA	NA
≥ 2	31	0	NA	NA	NA	NA	NA
≥ 3	15	0	NA	NA	NA	NA	NA
C. jejuni							
1	64	4	0.037	0.0029	0.20	0.0047	0.0088
2	26	7	NA	0.36	0.74	NA	0.99
≤ 2	90	11	NA	0.015	0.48	NA	0.045
3	14	7	NA	NA	0.30	NA	NA
≤ 3	104	18	NA	NA	0.76	NA	NA
4	20	4	NA	NA	NA	NA	NA
≥ 2	60	18	NA	NA	NA	NA	NA
≥ 3	34	11	NA	NA	NA	NA	NA

[a] Pos, positive; neg, negative.

[b] P values were determined by the two-sided Fisher's exact test. NA, not applicable.

Effect of delayed inoculation for MSM-guided culture.

Delayed inoculation of culture may prove detrimental for obtaining positive culture results, especially for *Campylobacter* species [9]. As the MSM introduced a delay of at least 1 day between the inoculation of the routine culture and MSM-guided culture, we assessed the effect of the delayed inoculation caused by the MSM on the outcomes for these cultures in comparison with the routine culture result. Table 5.3 shows the delay between the inoculation of the routine culture and the MSM-guided culture for those specimens positive by both routine culture and MSM. Delayed inoculation did not have any significant effect on the outcome of the MSM-guided culture for *S. enterica*. For *C. jejuni*, on the other hand, a delay of more than 1 day resulted in a significantly lower potential to obtain a positive culture result for the MSM-guided cultures.

Analysis of discrepant results between MSM and routine culture.

For nine cases identified as *C. jejuni* by the MSM, including one of the two cases where the *C. jejuni* strain isolated from the MSM approach could by specified only by PCR analysis, *Campylobacter* species other than *C. jejuni* were isolated by routine culture. These included *C. coli* (n = 7), and *C. lari* (n = 2). To further elucidate the status of these specimens, the isolates stored at –75°C were recultured and tested by PCRs specific for *C. jejuni*, *C. coli*, and *Campylobacter* species. In addition, the fecal DNA extracts from these specimens were also subjected to the same panel of PCRs. The results are shown in Table 5.4. The results showed that for three of the seven cases in which routine culture initially identified the organism as *C. coli*, the additional PCR results showed no evidence for the presence *C. coli* in these specimens. Although the three strains originally isolated by routine culture could not be recultured from their storage at –75°C, it seems likely that they were in fact hippurate-negative *C. jejuni* strains. For the four other specimens originally identified as *C. coli* by routine culture, additional testing confirmed the presence of *C. coli* in these specimens. However, both *C. jejuni* and *C. coli* were identified by the additional PCRs in all four specimens, as well as in one of the stored isolates which originated from routine culture. From this it may be concluded that these cases are in fact mixed infections rather than single infections.

For the two specimens originally identified as *C. lari* by routine culture, additional testing showed that one was in fact a mixed *C. jejuni-C. coli* infection, whereas the other was a single *C. jejuni* infection in which the strain was misidentified as *C. lari* due to a combination of a (false-) negative hipurate hydrolysis and nalidixic acid resistance phenotype.

Campylobacter spp. were also isolated by routine culture from seven specimens with negative MSM results. Identification of the strains in these seven specimens yielded *C. jejuni* on four occasions and *C. coli* on the remaining three. The isolated strains and the original stool DNA extracts from these specimens were subjected to the same testing strategy mentioned above for the specimens that were MSM positive and routine culture positive for non-*C. jejuni Campylobacter* spp., and the results are also shown in Table 5.4.

TABLE 5.4. Additional analysis of discrepant *C. jejuni* MSM/*Campylobacter* species routine culture results[a].

Discrepancy type and study identifier	Initial result by: MSM	Initial result by: Routine culture	Result of additional PCR testing performed with: MSM isolate	Result of additional PCR testing performed with: Routine culture isolate	Result of additional PCR testing performed with: Stool DNA extract	Final conclusion
MSM C. jejuni positive/routine culture non-jejuni Campylobacter spp. positive						
74	*C. jejuni*	*C. coli*	*C. jejuni*	NT	*C. jejuni*	*C. jejuni*
89	*C. jejuni*	*C. coli*	*C. jejuni*	NT	*C. jejuni*	*C. jejuni*
103	*C. jejuni*	*C. coli*	NSI	*C. coli*	*C. jejuni/C. coli*	Mixed infection
109	*C. jejuni*	*C. coli*	*C. coli*	*C. coli*	*C. jejuni/C. coli*	Mixed infection
183	*C. jejuni*	*C. coli*	*C. coli*	*C. jejuni/C. coli*	*C. jejuni/C. coli*	Mixed infection
385	*C. jejuni*	*C. lari*	*C. coli*	*C. coli*	*C. jejuni/C. coli*	Mixed infection
417	*C. jejuni*	*C. coli*	*C. jejuni*	NT	*C. jejuni*	*C. jejuni*
527	*C. jejuni*	*C. lari*	*C. jejuni*	*C. jejuni*	*C. jejuni*	*C. jejuni*
590	*C. jejuni*	*C. coli*	*C. coli*	NT	*C. jejuni/C. coli*	Mixed infection
MSM negative/routine culture positive						
331	negative	*C. jejuni*	NSI	*C. coli*	negative	*C. coli*
1492	negative	*C. jejuni*	NSI	NT	*C. coli*	*C. coli*
1598	negative	*C. jejuni*	NSI	*C. species*	*C. coli*	*C. coli*, presumably mixed with another *Campylobacter sp.*
1910	negative	*C. jejuni*	NSI	*C. jejuni*	negative	*C. jejuni*
MSM negative/routine culture non-jejuni Campylobacter spp. positive						
1302	negative	*C. coli*	NSI	*C. coli*	*C. coli*	*C. coli*
1403	negative	*C. coli*	NSI	NT	*C. coli*	*C. coli*
1873	negative	*C. coli*	NSI	*C. coli*	*C. coli*	*C. coli*

[a] NSI, no strain isolated; NT, not tested due to the absence of growth of the stored isolate.

Of the four cases identified by routine culture to be *C. jejuni*, the results for only one case could be confirmed by the additional PCR testing. In all three other cases, the PCRs identified *C. coli* rather than *C. jejuni*, although in one case another *Campylobacter* sp. which could not be identified as *C. coli* or *C. jejuni* may have been present. The last three specimens identified to contain *C. coli* by routine culture were confirmed to be *C. coli* positive by the additional PCR testing.

Other microbiological findings.

Additional microorganisms other than *S. enterica* and *Campylobacter* spp. were identified in 194 of the 2,067 stool specimens examined by routine diagnostic procedures. Among these 194 specimens, the following groups of microorganisms were identified as single infections: pathogenic bacteria (n = 20), viruses (n = 3), and intestinal parasites (n = 140). The following mixed infections were identified at the indicated frequencies: double infections by intestinal parasites, n = 21; double infections by a bacterial species and an intestinal parasite, n = 6; bacterial species and two intestinal parasites, n = 1; three intestinal parasites, n = 2; and four intestinal parasites, n = 1.

In addition, two other mixed infections involving *Salmonella* spp. and/or *C. jejuni* were identified during this study and consisted of one case of mixed infection with *S. enterica* serovar Typhi and *C. jejuni* and one case of mixed infection with serovar Typhi and serovar Saint Paul. Both of these cases were identified by the MSM and routine culture approaches.

DISCUSSION.

In the present study we have assessed the feasibility of an MSM for the detection of the two gastrointestinal bacterial pathogens *S. enterica* and *C. jejuni*. Stool specimens were first screened by real-time PCR, and subsequently, cultures were inoculated only after a positive or inhibited MSM result was obtained. The MSMs proved to be significantly more sensitive that the traditional culture approaches. The rates of detection of *S. enterica* and *C. jejuni* improved by 15% to 18%. Previous studies have also shown improvements in the rates of detection of gastrointestinal pathogens [7, 17, 22, 24, 26].

In our study, only 4 specimens of the 266 true-positive specimens were detected by routine culture and not by the MSM approach, and all these

specimens contained *C. jejuni*, based upon their initial identification. However, subsequent retesting of the isolated strains and/or stool DNA extracts showed no evidence for *C. jejuni* in three of these four specimens. This makes the results of the initial hippurate hydrolysis testing at least questionable. It has been reported by others that false-positive hippurate hydrolysis results can be obtained, and it has been hypothesized that this may be the result of the transfer of amino acids or peptides from the culture media or their production during the incubation [8, 19]. If this is the case, the MSM would have missed only a single *C. jejuni*-positive stool specimen among the 195 confirmed cases, increasing the sensitivity and the NPV of the *C. jejuni* MSM to 99.5% and 99.9%, respectively. However, we cannot rule out the possibility that the initial identification was correct and that in fact these three specimens were mixed *C. jejuni*-*C. coli* infections.

The main advantage of the MSM is the speed in which definitely negative samples can be identified at a confidence higher than that for routine culture. During our study, the MSM generated negative results within a mean 1.5 days after the specimen was received by the laboratory, which is nearly 2.5 days earlier than the time to negative results by routine culture. These data confirm the hypothesized speed of the MSM in routine practice. With regard to generating final positive results, the MSM was slightly slower than routine culture (6.9 to 6.3 days and 5.2 to 4.4 days, respectively), resulting in average delays of 1.7 and 2.0 days for *S. enterica* and *C. jejuni*, respectively. A delay of 1 day is expected for both MSMs, as the MSMs have to wait for the PCR results before cultures can be inoculated. The further delay was most likely caused by the fact that VITEK 2 system identification and resistance profiling for *S. enterica* strains positive by MSM was usually performed overnight, since the isolates were identified in the afternoon. This was mandatory due to the fact that the MSM approach was carried out by a single technician, and therefore, all culture handlings took place after the PCR was performed. In addition, MSM cultures were prone to increased hands-on time due to the fact that they were performed by technicians who normally do not perform routine cultures for *S. enterica* and *C. jejuni*. When these MSM cultures are performed by routine technicians, it is, however, expected that the total delay will be no more than the 1 day introduced by PCR. On the other hand, due to the high specificities and PPVs of the

MSMs, preliminary positive results based on the real-time PCR results can be reported to the attending physician with a high degree of confidence. These preliminary positive results were reported, on average, 3.8 and 2.9 days earlier than the final routine culture results for *S. enterica* and *C. jejuni*, respectively.

Another advantage of using the MSM approach is that it increases the overall sensitivity. The subsequent culturing upon a positive MSM result yielded in total 19 additional culture-positive specimens not detected by routine culture. This may be explained by the fact that a technician might be tempted to look more intensively for a suspicious colony because the MSM already flagged a specimen as positive, whereas in routine culture, the time pressure introduced by the overall workload may prevent such a more thorough search.

An interesting observation made during this study was the identification of several mixed *C. jejuni-C. coli* infections. Although in both separate approaches these mixed infections would not have been noticed, the discrepancy analysis was able to reveal their existence. Others have also reported mixed *C. jejuni-C. coli* infections in human patients with a similar prevalence [19, 24], as well as in cultures originating from food production animals or poultry and raw meat from retail sales outlets [8, 12, 20]. Our results confirm the findings described in those previous reports and indicate that mixed *Campylobacter* infections do occur in about 1 to 5% of the laboratory-confirmed cases of *Campylobacter*-associated gastroenteritis.

Although PCR inhibition can pose a serious problem for the molecular-based screening of stool specimens, the methods used in our study proved to be insensitive to PCR inhibition. This is important if the MSM approach is to be implemented in the routine laboratory, as inhibited specimens still need to be cultured, whereas one of the main goals of the MSM is to reduce the TAT and the workload involved with stool cultures. Furthermore, the inhibition rates reported in this work (3.7% after initial testing, 1.8% after retesting) are regarded to be excellent. Others have reported inhibitions rates of 8 to 15% at similar input levels [4, 6, 15] and 1 to 63% at lower input levels [15, 28].

On the basis of the data presented here, the introduction of an MSM for routine processing of stool specimens in the clinical microbiology laboratory

is feasible. However, to accommodate the introduction of an MSM in the routine laboratory, several adaptations to the format presented here may be necessary. First of all, to obtain an improved success rate with the subsequent isolation of *C. jejuni* from MSM-positive specimens, the delay in the inoculation of the stool specimens must be limited at most to 1 day after the specimen is received by the laboratory. To accommodate this, specimens received on a Friday and during the weekend should be inoculated for *C. jejuni* culture as soon as possible. On the other hand, the MSM approach could also be performed during the weekends and not only on weekdays, as was the case in our study. However, this will need a change in laboratory management, as most microbiology laboratories offer only limited services during the weekend. Furthermore, it is advisable to incorporate tests for other gastrointestinal pathogens into the MSM in an attempt to further decrease the culture workload involved with the testing of stool specimens. Multiplexing of the different MSMs seems to be mandatory because with the increase in the number of screened targets, the throughput per real-time PCR run is diminished, which may prove to be limiting from the perspective of the high workload needed to process all stool specimens sent to the laboratory, especially during the seasonal peaks. Automation of the DNA extraction process may also be considered to decrease the hands-on time required by the technicians performing the MSM.

In conclusion, our data indicate that an MSM approach for the screening of stool specimens for gastrointestinal pathogens in a routine clinical microbiology laboratory is feasible and that the introduction of MSM will result in faster reporting of the final results for negative specimens, as well as preliminary positive results, and with only minimal delay in the reporting of the final results for positive specimens. In addition, the MSM will significantly improve the performance of the screening of stool specimens with regard to sensitivity, whereas the specificity, PPV, and NPV will also be improved, although to a smaller extent. We are currently conducting further research on automated extraction and multiplex real-time PCR to accommodate the introduction of an MSM for stool specimens in our routine laboratory.

ACKNOWLEDGMENTS.

Hans Wilke of the Laboratory for Medical Microbiology, Twente Achterhoek, Enschede, The Netherlands, is kindly acknowledged for providing the primers and probe for the *C. jejuni* and *C. coli* confirmation real-time PCR assays. Wim Ang (Department of Medical Microbiology, Erasmus University Medical Center) is acknowledged for generously providing the Penner serotype *C. jejuni* strains. We also acknowledge Alex van Belkum for critical reading of the manuscript and his helpful discussions and suggestions.

This study was supported in part by bioMérieux Benelux BV.

REFERENCES.

1. **Abu Al-Soud, W., and P. Rådström.** 1998. Capacity of nine thermostable DNA polymerases to mediate amplification in the presence of PCR-inhibiting samples. Appl. Environ. Microbiol. **64:**3748-3753.
2. **Abu Al-Soud, W., and P. Rådström.** 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. J. Clin. Microbiol. **38:**4463-4470.
3. **Allos, B. M.** 1997. Association between *Campylobacter* infection and Guillain-Barré syndrome. J. Infect. Dis. **176:**S125-S128.
4. **Beld, M., R. Minnaar, J. Weel, C. Sol, M. Damen, H. van der Avoort, P. Wertheim-van Dillen, A. van Breda, and R. Boom.** 2004. Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. J. Clin. Microbiol. **42:**3059-3064.
5. **Best, E. L., E. J. Powell, C. Swift, K. A. Grant, and J. A. Frost.** 2003. Applicability of a rapid duplex real-time PCR assay for speciation of *Campylobacter jejuni* and *Campylobacter coli* directly from culture plates. FEMS Microbiol. Lett. **229:**237-241.
6. **Boom, R., C. Sol, J. Weel, K. Lettinga, Y. Gerrits, A. van Breda, and P. Wertheim-Van Dillen.** 2000. Detection and quantitation of human cytomegalovirus DNA in faeces. J. Virol. Methods **84:**1-14.
7. **Chiu, C.-H., and J. T. Ou.** 1996. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *invC*, by an enrichment broth culture-multiplex PCR combination assay. J. Clin. Microbiol. **34:**2619-2622.
8. **Denis, M., C. Soumet, K. Rivoal, G. Ermel, D. Blivet, G. Salvat, and P. Colin.** 1999. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Lett. Appl. Microbiol. **29:**406-410.
9. **De Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, N. J. van Leeuwen, A. I. M. Bartelds, and Y. T. H. P. van Duynhoven.** 2001. Gastroenteritis in sentinel general practices, The Netherlands. Emerg. Infect. Dis. **7:**82-89.
10. **De Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, N. J. van Leeuwen, J. Vinjé, and Y. T. H. P. van Duynhoven.** 2001. Etiology of gastroenteritis in sentinel general practices in The Netherlands. Clin. Infect. Dis. **33:**280-288.
11. **Dusch, H., and M. Altwegg.** 1995. Evaluation of five new plating media for isolation of *Salmonella* species. J. Clin. Microbiol. **33:**802-804.
12. **Englen, M. D., and P. J. Fedorka-Cray.** 2002. Evaluation of a commercial diagnostic PCR for the identification of *Campylobacter jejuni* and *Campylobacter coli*. Lett. Appl. Microbiol. **35:**353-356.
13. **Farrell, J. J., L. J. Doyle, R. M. Addison, L. B. Reller, G. S. Hall, and G. W. Procop.** 2005. Broad-range (pan) *Salmonella* and *Salmonella* serotype Typhi-specific real-time PCR assays. Am. J. Clin. Pathol. **123:**339-345.
14. **Guarrant, R. L., J. M. Hughes, N. L. Lima, and J. Crane.** 1990. Diarrhea in developed and developing countries: magnitude, special settings, and etiologies. Rev. Infect. Dis. **12:**S41-S50.
15. **Holland, J. J., L. Louie, A. E. Simor, and M. Louie.** 2000. PCR detection of *Escherichia coli* O157:H7 directly from stools: evaluation of commercial extraction methods for purifying fecal DNA. J. Clin. Microbiol. **38:**4108-4113.
16. **Hoorfar, J., P. Ahrens, and P. Rådström.** 2000. Automated 5' nuclease PCR assay for identification of *Salmonella enterica*. J. Clin. Microbiol. **38:**3429-3435.
17. **Iijima, Y., N. T. Asako, M. Aihara, and K. Hayashi.** 2004. Improvement in the detection rate of diarrhoeagenic bacteria in human stool specimens by a rapid real-time PCR assay. J. Med. Microbiol. **53:**617-622.
18. **Jensen, A. N., M. T. Andersen, A. Dalsgaard, D. L. Baggesen, and E. M. Nielsen.** 2005. Development of real-time PCR and hybridization methods for detection and identification of thermophilic *Campylobacter* spp. in pig faecal samples. J. Appl. Microbiol. **99:**292-300.
19. **Kolackova, I., and R. Karpiskova.** 2005. Species level identification of thermotolerant campylobacters. Vet. Med. Czech. **50:**543-547.

20. **Kramer, J. M., J. A. Frost, D. R. A. Wareing, and F. J. Bolton.** 2000. *Campylobacter* contamination of raw meat and poultry at retail sale: identification of multiple types and comparison with isolates from human infection. J. Food Prot. **63:**1654-1659.
21. **Kreader, C. A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. J. Clin. Microbiol. **34:**1102-1106.
22. **Kulkarni, S. P., S. Lever, J. M. J. Logan, A. J. Lawson, J. Stanley, and M. S. Shafi.** 2002. Detection of Campylobacter species: a comparison of culture and polymerase chain reaction based methods. J. Clin. Pathol. **55:**749-753.
23. **LaGier, M. J., L. A. Joseph, T. V. Passaretti, K. A. Musser, and N. M. Cirino.** 2004. A real-time multiplexed PCR assay for rapid detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli*. Mol. Cell. Probes **18:**275-282.
24. **Lawson, A. J., J. M. J. Logan, G. L. O'Neill, M. Desal, and J. Stanley.** 1999. Large-scale survey of *Campylobacter* species in human gastroenteritis by PCR and PCR-enzyme-linked immunosorbent assay. J. Clin. Microbiol. **37:**3860-3864.
25. **Logan, J. M. J., K. J. Edwards, N. A. Saunders, and J. Stanley.** 2001. Rapid identification of *Campylobacter* spp. by melting peak analysis of biprobes in real-tme PCR. J. Clin. Microbiol. **39:**2227-2232.
26. **Maher, M., C. Finnegan, E. Collins, B. Ward, C. Carroll, and M. Cormican.** 2003. Evaluation of culture methods and a DNA probe-based PCR assay for detection of *Campylobacter* species in clinical specimens of feces. J. Clin. Microbiol. **41:**2980-2986.
27. **Malorny, B., E. Paccassoni, P. Fach, C. Bunge, A. Martin, and R. Helmuth.** 2004. Diagnostic real-time PCR for the detection of *Salmonella* in food. Appl. Environ. Microbiol. **70:**7046-7052.
28. **Monteiro, L., D. Bonnemaison, A. Vekris, K. G. Petry, J. Bonnet, R. Vidal, J. Cabrita, and F. Megraud.** 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J. Clin. Microbiol. **35:**995-998.
29. **Nogva, H. K., A. Bergh, A. Holck, and K. Rudi.** 2000. Application of the 5'-nuclease PCR assay in evaluation and development of methods for quantitative detection of *Campylobacter jejuni*. Appl. Environ. Microbiol. **66:**4029-4036.
30. **Rudi, K., H. K. Høidal, T. Katla, B. K. Johansen, J. Nordal, and K. S. Jakobsen.** 2004. Direct real-time PCR quantification of *Campylobacter jejuni* in chicken fecal and cecal samples by integrated cell concentration and DNA purification. Appl. Environ. Microbiol. **70:**790-797.
31. **Ruiz, J., M. L. Nunez, J. Diaz, I. Lorente, J. Perez, and J. Gomez.** 1996. Comparison of five plating media for isolation of *Salmonella* species from human stools. J. Clin. Microbiol. **34:**686-688.
32. **Schuurman, T., A. Roovers, W. K. van der Zwaluw, A. A. van Zwet, L. J. M. Sabbe, A. M. D. Kooistra-Smid, and Y. T. H. P. van Duynhoven.** 2007. Evaluation of 5'-nuclease and hybridization probe assays for the detection of Shiga toxin-producing *Escherichia coli* in human stools. J. Microbiol. Methods doi:10.1016/j.mimet. 2007.05.016.
33. **Van Belkum, A., N. van den Braak, P. Godschalk, W. Ang, B. Jacobs, and M. Gilbert.** 2001. A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. Nat. Med. **7:**752-753.
34. **van den Brandhof, W. E., G. A de Wit, M. A. S. de Wit, and Y. T. H. P. van Duynhoven.** 2004. Costs of gastroenteritis in The Netherlands. Epidemiol. Infect. **132:**211-221.
35. **Van Hees, B. C., M. J. Veldman-Ariesen, B. M. de Jongh, M. Tersmette, and W. van Pelt.** 2007. Regional and seasonal differences in incidence and antibiotic resistance of *Campylobacter* from a nationwide surveillance study in The Netherlands: an overview of 2000-2004. Clin. Microbiol. Infect. **13:**305-310.
36. **Van Pelt, W., M. A. de Wit, W. J. Wannet, E. J. Ligtvoet, M. A. Widdowson, and Y. T. van Duynhoven.** 2003. Laboratory surveillance of bacterial gastroentertic pathogens in The Netherlands, 1991-2001. Epidemiol. Infect. **130:**431-441.

37. **Widjojoatmodjo, M. N., A. C. Fluit, R. Torensma, G. P. H. Verdonk, and J. Verhoef.** 1992. The magnetic immuno polymerase chain reaction assay for direct detection of *Salmonellae* in fecal samples. J. Clin. Microbiol. **30:**3195-3199.
38. **Yuki, N.** 2001. Infectious origins of, and molecular mimicry in, Guillain-Barré and Fisher syndromes. Lancet Infect. Dis. **1:**29-37.

CHAPTER 6

Evaluation of 5′-Nuclease and Hybridization Probe Assays for the Detection of Shiga Toxin-Producing *Escherichia coli* in Human Stools

Tim Schuurman [1]
Alexander Roovers [2]
W. Kim van der Zwaluw [3]
Anton A. van Zwet [4]
Luc J.M. Sabbe [2]
A. Mirjam D. Kooistra-Smid [1]
Yvonne T.H.P. van Duynhoven [3]

[1] Laboratory for Infectious Diseases, Department of Research & Development, Groningen
[2] Oosterschelde Hospitals, Department of Medical Microbiology and Immunology, Goes
[3] National Institute of Public Health and the Environment, Center for Infectious Disease Control, Bilthoven
[4] Hospital Rijnstate, Department of Medical Microbiology, Arnhem
The Netherlands

Journal of Microbiological Methods. 2007. **70:**406-415

ABSTRACT.

5′-Nuclease and a hybridization probe assays for the detection of Shiga toxin-producing *Escherichia coli* were validated with regard to selectivity, analytical sensitivity, reproducibility and clinical performance. Both assays were capable of detecting the classical stx_1 and stx_2 genes when challenged with reference strains of *E. coli* (n = 40), although 1 to 4 minority sequence variants, whose clinical relevance is limited (stx_{1c}, stx_{1d}, and stx_{2f}), were detected less efficiently or not at all by one or both assays. No cross reaction was observed for both assays with 37 strains representing other gastrointestinal pathogens, or normal gastrointestinal flora. Analytical sensitivity ranged from 3.07 to 3.52 $\log_{10}$ and 3.42 to 4.63 $\log_{10}$ CFU/g of stool for 5′-nuclease and hybridization probe assay, respectively. Reproducibility was high with coefficients of variation of ≤ 5% for both inter- and intra-assay variation. Clinical performance was identical with a panel of archived positive specimens (n = 19) and a prospective panel of stools associated with bloody diarrhea (n = 115). In conclusion, both assays proved to be sensitive and reproducible.

INTRODUCTION.

The group of Shiga toxin-producing *Escherichia coli* (STEC), also referred to as Vero toxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC), is one of the six groups of *E. coli* that can cause diarrhea in humans. The other 5 groups are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) [30]. *E. coli* strains belonging to the group of STEC are characterized by the presence of at least one of the two genes, stx_1 and stx_2, that encode the Shiga toxins (Stx1 and Stx2). Infections caused by STEC can range from mild, self-limiting diarrhea to severe hemorrhagic colitis (HC) and hemolytic–uremic syndrome (HUS). Most of the severe complications of STEC infection are due to an infection by *E. coli* O157:H7/H$^-$. Its first description dates back to 2 outbreaks in Oregon and Michigan. Since the first report of infection caused by this serotype in 1982 [35], many outbreaks and sporadic cases have been reported. Therefore, laboratory surveillance for STEC has focused on the detection of *E. coli* O157:H7/H$^-$, and is based on the incapacity of *E. coli* O157:H7/H$^-$ to ferment sorbitol during stool culture on sorbitol MacConkey agar (SMAC). However, SMAC culture does not detect sorbitol-fermenting (SF) *E. coli* O157:H$^-$, and non-O157 STEC serotypes, which generally ferment sorbitol. This SF *E. coli* O157:H$^-$ clone, is widespread in Germany and the Czech Republic and has been associated with HUS [7]. Furthermore, non-O157 strains are also increasingly associated with diarrhea, HC and HUS in several European countries, Argentina, Chile, South Africa, the United States and Australia [24, 30]. In fact, in several European countries, including Belgium, Czech Republic, Denmark, Finland, and Spain, non-O157 strains dominate over O157 strains in STEC-associated diarrheal illnesses. In addition, recent reports suggest that although non-O157 strains are more likely to cause watery diarrhea than bloody diarrhea, some serogroups (O26, O103, O111, and O145) may be more virulent and may therefore be more likely to precipitate HUS. Therefore, detection of non-O157 STEC strains is becoming more and more necessary in routine clinical microbiology laboratories, not only from a diagnostic perspective, but also from an epidemiological point of view.

To facilitate the detection of both O157 and non-O157 STEC, alternatives for conventional SMAC screening need to be developed and implemented. Accepted alternatives for SMAC screening of stools are detection of the Shiga toxins in stool by enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) [15, 26, 32], and real-time PCR [4, 6, 23, 29, 32, 33]. EIA and ELISA are rapid and simple to perform, without the need for specialized laboratory equipment. However, the sensitivity and specificity of EIA and ELISA are debated [2, 3, 16, 27, 32]. Therefore, real-time PCR amplification of *stx*$_1$ and *stx*$_2$ provides an interesting alternative for screening stool specimens for STEC. A drawback of PCR amplification assays with stool samples is the significant risk for PCR inhibition. However, by using an effective sample preparation strategy and amplification facilitators, such as bovine serum albumin (BSA), the inhibitory effects can be minimized [1, 28].

The current burden of non-O157 STEC-associated diarrheal disease in The Netherlands is unknown, although two studies in the late nineties suggested that non-O157 strains are also dominant in our country [37]. This lack of knowledge about the current Dutch non-O157 STEC prevalence, combined with the reported non-O157 STEC prevalence rates in the surrounding countries, and the increasing association of the capability of non-O157 strains to cause severe disease, has prompted us to conduct a one-year nationwide surveillance to assess the prevalence of STEC (both O157 and non-O157) in The Netherlands. This survey will be real-time PCR based, with subsequent isolation of STEC from the PCR-positive stools.

Several real-time PCR assays have been described for detection of STEC in the literature [4, 6, 18, 20, 23, 32, 33]. None of the 5′-nuclease (TaqMan) and hybridization probe (LightCycler) assays, which are the 2 dominant real-time PCR chemistries used by most laboratories in The Netherlands, have been validated against an assay of different chemistry. In case of large surveys, it is especially important that the methods utilized have similar performance characteristics, and that the differences in performance are well documented. Therefore, as the selected methods are intended to be used by eight independent laboratories throughout The Netherlands, we conducted a thorough validation study with a hybridization probe based LightCycler assay

(LC) and a 5′-nuclease based TaqMan (TM) with regard to selectivity, analytical performance, analytical reproducibility, and clinical performance.

MATERIALS AND METHODS.

Bacterial and fungal strains.

A panel of 40 well characterized *E. coli* strains and a total of 37 bacterial and fungal strains representing other gastrointestinal pathogens and normal gastrointestinal flora were used in this study. All *E. coli* strains are listed in Table 6.1, whereas other organisms are listed in Table 6.2. All organisms were grown on the appropriate media and under appropriate conditions for 16 to 48 h and harvested (approximately 10^9 CFU) into 1 ml of physiological saline. Crude chromosomal DNA extracts were prepared from the strains by heat lysis for 10 min at 95°C followed by centrifugation at 16,000 × g. for 1 min to sediment debris.

Stool specimens.

Clinical stool specimens were collected by the Laboratory for Infectious Diseases (LvI), Groningen, The Netherlands, the Laboratory for Medical Microbiology and Immunology, Oosterschelde Hospitals (LMMI-OH), Goes, The Netherlands, and the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

Three different panels of stool specimens were collected: i) 8 unformed stools and 5 watery, stools were collected at the LvI, and shown to be negative by 5′-nuclease PCR for STEC. From these stools, 2 pools (unformed and watery) were made to serve as a background stool matrices in spiking experiments, ii) 19 stools known to contain STEC by culture (SMAC, n = 10), real-time PCR (TaqMan, n = 4, or LightCycler, n = 4), or cytotoxicity testing (Vero cell cytotoxicity assay, n = 1) were collected by the RIVM (n = 8), the LvI (n = 7) and the LMMI-OH (n = 4), and iii) a prospectively collected panel at the LvI, consisting of 115 stool specimens for which conventional testing was performed for bacterial and/or parasitological gastrointestinal pathogens. Of these 115 specimens, 90 were from patients with a recent history of bloody diarrhea (as reported by the general practitioners [GP] requesting bacterial and/or parasitological examination of the stool specimen), but without visible blood in the sampled

stool specimen, 9 showed visible blood present in the collected stool specimen, and for the remaining 16 specimens both criteria were present.

TABLE 6.1. *Escherichia coli* strains used in this study[a].

			stx gene present[b]		TaqMan	LightCycler PCR	
Species	Serotype	Origin	*stx*$_1$	*stx*$_2$	PCR	*stx*$_1$	*stx*$_2$
E. coli	O157:H7	RIVM 1120300011	-	+	+	-	+
	O157:H-	RIVM 1120300015	-	+	+	-	+
	O91:H-	RIVM 1120200026	+	+	+	+	+
	O70:H-	RIVM 1120200036	+	+	+	+	+
	O157:H-	RIVM 1120400083	+	+	+	+	+
	O166:H*	RIVM 1120200134	+	+	+	+	+
	Ont.:H*	RIVM 1120200241	-	+	+	-	+
	O121:H*	RIVM 1120200242	-	+	+	-	+
	O26:H-	RIVM 1120200245	+	-	+	+	-
	O157:H7	RIVM 1120300271	+	+	+	+	+
	O113:H*	RIVM 1120200280	+	+	+	+	+
	O118:H*	RIVM 1120200302	+	-	+	+	-
	O87:H16	RIVM 1120100407	-	+	+	-	+
	O87:H16	RIVM 1120100407R	-	+	+	-	+
	O5:H-	RIVM 1120100461	+	+	+	+	+
	O111:H-	RIVM 95NR1	+	+	+	+	+
	O113:H21	RIVM 98NK2	-	+	+	-	+
	O145:H*	RIVM 1129800005	+	-	+	+	-
	O103:H2	RIVM 1120000131	+	+	+	+	+
	O76:H19	RIVM 1120000240	+	-	+	+	-
	O146:H-	RIVM 1120100212	+	+	+	+	+
	O26:H*	RIVM 1120100321	+	-	+	+	-
	O26:H11	RIVM ECOL 401	+	-	+	+	-
	O157:H7	RIVM 1120500075	-	+	+	-	+
	O157:H7	RIVM ECOL 411	+	+	+	+	+
	O157:H7	ATCC 35150	+	+	+	+	+
	O128:NM	IHUM 3560/96[c]	+ (*stx*$_{1c}$)	-	+[L]	+	-
	O78:NM	IHUM 3653/97	+ (*stx*$_{1c}$)	-	+[L]	+	-
	O8:NM	IHUM 7139/96	+ (*stx*$_{1d}$)	-	-	+	-
	O157:NM	IHUM E32511/HSC	-	+ (*stx*$_{2c}$)	+	-	+
	Ont:H12	IHUM EH250	-	+ (*stx*$_{2d\text{-}n\text{-}act}$)	+	-	+
	O91:H21	IHUM B2F1	-	+ (*stx*$_{2d\text{-}act}$)	+	-	+
	Ont:NM	IHUM 2771/97	-	+ (*stx*$_{2e}$)	+	-	+
	O128:H2	IHUM T4/97	-	+ (*stx*$_{2f}$)	-	-	-
	O145:H34	RIVM 1120200176	-	-	-	-	-
	O103:H2	RIVM 1120200177	-	-	-	-	-
	O111:H*	RIVM 1120200277	-	-	-	-	-
	O157:H16	RIVM 1120200336	-	-	-	-	-
	O110:H*	RIVM 1120200496	-	-	-	-	-
	O23:H15	ATCC 25922	-	-	-	-	-

[a] nt, non-typable; H*, H-antigen not typed; NM, non-motile; RIVM, National Institute for Public Health and the Environment; ATCC, American Type Culture Collection; IHUM, Institue of Hygene and National Consulting Laboratory on Hemolytic Uremic Syndrome University of Münster Germany; +, positive; -, negative; +[L], strain detected at lower efficiency; n-act, non-activatable; act, activatable.

[b] Variant toxins are depicted in parathesis.

[c] IHUM type strains were kindly donated by Prof. H. Karch of the University of Münster.

TABLE 6.2. Non-*E. coli* species used for exclusivity testing[a].

Species	Origin	TaqMan PCR	LightCycler PCR	
			stx$_1$	*stx*$_2$
Bacteroides fragilis	ATCC 25285	-	-	-
Bacteroides melaninogenicus	ATCC 25845	-	-	-
Campylobacter jejuni	ATCC 29428	-	-	-
Campylobacter coli	LvI 176	-	-	-
Candida albicans	ATCC 10231	-	-	-
Candida kruzei	ATCC 14243	-	-	-
Citrobacter diversus	LvI 197	-	-	-
Citrobacter freundii	ATCC 8090	-	-	-
Clostridium difficile	ATCC 9689	-	-	-
Clostridium perfringens	ATCC 13124	-	-	-
Corynebacterium diphtheria	ATCC 11913	-	-	-
Corynebacterium pseudodiphteriticum	ATCC 10700	-	-	-
Enterobacter aerogenes	ATCC 13048	-	-	-
Enterobacter agglomerans	LvI 74	-	-	-
Enterobacter amnigenus	LvI 137	-	-	-
Enterobacter cloacae	ATCC 13047	-	-	-
Enterococcus faecalis	ATCC 51299	-	-	-
Hafnia alvei	LvI 112	-	-	-
Klebsiella oxytoca	ATCC 43863	-	-	-
Klebsiella pneumoniae	ATCC 13883	-	-	-
Listeria monocytogenes	ATCC 19117	-	-	-
Peptostreptococcus anaerobius	ATCC 27337	-	-	-
Proteus mirabilis	ATCC 12453	-	-	-
Proteus vulgaris	ATCC 13315	-	-	-
Pseudomonas aeruginosa	ATCC 27853	-	-	-
Salmonella enterica serovar enteritidis	ATCC 13076	-	-	-
Salmonella enterica serovar typhimurium	ATCC 14028	-	-	-
Serratia liquefaciens	ATCC 27592	-	-	-
Serratia marcescens	ATCC 13880	-	-	-
Shigella boydii	ATCC 9207	-	-	-
Shigella dysenteriae[b]	LvI 168	-	-	-
Shigella flexneri	ATCC 12022	-	-	-
Shigella sonnei	ATCC 25931	-	-	-
Staphylococcus aureus	ATCC 25923	-	-	-
Staphylococcus epidermidis	ATCC 155	-	-	-
Streptococcus pneumoniae	ATCC 49619	-	-	-
Yersinia enterocolitica	ATCC 23715	-	-	-

[a] ATCC, American Type Culture Collection; LvI, Laboratory for Infectious Diseases Clinical Isolates; -, negative.
[b] serotype 2 (not carrying *stx*).

Preparation of spiked stool specimens for sensitivity and reproducibility testing.

The pooled unformed stool specimens were aliquoted and subsequently seeded with 10-fold serial dilutions of *E. coli* O26:H11 (RIVM ECOL 401, *stx*$_1$ positive) ranging from 7.08 $\log_{10}$ to 1.08 $\log_{10}$ CFU/g of feces. The pooled watery stool specimens were also aliquoted and subsequently seeded with *E. coli* O157:H7 (RIVM 1120500075, *stx*$_2$ positive) ranging from 6.88 $\log_{10}$ to 0.88 CFU/g of feces. In addition a single unseeded (0 CFU/g) specimen was included for each stool background (unformed and watery). Both panels

were stored in two 600 µl and eight 150 µl aliquots at −20°C. Half of each panel (1 × 600 µl and 4 × 150 µl of each panel) was shipped to the laboratory in Goes by courier service on dry ice and on arrival, these specimens were immediately stored again at −20°C. To mimic the transport conditions, the other half of both panels was placed on dry ice until notification that the courier had delivered the specimens to the laboratory in Goes.

DNA extraction.

DNA was extracted from the stool specimens using the semi-automated NucliSENS miniMAG instrument (bioMérieux, Boxtel, The Netherlands) in combination with the NucliSENS Magnetic Extraction Reagents (bioMérieux) according to the manufacturer's instructions. The NucliSENS miniMAG is a semi-automated bench-top instrument for the extraction of nucleic acids from 12 specimens at a time, using a magnetic bead-based adaptation of the silica-guanidiniumthiocyante procedure originally described by Boom *et al.* [9]. For fecal specimens, the pre-extraction protocol stool samples, release 1.0 for the NucliSENS easyMAG was used [8] and subsequently 100 µl of fecal suspension (33–50% [wt/vol]) was used as input with the miniMAG. In addition, approximately 6000 copies of the phocin herpes virus (PhHV) was co-purified with the fecal suspension, and served as an internal control. DNA was eluted in 100 µl of elution buffer.

Triplex 5′-nuclease TaqMan assay for STEC.

All TaqMan experiments were performed by two technicians at the department of R&D, Laboratory for Infectious Diseases in Groningen. Primers and probes targeting the *stx*$_1$ gene, based on the sequences reported by Jinneman *et al.* [23] , were modified to a minor groove binder (MGB) primer/probe set to accommodate the incorporation of a non-fluorescent quencher (NFQ) for multiplex PCR. For the *stx*$_2$ gene, including the variants *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$, and *stx*$_{2g}$, a new primer/probe design was conducted with the Primer Express v 1.5 software package (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) after alignment of 43 *stx*$_2$ gene sequences (comprising *stx*$_2$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$, *stx*$_{2f}$, and *stx*$_{2g}$) with the Auto Assembler software v 2.1 software package (Applied Biosystems). Both the *stx*$_1$ and the newly designed *stx*$_2$ targeting primer/probe sets were checked using alignments of *stx*$_1$ and *stx*$_2$ gene sequences (n = 16 [*stx*$_1$], n =

10 [stx_{1c}], n = 4 [stx_{1d}], and n = 43 [stx_2 and variants]), and subsequently with the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/).

For the PhHV internal control target, the primers described by Niesters [31] were used, whereas the probe was modified to a MGB-probe to accommodate labeling with the fluorophore NED™ and NFQ for multiplex PCR. All primers and probes for the TaqMan assay were purchased from Applied Biosystems, and are listed in Table 6.3.

TaqMan PCR was carried out as a triplex PCR on the Applied Biosystems 7500 Real-Time PCR System (AB 7500) with the use of the TaqMan Universal PCR Master Mix (Applied Biosystems). Each 25 µl reaction consisted of 5 µl of extracted DNA, 1 x TaqMan Universal PCR Master Mix, the forward primers stx1F934_mod, stx2F_LvI, and PhHV-267s and the reverse primer PhHV-337as at 300 nM each, the reverse primers stx1R1042_G, stx1R1042_modC, stx2R_G_LvI, and stx2R_A_LvI at 150 nM each, the TaqMan MGB probes stx1P990_mod_MGB, stx2P_LvI_MGB, and PhHV-1-MGB at 100 nM each, and 2.5 µg bovine serum albumin (Roche Diagnostics Nederland B.V., Almere, The Netherlands).

Reactions were run under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min with the AB 7500 running with the 9600 emulation mode disabled. Data were acquired and analyzed with the 7500 System Sequence Detection Software v 1.2.3 (Applied Biosystems).

LightCycler assay for STEC.

All LightCycler experiments were performed by two technicians at the department of medical microbiology and immunology, Oosterschelde Hospitals in Goes. Amplification on the LightCycler (Roche Diagnostics Nederland B.V.) was carried out as described by Bellin *et al.* [6], with minor modifications. Briefly, reactions were performed using the LightCycler FastStart DNA Master HybProbe Kit (Roche Diagnostics Nederland B.V.) and the primers, and probes listed in Table 6.3. The 20 µl reaction consisted of 5 µl of extracted DNA, 2 µl of 10 × LightCycler FastStart DNA Master for hybridization probes, the primers StxA1 598, StxA1 1015, StxA2 679, StxA2 942 (MWG-Biotech, Ebersberg, Germany), the probes StxA2 FL 769 and StxA2 LC 799 R6 (TIB MOLBIOL, Berlin, Germany) at 1.0 µM each, the probes StxA1 FL 724 and StxA1 LC 693 R7 (TIB MOLBIOL) at 0.5 µM each,

TABLE 6.3. Primer and probe sequences used in the STEC real-time PCRs.

Application and target	Sequence name	Sequence[a]	Tm (°C)	Reference
TaqMan, *stx*$_1$	stx1F934_mod	5'-TGG CAT TAA TAC TGA ATT GTC ATC ATC-3'	59.2	[23], this study
	stx1R1042_G	5'-GCG TAA TCC CAC GGA CTC TTC-3'	59.6	[23]
	stx1R1042_modC	5'-GCG TAA TCC CAC GCA CTC TT-3'	58.8	[23], this study
	stx1P990_mod_MGB	5'-FAM-TTC CTT CTA TGT GTC CGG CAG-NFQ-MGB -3'	69.0	[23], this study
stx$_2$	stx2F_Lvl	5'-CCG GAA TGC AAA TCA GTC GT-3'	59.5	This study
	stx2R_G_Lvl	5'-ACC ACT GAA CTC CAT TAA CGC C-3'	59.0	This study
	stx2R_A_Lvl	5'-TAC CAC TAA ACT CCA TTA ACG CCA-3'	58.7	This study
	stx2P_Lvi_MGB	5'-FAM-ACT CAC TGG TTT CAT CAT A-NFQ-MGB-3'	68.9	This study
PhHV (internal control)	PhHV-267s	5'-GGG CGA ATC ACA GAT TGA ATC-3'	58.1	[31]
	PhHV-337as	5'-GCG GTT CCA AAC GTA CCA A-3'	58.1	[31]
	PhHV-1-MGB	5'-NED-CGC CAC CAT CTG GAT-NFQ-MGB-3'	70.0	[31], this study
LightCycler, *stx*$_1$	StxA1 598	5'-AGT CGT ACG GGG ATG CAG ATA AAT-3'	56.9	[6]
	StxA1 1015	5'-CCG GAC ACA TAG AAG GAA ACT CAT-3'	55.3	[6]
	StxA1 FL 724	5'-CTG TCA CAG TAA CAA ACC GTA ACA TCG CTC-Fl-3'	65.5	[6]
	StxA1 LC 693 R7	5'-LCR$_{705}$-TGC CAC AGA CTG CGT CAG TGA GGT-ph-3'	67.5	[6]
stx$_2$	StxA2 679	5'-TTC CGG AAT GCA AAT CAG TC-3'	52.5	[6]
	StxA2 942	5'-CGA TAC TCC GGA AGC ACA TTG-3'	54.6	[6]
	StxA2 FL 769	5'-MAG AGC AGT TCT GCG TTT TGT CAC TGT CA-Fl-3'	65.0	[6]
	StxA2 LC 799 R6	5'-LCR$_{640}$-AGC AGA AGC CTT ACG CTT CAG GC-ph-3'	63.3	[6]
PhHV (internal control)	PhHV-267s	5'-GGG CGA ATC ACA GAT TGA ATC-3'	58.1	[31]
	PhHV-337as	5'-GCG GTT CCA AAC GTA CCA A-3'	58.1	[31]
	PhHV-305tq	5'-FAM-TTT TTA TGT GTC CGC CAC CAT CTG GAT C-TAMRA-3'	68.8	[31]

[a] FAM, 6-carboxyfluorescein; NFQ-MGB, non fluorescent quencher minor groove binder; NED, 2'-chloro-5'-fluoro-7',8'-fused phenyl-1,4-dichloro-6-carboxy-fluorescein; Fl, fluorscein; LCR, LightCycler Red; M: C or A; ph, phosphate; TAMRA, 6-carboxytetramethylrhodamine.

and 4.0 mM $MgCl_2$. For the PhHV internal control, a separate 20 µl reaction was run with the following composition: 5 µl of extracted DNA, 2 µl of 10 × LightCycler FastStart DNA Master for hybridization probes, the primers PhHV-267s, and PhHV-337as (TIB MOLBIOL) at 0.5 µM each, the probe PhHV-305tq (TIB MOLBIOL) at 0.2 µM each, and 4.0 mM $MgCl_2$. The amplification was carried out on a LightCycler 2.0 instrument (Roche Diagnostics Nederland B.V.) with the following thermal cycling profile: 95°C for 2 min, followed by 45 cycles of 95°C for 1 s, 59°C for 5 s (reached with a touchdown from 64°C over the course of the first 5 cycles), and 72°C for 20 s. The temperature transition rate was set at 20°C/s. Amplification was followed by a melting curve analysis to discriminate between detection of stx_1 and/or stx_2.

Sensitivity and reproducibility testing.

The experiments to assess the intra- and interassay variation were performed as follows: i) on day 1, both laboratories extracted the 600 µl aliquot of each dilution of the unformed stool panel in 5-fold and analyzed these DNA extracts in a single real-time PCR run on the same day. On the 4 following days (days 2, 3, 4, and 5), a 150 µl aliquot of each dilution of the unformed stool panel was extracted and tested. The watery stool panel was analyzed in the same manner as the unformed stool panel, starting at day 2 of the experiment with the 5-fold analysis of the 600 µl aliquot of each dilution, followed by the 150 µl aliquots of each dilution at days 3, 4, 5, and 6. Sensitivity was assessed by determining the detection probability of each assay for each panel.

RESULTS.

Primer/probe design.

The stx_1 primer/probe set was shown to be specific for stx_1, but showed 2 mismatches in the probe for stx_{1c}, which would probably result in less efficient detection of this stx_1 variant. The stx_{1d} variant has several mismatches (forward n = 3, reverse n = 3, probe n = 2) with both the primer and probe sequences and will therefore not be detected using this primer/probe set. Furthermore, the stx_{1d} variant was deliberately not targeted by the stx_1 primer/probe set, as only a single human isolate of this variant

has been reported to date [25]. BLAST analysis confirmed the specificity of the *stx*$_1$ primer/probe set.

For *stx*$_2$, a new primer/probe design was conducted to include all the *stx*$_2$ variants except *stx*$_{2f}$. Although this variant has been frequently isolated from the feces of pigeons, to date, only 4 human isolates have been reported [14, 21, 22]. Both the *stx*$_2$ gene alignment and BLAST analysis confirmed the specificity of the primer/probe set for *stx*$_2$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$, and *stx*$_{2g}$.

Selectivity.

Selectivity was defined as a measure of the degree of response from target and non-target microorganisms and comprises inclusivity and exclusivity. Inclusivity describes the ability of a method (here real-time PCR) to specifically detect the target pathogen from a wide range of strains, whereas exclusivity is the lack of response from a relevant range of closely related, non-target strains. According to the new International Organization for Standardization (ISO) standard, the terms “inclusivity” and “exclusivity” should replace the traditional terms “specificity” and “sensitivity,” which should only be used to express results from diagnostic samples [19].

To assess the selectivity, DNA from 40 well-characterized *E. coli* strains and 37 other bacterial and fungal strains were tested in both real-time PCR assays. Results for inclusivity and exclusivity are shown in Table 6.1 and Table 6.2, respectively. Both assays showed 100% exclusivity, resulting in no false positive results. However, inclusivity was 94% for TM and 97% for LC. As expected, the TM assay did not detect the *stx*$_{1d}$ and *stx*$_{2f}$ variants, whereas the *stx*$_{1c}$ variant was detected, albeit at slightly lower efficiency. The LC assay, as expected, did not detect the *stx*$_{2f}$ variant.

Variation of DNA extraction and real-time PCR in spiked stool specimens.

To assess the reproducibility of the entire procedure, 2 panels of spiked stool specimens were tested on 2 locations as described in the materials and methods. The data, summarized in Table 6.4, indicate that the performance of the combination of NucliSENS miniMAG DNA extraction with both real-time PCR assays is highly reproducible with coefficients of variation (CV) ≤ 5%.

TABLE 6.4. Inter- and intra-assay variations in spiked stool specimens[a].

Stool specimen type and Log_{10} CFU/g	Variation (mean Ct/Cp value ± standard deviation [% CV])			
	Intra-assay		Interassay	
	TaqMan	LightCycler	TaqMan	LightCycler
Unformed				
7.08	26.13 ± 0.53 (2.0)	24.49 ± 0.32 (1.3)	24.63 ± 0.55 (2.2)	24.29 ± 0.66 (2.7)
6.08	29.51 ± 0.64 (2.2)	26.47 ± 0.81 (3.0)	28.61 ± 0.65 (2.3)	26.76 ± 0.81 (3.0)
5.08	32.63 ± 0.87 (2.7)	30.08 ± 0.27 (0.9)	32.25 ± 0.81 (2.5)	30.09 ± 0.14 (0.5)
4.08	35.57 ± 1.21 (3.4)	NA[b]	35.35 ± 0.66 (1.9)	NA
3.08	39.27 ± 1.64 (4.2)	NA	39.58 ± 0.94 (2.4)	NA
2.08 – 0	NA	NA	NA	NA
Watery				
6.88	23.26 ± 0.09 (0.4)	18.69 ± 0.75 (4.0)	23.26 ± 0.18 (0.8)	18.94 ± 0.50 (2.6)
5.88	27.09 ± 0.18 (0.7)	21.81 ± 0.48 (2.2)	27.04 ± 0.41 (1.5)	22.28 ± 0.65 (2.9)
4.88	30.68 ± 0.30 (1.0)	24.57 ± 0.85 (3.4)	30.11 ± 0.44 (1.5)	25.10 ± 0.86 (3.4)
3.88	33.75 ± 0.97 (2.9)	28.02 ± 0.67 (2.4)	34.07 ± 0.41 (1.2)	28.17 ± 0.51 (1.8)
2.88	37.94 ± 1.89 (5.0)	NA	37.36 ± 1.82 (4.9)	NA
1.88 - 0	NA	NA	NA	NA

[a] Unformed and watery pooled stools were supplemented with serial tenfold dilutions of STEC O26:H11, RIVM ECOL 401 (range 0 – 7.08 log_{10} CFU/g, unformed stool) or STEC O157:H7, RIVM 1120500075 (range 0 – 6.88 log_{10} CFU/g, watery stool). DNA extraction and real-time PCR was done for these specimens to determine intra-assay (n = 5) and interassay (n = 5) variations.
[b] NA, Not applicable because of STEC concentrations below the detection limit of the assay.

Analytical sensitivity and linearity.

The analytical sensitivity was determined by plotting the detection probability against the log_{10} CFU/g of feces for all 9 replicates of each dilution for both panels of the variation experiments, as is shown in Figure 6.1A. At the 95% detection probability the detection limits were determined to be 3.07 log_{10} for TM and 3.42 log_{10} CFU/g of feces for LC when testing the watery stool matrix, whereas with the unformed stool matrix the detection limits were 2.8 and 16.2-fold higher at 3.52 log_{10} and 4.63 $log1_0$ CFU/g of feces for TM and LC respectively. At lower STEC concentrations, TM showed inconsistent detection (2 out of 9, and 6 out of 9 replicates) at 3.08 log_{10}, and 2.88 log_{10} CFU/g of feces, respectively for the unformed and watery stool panels. This is in concordance with the expected distribution predicted by the Poisson distribution. None of the lower dilutions tested positive for the LC assay.

Both assays also showed good linearity across the range of measurements with slopes between −2.8349 and −3.5896 and r^2 values > 0.98, resulting in PCR efficiencies between 1.90 and 2.25 as calculated from the formula $E = 10^{-1/slope}$ (Fig. 6.1B).

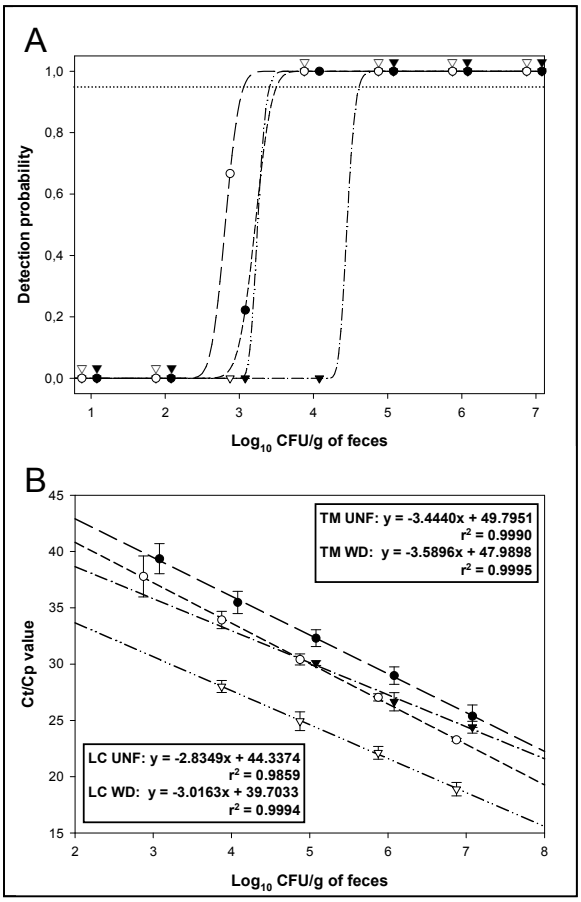


Figure 6.1. **Detection limits A) and linearity B) of TM and LC assays.**

A) Detection probability plotted against STEC load (log_{10} CFU/g of feces) for TM unformed (closed circles, short dashed line), TM watery (open circles, long dashed line), LC unformed (closed triangles, dash-dotted line), and LC watery (open triangles, dash-dot-dotted line) stool panels. Dotted horizontal line represents the 95% detection probability. For clarity, the open and closed triangles have been plotted above the open and closed circles in case they have the same detection probability.

B) Standard curves for mean Ct/Cp value plotted against STEC load (log_{10} CFU/g of feces) for TM unformed (closed circles, long dashed line), TM watery (open circles, short dashed line), LC unformed (closed triangles, dash-dotted line), and LC watery (open triangles, dash-dot-dotted line) stool panels. Error bars represent the standard deviations of the mean Ct/Cp values.

Detection of STEC in clinical stool specimens.

To define the performance of both real-time PCR assays with clinical stool specimens, a panel of 19 archived stools known to contain STEC was first tested. All 19 stools reacted positive in both real-time PCR assays, and speciation of *stx*$_1$ and/or *stx*$_2$ by the LightCycler was in complete concordance for the 7 specimens, from which known virulence profiles were obtained before. Among the remaining 12 specimens, LC speciation resulted in 2 *stx*$_1$, 5 *stx*$_2$, and 5 *stx*$_1$/*stx*$_2$ positive specimens.

After validation of both real-time PCR assays with archived stools, a panel of 115 stools obtained from patients with either a recent history of BD or acute BD at the time of specimen collection was analyzed. The results for

both real-time PCR assays are shown in Table 6.5, as well as other microbiological testing results for these specimens. STEC were detected by both real-time PCR assays in 3 specimens, 2 of which were also positive for *E. coli* O157 by SMAC culture, whereas for the other PCR positive specimen no SMAC culture was performed. Other microbiological tests did not detect other pathogens from this patient's stool, nor from the 2 other *E. coli* O157 positive stools. LC stx gene speciation yielded stx_2 for both specimens positive for *E. coli* O157 by SMAC culture, whereas stx_1 was detected in the remaining specimen. Mild inhibition (i.e. Ct/Cp value for the PhHV internal control outside the range mean + 2 standard deviations, but still detectable) of the real-time PCR was observed for 3 stools with TM and 7 stools with LC. For a single additional stool specimen the TM PhHV Ct-value was detected as completely inhibited (i.e. PhHV undetected). However, this stool proved to be strongly positive for STEC. As a consequence of that, the PhHV Ct-value of this specimen was not regarded as inhibited, but rather as a low signal due to competition with the high amount of STEC DNA in the multiplex amplification reaction. Overall, final PCR inhibition rates in the BD panel were not statistically different with 2.6% for TM, and 6.1% for LC (2-sided Fisher exact test, $p > 0.2$). Furthermore, no significant relation was found between the macroscopic presence of blood in the stool and PCR inhibition for both assays (PCR inhibition for macroscopic blood present vs macroscopic blood not present, 8.0% vs 5.6% for LC, and 4.0% vs 2.2% for TM, 2-sided Fisher exact test, $p > 0.5$).

Among the 112 stools negative for STEC, other (possible) pathogens and non-pathogens were identified in 35 specimens. The pathogen most associated with (a history of) BD was *Campylobacter jejuni* with an overall prevalence of 20% (23/115), including a single mixed infection with *C. jejuni* and *Giardia lamblia*. *Salmonella enterica* was the second most prevalent pathogen with a prevalence of 3.5% (4/115), followed by STEC with 2.6% (3/115). Among the remaining 8 stools, known gastrointestinal pathogens were identified in 2 stools (*Clostridium difficile* [1×] and a *Shigella* spp. or enteroinvasive *E. coli* [1×]), whereas in the remaining 6 stools only yeasts, or possibly pathogenic or non-pathogenic parasites (Table 6.5) were detected.

TABLE 6.5. STEC PCR and microbiological test results for bloody diarrheal stools.

	Real-time PCR						
	TaqMan		LightCycler		Other microbiological test results[b]		
Specimen type[a]	Pos	Neg	Pos	Neg	*S. enterica*[c]	*C. jejuni*[c]	Others[d]
VB	0	9[h]	0	9[i]	1	2[e,f]	2[f,g]
VB and history of BD	0	16	0	16[i]	1	5	0
History of BD	3	87[h]	3[i]	87[i]	2	16[j]	7[k]
All specimens	3	112[h]	3[i]	112[i]	4	23[e,f,j]	9[f,g,k]

[a] BD, bloody diarrhea; VB, visible blood.

b Testing includes culture for *Salmonella enterica*., *Campylobacter* spp., *Yersinia* spp., *Shigella* spp., parasitological microscopical examination, and PCR testing for *Salmonella enterica*, *Campylobacter jejuni*, *Giardia lamblia*, and *Shigella* spp., and *Clostridium difficile* toxin testing (Vero cell cytotoxicity assay and ImmunoCard Toxins A & B [ICTAB], Meridian Biosience Europe)

[c] Positive by both PCR and culture, unless stated otherwise.

[d] Others include pathogens, possible pathogens and non-pathogens.

[e] One specimen positive by PCR only.

[f] Double infection *C. jejuni* (culture and PCR) and *G. lamblia* (PCR only).

[g] *G. lamblia* (PCR only) (1x), and Yeast (1x).

[h] For 3 (1 VB and 2 history of DB) of the 112 negative specimens mild PCR inhibition was observed.

[i] For 3 positive (history of BD) and 4 negative (1 VB, 1 VB and history of BD, and 2 history of BD) specimens mild PCR inhibition was observed.

[j] 3 Specimens positive by PCR only.

[k] *Clostridium difficile* toxin (Vero cell cytotoxicity assay and ICTAB) (1x), *Shigella* spp. or entero-invasive *E. coli* (ipaH PCR positive only) (1x), *Blastocystis hominins* (1x), *Entamoeba coli* and *Endolimax nana* (1x), *Entamoeba coli* (1x), and Yeast (2x).

DISCUSSION.

We have validated 2 real-time PCR assays for the detection of STEC in stool specimens. When challenged with a panel of well-characterized strains, both assays

showed excellent exclusivity. On the other hand, however, the assays did not detect all STEC strains. As expected, both assays could not detect the *stx*$_{2f}$ variant, whereas the TM assay also missed the *stx*$_{1d}$ variant. As stated before, these sequence variants of *stx*$_1$ and *stx*$_2$ only constitute a very small proportion of all circulating STEC strains found in humans [14, 21, 22, 25], and furthermore, their toxicity may also be limited [11]. In addition, the most prevalent variant, *stx*$_{1c}$ (3.6%), is frequently found in conjunction with *stx*$_{2d}$ [25]. And finally, these variant strains have to date been associated mostly with uncomplicated diarrhea or asymptomatic carriage [25, 29], although 6 HUS-associated *stx*$_{1c}$ positive strains have been documented, of which 3 also carried *stx*$_2$ [10, 13]. Therefore, not detecting these strains currently does not seem to have clinical consequences.

The 5′-nuclease assay was shown to have a higher analytical sensitivity (at the 95% detection probability) as compared to the LC assay for both watery and unformed stool. At lower STEC concentrations the TM assay was

still able to generate positive results, although at less consistent detection rates (67% and 22%, respectively), whereas the LC could not. The difference in sensitivity may at least in part be explained by the use of BSA in the TM amplification reactions, since BSA is known to be a very efficient PCR facilitator [1, 28]. This hypothesis may be supported by the fact that the largest difference in sensitivity was observed in the unformed stool panel. In fact, in watery stool specimens inhibitory substances are likely to be more diluted, compared to unformed or even solid stool specimens. Adding BSA to the LC assay may improve the analytical sensitivity, but this was not attempted in this study. When the obtained analytical sensitivities are compared to those of other published real-time PCR assays targeting stool, the TM assay has one of the highest reported analytical sensitivities without prior enrichment of the specimen, whereas the LC assay also compares favorably with most of the reported assays (Table 6.6, [4, 5, 17, 20, 33, 34, 36]).

TABLE 6.6. Limit of detection (LOD) of published real-time PCRs with stool specimens.

Reported LOD (CFU/g feces)	Target / Assay type	Reference
1.0×10^4	STEC / LightCycler, hybprobes	[33]
1.0×10^5	STEC / Smartcycler, molecular beacons	[4]
5.0×10^4	*C. difficile* / Smartcycler, molecular beacons	[5]
1.0×10^4	*S. enterica* / ABI 7700, TaqMan probes	[20]
1.0×10^4	STEC / ABI 7700, TaqMan probes	[17]
1.0×10^5	*C. difficile* / iCycler, TaqMan probes	[36]
$1.0 \times 10^5 – 1.0 \times 10^6$	ETEC / LightCycler, hybprobes	[34]
$1.0 \times 10^3 – 3.3 \times 10^3$	STEC / ABI 7500, TaqMan probes	This study
$2.6 \times 10^3 – 4.3 \times 10^4$	STEC / LightCycler, hybprobes	This study

Furthermore, both assays have an analytical sensitivity at least 1–2 $\log_{10}$ more sensitive as compared to SMAC screening [4], which is the current standard in STEC O157 testing in The Netherlands [37]. Not surprisingly, all 12 specimens known to contain STEC O157 by SMAC screening were detected by both real-time PCR assays. In addition, a sequential stool received 3 days after the primary stool, from a patient with a positive result for STEC O157 by SMAC culture in the previous specimen, was detected by both real-time PCR assays, whereas the SMAC screening did not yield a positive result.

The clinical performance of both assays was completely identical with regard to the numbers of STEC detected in both archived specimens and stools from the prospective BD panel.

Although no attempt was made to culture STEC from the PCR positive stools, with exception of those specimens where SMAC culture was indicated by the routine diagnostic procedures, some stools were regarded to contain non-O157 STEC. The most likely candidates are stools solely positive for *stx*$_1$, and stools positive for *stx*$_1$/*stx*$_2$ or *stx*$_2$ alone, without the presence of the gene encoding intimin (eae). This is because the majority (97.6%) of STEC O157 strains isolated in The Netherlands are usually *stx*$_2$/*eae* or *stx*$_1$/*stx*$_2$/*eae* positive [37]. In this study, 4 specimens were suspected to contain non-O157 STEC, based on the aforementioned assumption (data not shown).

The BD panel was chosen for evaluation of the STEC real-time PCR assays based on the fact that BD is a very common symptom of STEC O157 infection, as was demonstrated in the enhanced surveillance of STEC O157 in The Netherlands, where for 85% of the laboratory-confirmed cases, the presence of blood in the stool was reported [37]. Therefore, BD or a recent history of BD is used as one of the primary selection criteria by nearly all laboratories for testing for STEC O157 in The Netherlands [37]. Nevertheless, for non-O157 STEC the presence of blood in the stool might be less common, as other virulence markers such as eae and enterohemolysin A (*hlyA*) are generally less prevalent in these STEC.

In the BD panel, STEC were the 3rd most prevalent microorganism detected, after *C. jejuni* and *S. enterica*. Our results are in line with a recent GP-based study from The Netherlands [12], where *Campylobacter* species were also most frequently isolated from patients with BD (32.6%), followed by *S. enterica* (7.4%).

The low inhibition rates observed in this study are excellent, especially since 21.7% of the specimens showed macroscopic presence of blood.

In conclusion, we have validated two real-time PCR assays utilizing different chemistries. Although the LC assay obtained a lower analytical sensitivity and proved slightly more vulnerable to PCR inhibition, this did not result in differences in clinical performance with the clinical stool specimens tested in this study. Therefore, with the performance characteristics of both assays well documented, these methods are both suitable for use within multi-center studies to assess the prevalence of STEC-associated diarrhea not only in The Netherlands, but also elsewhere.

ACKNOWLEDGMENTS.

The authors would like to thank Willeke Theune and Richard de Boer for their technical assistance during this study. The group of Helge Karch at the Institute of Hygiene and National Consulting Laboratory on Hemolytic Uremic Syndrome University of Münster Germany is acknowledged for kindly providing the reference strains containing the *stx*-gene variants. Alex van Belkum is acknowledged for critically reviewing the manuscript.

This work was financially supported by a grant from the Inspectorate for Health Care in The Netherlands.

REFERENCES.

1. **Abu Al-Soud, W., and P. Rådström.** 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. J. Clin. Microbiol. **38:**4463–4470.
2. **Ball, H. J., D. Finlay, A. Zafar, and T. Wilson.** 1996. The detection of verocytotoxins in bacterial cultures from human diarrhoeal samples with monoclonal antibody-based ELISAs. J. Med. Microbiol. **44:**273–276.
3. **Beutin, L., S. Zimmermann, and K. Gleier.** 1996. *Pseudomonas aeruginosa* can cause false-positive identification of verotoxin (Shiga-like toxin) production by a commercial enzyme immune assay system for the detection of Shiga-like toxins (SLTs), Infection **24:**267–268.
4. **Bélanger, S. D., M. Boissinot, C. Menard, F. J. Picard, and M. G. Bergeron.** 2002. Rapid detection of Shiga toxin-producing bacteria in feces by multiplex PCR with molecular beacons on the SmartCycler. J. Clin. Microbiol. **40:**1436–1440.
5. **Bélanger, S. D., M. Boissinot, C. Clairoux, F.J. Picard, and M. G. Bergeron.** 2003. Rapid detection of *Clostridium difficile* in feces by real-time PCR. J. Clin. Microbiol. **41:**730–734.
6. **Bellin, T., M. Pulz, A. Matuseek, H.-G. Hempen, and F. Gunzer.** 2001 Rapid detection of enterohemorrhagic *Escherichia coli* by real-time PCR with fluorescent hybridization probes. J. Clin. Microbiol. **39:**370–374.
7. **Bielaszewska, M., H. Schmidt, M. A. Karmali, R. Khakhria, J. Janda, K. Blahova, and H. Karch.** 1998. Isolation and characterization of sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H^- strains in the Czech Republic. J. Clin. Microbiol. **36:**2135–2137.
8. **bioMérieux.** 2007. Pre-extraction protocol stool samples, release 1.0. bioMérieux Benelux BV, Boxtel, the Netherlands.
9. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990 Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
10. **Brett, K. N., V. Ramachandran, M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic.** 2003. Stx1c is the most common Shiga toxin 1 subtype among Shiga toxin-producing *Escherichia coli* isolates from sheep but not among isolates from cattle. J. Clin. Microbiol. **41:**926–936.
11. **Bürk, C., R. Dietrich, G. Açar, M. Moravek, M. Bülte, and E. Märtlbauer.** 2003. Identification and characterization of a new variant of Shiga toxin 1 in *Escherichia coli* ONT:H19 of bovine origin. J. Clin. Microbiol. **41:**2106–2112.
12. **de Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, N. J. van Leeuwen, J. Vinjé, and Y. T. H. P. van Duynhoven.** 2001. Etiology of gastroenteritis in sentinel general practices in The Netherlands. Clin. Infect. Dis. **33:**280–288.
13. **Friedrich, A. W., J. Borell, M. Bielaszewska, A. Fruth, H. Tschäpe, and H. Karch.** 2003. Shiga toxin 1c-producing *Escherichia coli* strains: phenotypic and genetic characterization and association with human disease. J. Clin. Microbiol. **41:**2448–2453.
14. **Gannon, V. P. J., C. Teerling, S. A. Masri, and C. L. Gyles.** 1990. Molecular cloning and nucleotide sequence of another variant of the *Escherichia coli* Shiga-like toxin II family. J. Gen. Microbiol. **136:**1125–1135.
15. **Gavin, P. J., L. R. Peterson, A. C. Pasquariello, J. Blackburn, M. G. Hamming, K. J. Kuo, and R. B. Thomson Jr.** 2004. Evaluation of performance and potential clinical impact of ProSpecT Shiga toxin *Escherichia coli* microplate assay for detection of Shiga toxin-producing *E. coli* in stool samples. J. Clin. Microbiol. **42:**1652–1656.
16. **Gerritzen, A.** 1998. Comparison of two enzyme immuno assays and verocell cytotoxicity for detection of verotoxins in human feces. J. Lab. Med. **22:**704–712.
17. **Hsu, C. F., T. Y. Tsai, and T. M. Pan.** 2005. Use of the duplex TaqMan PCR system for detection of Shiga-like toxin-producing *Escherichia coli* O157. J. Clin. Microbiol. **43:**2668–2673.

18. **Hiroshi, F., T. Yoshie, and S. Ryotaro**. 2003. Duplex real-time SYBR Green PCR assays for detection of 17 species of food- or waterborne pathogens in stools J. Clin. Microbiol. **41:**5134–5146.
19. **Hoorfar, J., and N. Cook.** 2002. Critical steps in standardization of PCR. Methods Mol. Biol. **216:**51–64.
20. **Iijima, Y., N. T. Asako, M. Aihara, and K. Hayashi.** 2004. Improvement in the detection rate of diarrhoeagenic bacteria in human stool specimens by a rapid real-time PCR assay. J. Med. Microbiol. **53:**617–622.
21. **Isobe, J., K. Kimata, M. Shimojima, S. Hosorogi, D. Tanaka, and Y. Gyobu.** 2004. Isolation of *Escherichia coli* O128:HNM harboring stx2f gene from diarrhea patients. Kansenshogaku Zasshi **78:**1000–1005.
22. **Jenkins, C., G. A. Willshaw, J. Evans, T. Cheasty, H. Chart, D. J. Shaw, G. Dougan, G. Frankel, and H. R. Smith.** 2003. Subtyping of virulence genes in verocytotoxin-producing *Escherichia coli* (VTEC) other than serogroup O157 associated with disease in the United Kingdom. J. Med. Microbiol. **52:**941–947.
23. **Jinneman, K. C., K. J. Yoshitomi, and S. D. Weagant.** 2003. Multiplex real-time PCR method to identify Shiga toxin genes *stx1* and *stx2* and *Escherichia coli* O157:H7/H$^-$ serotype. Appl. Environ. Microbiol. **69:**6327–6333.
24. **Johnson, K .E., C. M. Thorpe, and C. L. Sears.** 2006. The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. Clin. Infect. Dis. **43:**1587–1595.
25. **Karch, H., A. W. Friedrich, A. Gerber, L. B. Zimmerhackl, M. A. Schmidt, and M. Bielaszewska.** 2006. New aspects in the pathogenesis of enteropathic hemolytic uremic syndrome. Semin. Thromb. Hemost. **32:**105–112.
26. **Kehl, K. S., P. Havens, C. E. Behnke, and D. W. K. Acheson.** 1997. Evaluation of the premier EHEC assay for detection of shiga toxin-producing *Escherichia coli*. J. Clin. Microbiol. **35:**2051–2054.
27. **Klein, E. J., J. R. A. Stapp, M. Neill, J. M. Besser, M. T. Osterholm, P. I. Tarr, R. B. Thomson Jr., P. J. Gavin, and L.R. Peterson.** 2004. Shiga toxin antigen detection should not replace sorbitol MacConkey agar screening of stool specimens. J. Clin. Microbiol. **42:**4416–4417.
28. **Kreader, C. A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. J. Clin. Microbiol. **34:**1102–1106.
29. **Kuczius, T., M. Bielaszewska, A. W. Friedrich, and W. Zhang.** 2004. A rapid method for the discrimination of genes encoding classical Shiga toxin (Stx) 1 and its variants, Stx1c and Stx1d, in *Escherichia coli. Mol*. Nutr. Food Res. **48:**515–521.
30. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201
31. **Niesters, H. G.** 2002. Clinical virology in real time. J. Clin. Virol. **25:**S3–S12.
32. **Pulz, M., A. Matussek, M. Monazahian, A. Tittel, E. Nikolic, M. Hartmann, T. Bellin, J. Buer, and F. Gunzer.** 2003. Comparison of a Shiga toxin enzyme-linked immunosorbent assay and two types of PCR for detection of Shiga toxin-producing *Escherichia coli* in human stool specimens. J. Clin. Microbiol. **41:**4671–4675.
33. **Reischl, U., M. T. Youssef, J. Kilwinski, N. Lehn, W. L. Zhang, H. Karch, and N. A. Strockbine.** 2002. Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. J. Clin. Microbiol. **40:**2555–2565.
34. **Reischl, U., M. T. Youssef, H. Wolf, E. Hyytia-Trees, and N. A. Strockbine.** 2004. Real-time fluorescence PCR assays for detection and characterization of heat-labile I and heat-stable I enterotoxin genes from enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **42:**4092–4100.
35. **Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308:**681–685.
36. **van den Berg, R. J., L. S. Bruijnesteijn van Coppenraet, H. Gerritsen, H. P. Endtz, E. R. van der Vorm, and E. J. Kuijper.** 2005. Prospective multicenter evaluation of a new immunoassay and real-time PCR for rapid diagnosis of

Clostridium difficile-associated diarrhea in hospitalized patients. J. Clin. Microbiol. **43:**5338–5340.

37. **van Duynhoven, Y. T. H. P., C. M. de Jager, A. E. Heuvelink, W. K. van der Zwaluw, H. M. E. Maas, W. van Pelt, and W. J. B. Wannet.** 2002. Enhanced laboratory-based surveillance of shiga-toxin-producing *Escherichia coli* O157 in The Netherlands. Eur. J. Clin. Microbiol. Infect. Dis. **21:**513–522.

CHAPTER 7

Prevalence, Characterization and Clinical Profiles of Shiga Toxin-Producing *Escherichia coli* in The Netherlands

Y. T. H. P. van Duynhoven [1]
I. H. M. Friesema [1]
T. Schuurman [2]
A. Roovers [3]
A. A. van Zwet [4]
L. J. M. Sabbe [3]
W. K. van der Zwaluw [1]
D. W. Notermans [1]
B. Mulder [5]
E. J. van Hannen [6]
F. G. C. Heilmann [7]
A. Buiting [8]
R. Jansen [9]
A. M. D. Kooistra-Smid [2]

[1] National Institute of Public Health and the Environment, Center for Infectious Disease Control, Bilthoven
[2] Laboratory for Infectious Diseases, Department of Research & Development, Groningen
[3] Oosterschelde Hospitals, Department of Medical Microbiology and Immunology, Goes
[4] Department of Medical Microbiology, Hospital Rijnstate, Arnhem
[5] Laboratory for Medical Microbiology Twente Achterhoek, Enschede
[6] Department of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein
[7] Department of Medical Microbiology and Infection control, Gelre hospitals , Apeldoorn
[8] Department of Medical Microbiology, St. Elisabeth Hospital, Tilburg
[9] Regional Public Health Laboratory, Haarlem
The Netherlands

Clinical Microbiology and Infection 2008. **14:**437-445.

ABSTRACT.

In The Netherlands, traditionally detection of Shiga toxin-producing *Escherichia coli* (STEC) is limited to serogroup O157. To assess the relative importance of STEC, including non-O157 serogroups, a nationwide study was performed. Stool samples submitted for investigation of enteric pathogens or diarrheal patients were screened with real-time PCR for the presence of the Shiga toxin genes. Patients were selected if their stool contained blood on macroscopic examination, if they had a history of bloody diarrhea, were diagnosed with HUS, or were below 6 years of age, independently of bloody aspect. PCR-positive stools were forwarded for STEC isolation and typing. In total, 4,069 stools were examined, with 68 PCR positive results (1.7%). The prevalence was highest for stools containing macroscopic blood (3.5%), followed by stools of patients with a history of bloody diarrhea (2.4%). Among young children, the prevalence was not higher (1.0%) than among random samples of non-bloody, diarrheal patients (1.4%). For 25 (38%) PCR positive stools, STEC could be isolated. Eleven O-serogroups were observed, including 5 STEC O157 strains. STEC was found in 1 in 70 uncomplicated gastroenteritis patients, and was more common in patients with (a history of) bloody diarrhea (1 in 35), but not in children below six years. As serogroup O157 represented only a fifth of the STEC isolates, laboratories should be encouraged to use techniques enabling them to detect the non-O157 serogroups, in tandem with culture for isolation and subsequent characterization of the STEC strain for public health surveillance and detection of outbreaks.

INTRODUCTION.

Infections caused by Shiga toxin-producing *Escherichia coli* **(**STEC) can range from mild, self-limiting diarrhea to severe complications such as hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) [48]. Mortality, occurring mainly in the acute phase of HUS, and long-term renal disorders, end stage renal disease and diabetes, develop in a small proportion of HUS cases [22, 47]. This causes a relatively high average disease burden per case compared to other common enteric pathogens [24]. This combined with the proven potential to cause massive outbreaks, placed STEC on the public health agenda of many developed countries. In The Netherlands, in the late nineties gastroenteritis due to STEC was very rare and dominated by STEC non-O157 over STEC O157 [12, 13]. Exposure of humans to non-O157 strains is probably more frequent because these strains are more prevalent among animals and in food products than STEC O157 [26, 27].

Recently, in several European countries using expanding testing regime targeting all STEC, STEC non-O157 are increasingly shown to be associated with diarrhea and HUS [7, 21, 23, 43, 50]. The continued predominance of STEC O157 among bloody diarrheal and HUS cases in the United Kingdom, Ireland and the United States, demonstrates that regional differences occur [10, 33, 34], although this might still be (partially) a diagnostic artifact. Outbreaks caused by STEC non-O157 are more regularly reported in recent years [1, 9, 18, 35, 37, 44, 53]. Trend information of STEC in The Netherlands is limited to non-sorbitol fermenting STEC O157, because traditionally the detection method for STEC O157 is culture on sorbitol McConckey agar (SMAC) or on SMAC with cefixime and tellurite (CT-SMAC). In 2000, 88% of the Dutch medical microbiological laboratories used these methods and only 6% tested for other STEC serogroups as well [51]. Comparison of Dutch HUS cases in the early nineties with limited data for the year 2000 suggests that also in the Netherlands the importance of STEC of serogroup O157 in diarrhea-associated HUS cases might be decreasing (78% versus 63%) [25, 49]. Rather than the O-serogroup, the combined presence of Shiga toxin 2 (*stx*$_2$) or *stx*$_{2c}$ and to a somewhat lesser extent the *E. coli* attaching and effacing (*eae*) seem important predictors of HUS [10, 19, 20, 30]. To assess the current relative importance of all STEC, and more

specific of the non-O157 serogroups in The Netherlands, a nationwide multi-center study was performed from October 2005 to November 2006, using standardized real-time PCR (rt-PCR) assays.

MATERIALS AND METHODS.

Study population.

Eight large public health laboratories, representing all regions of the country with an estimated coverage of 5.2 million inhabitants (32% of the Dutch population), participated in the study. From 24 October 2005 to 13 November 2006, stool samples that were submitted to these laboratories for investigation of enteric pathogens or from evident diarrheal patients were screened for STEC using rt-PCR assays if the stool contained blood on macroscopic examination, if the patient had a history of bloody diarrhea or if the patient was below 6 years of age, independently of bloody aspect of the stool. The aim was to include every week four macroscopic bloody stools and/or stools from patients with a history of bloody diarrhea and six stools from children below six years of age per laboratory. Stool samples from HUS cases were also included. By this, each laboratory weekly tested ten samples, adding randomly chosen stool samples of diarrheal patients, not meeting the criteria, to complete this number.

Molecular Screening for STEC.

Molecular screening for STEC was performed on the stool specimens as described previously [46] with minor modifications. Briefly, DNA was extracted by the NucliSENS miniMAG system (bioMérieux, Boxtel, The Netherlands) from 200 µl of a 20% (wt/v) fecal suspension, in the presence of approximately 6000 copies of the phocin herpes virus-1 (PhHV), and eluted in 100 µl. Real-time PCRs using TaqMan-, or LightCycler-based chemistries were performed as described previously [46]. Real-time amplification was performed with the following real-time PCR instruments: ABI PRISM 7000 Sequence Detection System (3 laboratories), ABI 7500 Real-Time PCR System (2 laboratories), ABI PRISM 7900 (1 laboratory) (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), LightCycler 1.5 (1 laboratory), and LightCycler 2.0 (1 laboratory) (Roche Diagnostics Nederland B.V., Almere, The Netherlands). Stools testing positive by rt-PCR were stored at 4°C until the transport, except for the first five months of the

study, when they were stored at -20°C. Storage conditions were changed because of improved recovery of coliform bacteria at 4°C compared to -20°C during an experiment at one of the participating laboratories (data not shown). Stools positive by rt-PCR were also retested with the other rt-PCR platform (TaqMan positive results with the LightCycler and vice versa) to confirm the results. Stool samples from two laboratories were also tested for the presence of the stx_{2f} variant starting in September (one laboratory) and October 2006 (second laboratory).

Standardized overviews of the testing results were forwarded weekly to the National Institute of Public Health and the Environment (RIVM) for data entry. These included the rt-PCR STEC results, the test values of positive and negative controls, the phocine herpes virus-1 (PhHV) internal control values and results of the other enteric pathogens examined in the stool. Inhibition of the PCR reaction for the TaqMan assay was defined as a cyclic threshold value above 33.7 (two times the standard deviation above the mean value after running 676 samples) and for the LightCycler as a crossing point value above 31.5 (two times the standard deviation above the mean value after running 269 samples).

STEC isolation and typing.

The stored stool samples which tested positive were forwarded every month to the RIVM for STEC isolation and, if successful, typing. Stools were cultured on Statens Serum Institute Enteric medium, on which *E. coli* appears as red colonies and can be distinguished from other *Enterobacteriaceae* [8]. Suspect *E. coli* colonies were tested by PCR for the presence of Shiga toxin 1 (stx_1), Shiga toxin 2 (stx_2), *E. coli* attaching and effacing (*eae*)- and the EHEC hemolysin (*ehly*) genes [39]. Attempts to isolate *stx*-positive colonies were made up to a maximum of 50 colonies per stool. If no growth was observed on SSI Enteric medium, stools were incubated in Brain Heart Infusion broth (BHI) prior to culturing on SSI Enteric medium. If *stx*-positive colonies were found, O- and H-typing was performed for two randomly chosen *stx*-positive colonies.

Clinical questionnaire.

For all STEC-positive patients, a brief standardized questionnaire was sent to the physician requesting the test. This questionnaire addressed occurrence of symptoms, development of HUS, use of antibiotics prior to

stool sampling, hospitalization, occurrence of similar symptoms in household members in the week before and after their own onset of illness and the main reasons for requesting laboratory diagnostics.

Data analyses.

All data from the laboratories and clinical questionnaires were entered into a MS Access database and, together with the typing data, exported to the SAS System, release 9.1, for analysis. Frequencies and cross-tabulations were made for descriptive analysis. Differences in positivity rates variables between subgroups or subcategories of categorical variables were tested using the chi-square test or Fisher's exact test. Differences for continuous variables were tested with the Wilcoxon rank-sum test.

RESULTS.

Collected stools, inhibition and positive test results.

In total, 4,292 stools were examined during the study period. Inhibition of the PCR reaction was observed for 223 (5.2%) stool samples. PCR of stool samples collected from patients with macroscopic bloody stools or a history of bloody diarrhea were most often inhibited (9.6%). These inhibited reactions were excluded from further analyses.

Overall, 68 (1.7%) of the remaining 4,069 tested stools yielded a positive result in the rt-PCR (Table 7.1). The prevalence ranged from 0.6% to 2.6% between laboratories. The highest prevalence was found for stools containing blood on macroscopic examination (3.5%), followed by stools of patients with a history of bloody diarrhea (2.4%). Among young children, prevalence was not higher than among a random sample of non-bloody, diarrheal patients (Table 7.1). Among children aged below six years with a history of bloody diarrhea or visible bloody stool, the prevalence was 1.5%. No association between the prevalence and gender (male 1.9%, female 1.5%) or degree of urbanization (ranged between 1.5% and 2.1%) was observed. Positivity rates ranged per month between 0% (February 2006) and 4.8% (November 2006) and were significantly higher for the period July to November 2006 compared to October 2005-June 2006 (overall 2.7% and 1.1%, respectively, Chi-square = 13.7, $p = 0.0002$) (Fig. 7.1).

TABLE 7.1. Result of rt-PCR tests for STEC related to inclusion criterion, The Netherlands, 24 October 2005 to 13 November 2006.

	Number of specimens	
Criterion for inclusion in study	Tested	Positive (%)
Macroscopic blood	545	19 (3.5)
History of bloody diarrhea	830	20 (2.4)
Either macroscopic blood or history of bloody diarrhea	1,267	37 (2.9)
Children aged below six years	1,865	18 (1.0)
Children aged below four years	1,421	13 (0.9)
Children aged below two years	817	7 (0.9)
HUS patients	3	0 (0)
Random diarrheal stool sample	1,059	15 (1.4)
Total[a]	4,069	68 (1.7)

[a] Categories do not add up to 4,069 because patients can have multiple inclusion criteria.

For four positive stools, insufficient specimen was left for retesting with the other rt-PCR platform. For the remaining 64 samples, the alternate rt-PCR platform confirmed the positive test result for 57 (89.1%), consisting of 51 stool samples (79.7%) and 6 DNA extracts (9.4%). For the remaining 7 samples (10.9%), 6 re-tests by LightCycler and 1 re-test by TaqMan were negative (median Ct/CP value 33.7). Two DNA extracts of the LightCycler-negative retests were repeatedly positive in the TaqMan assay and for one of these an STEC was cultured from the stool.

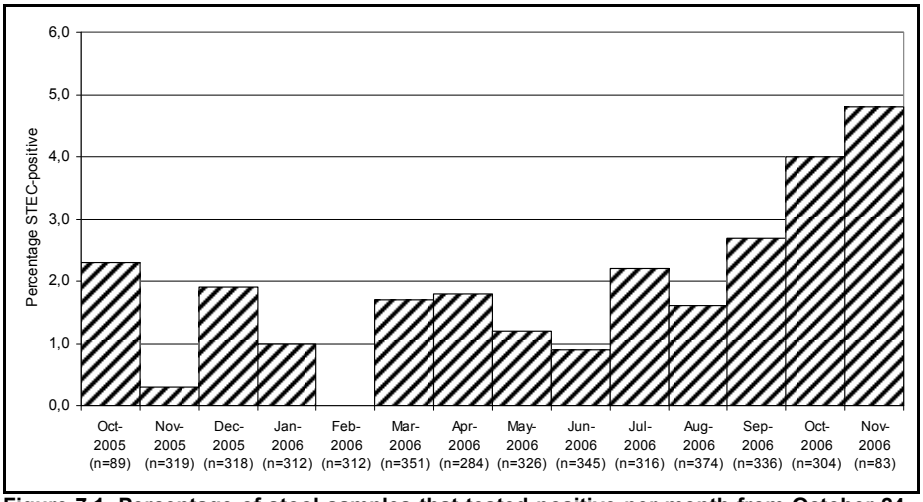


Figure 7.1. Percentage of stool samples that tested positive per month from October 24, 2005 to November 13, 2006, in The Netherlands.

STEC-positive patients.

Among the 68 STEC-positive patients, two showed mixed infections with *Campylobacter* and one with *Yersinia.* For 62 (91%) of the patients, the clinical questionnaire was returned. Common symptoms were diarrhea (85%), abdominal pain (55%), abdominal cramps (50%), and blood in stool (50%). Other symptoms such as nausea (21%), fever (19%), vomiting (13%) and decreased diuresis or anuria (5%) were less often reported. No HUS was reported. Twelve cases (19%) were hospitalized for gastrointestinal symptoms, one of which was admitted to an intensive care unit following surgery of the small intestine due to ischemia. After a stay of 138 days at the ICU this elderly woman died because of recurrent sepsis and renal failure. At least 3 cases were known to suffer from colitis or Crohn's disease, and in addition, one was diagnosed with adenocarcinoma during the hospital stay, one had a history of alcohol abuse and one terminal patient was diagnosed with systemic amyloidosis.

For twelve cases (19%), antibiotics were prescribed. In seven cases this was not for diarrheal disease, but for other complaints, such as urosepsis, urinary tract infection or respiratory infection. For 4 (24%) of the 17 cases aged below six years, similar complaints were reported for other household members (mother and father, mother, sister and brother) in the 7-day period before or after onset of their illness. For none of the cases aged six years or older, ill household members were reported.

The most common reasons for requesting laboratory investigation were severity (35%) or duration (32%) of symptoms (61% one of both reasons) or specific symptoms (25%), mainly blood in stool. Also, a travel-history (destinations Hungary, Turkey, Morocco, Tunis and India) was reported as an indication for 16% of the cases.

Two stool samples were lost during transport to the RIVM. Isolation of the STEC was successful for 25 (38%) of the 66 remaining rt-PCR positive stools. The cyclic threshold/crossing point values for the 41 stool samples where isolation of STEC was unsuccessful were significantly higher (median 29.5, 25^{th} percentile 25.7, 75^{th} percentile 35.0) than the values for stools that cultured STEC (median 24.0, 25^{th} percentile 20.0, 75^{th} percentile 29.5) (Wilcoxon rank-sum test, $p = 0.01$). For the stools where culture of STEC failed, 11 showed no growth at all on SSI medium or BHI and for 30 stools,

no *stx*-positive colonies grew. For 2 of the latter category, *stx*-negative but *eae*-positive *E. coli* were isolated (O136:H51 and O-nontypable:H49). Of the 11 stools that showed no growth, 7 (64%) were stored at -20°C (38% of all tested stool samples were stored at -20°C). Among the 25 successfully isolated STEC, eleven different O-serogroups were observed, in addition to some O-non-typable STEC strains. Most common were O157 (n = 5), O103 (n = 3), O8 (n = 3), O26 (n = 2), O91 (n = 2), and O174 (n = 2) (table 7.2). The *eae*- and *ehly*-gene were present in 52% and 72% of the isolated STEC, respectively. In this small subgroup of patients with isolated STEC, no association was observed between the presence of *eae* and *ehly* genes and young age (below six years) (data not shown). Also, no clear association was observed for these virulence genes with criteria for inclusion in the study, except for a higher occurrence of the *eae*-gene among the 8 STEC from patients with macroscopic blood in stool (88%) versus 35% among the 17 cases without macroscopic blood in stool (Fisher's exact test, $p = 0.03$).

TABLE 7.2. Distribution of O-, H-types and presence of *stx*-, *eae*- and *ehly*-genes among 25 STEC strains isolated from real time PCR-positive stools.

Serotype		Virulence profile					
O-type	H-type	*stx*$_1$	*stx*$_2$	*eae*	*ehly*	n	Diarrheal aspect[a]
O8	H9	Neg	Pos	Neg	Neg	2	B[b]
O8	H19	Neg	Pos	Neg	Neg	1	B
O26	H11	Pos	Neg	Pos	Pos	2	1 B, 1 NB
O54	H21	Neg	Pos	Neg	Neg	1	NB
O78	H$^-$	Pos	Neg	Neg	Pos	1	B
O80	H$^-$	Neg	Pos	Pos	Pos	1	NB
O91	H21	Pos	Pos	Neg	Pos	1	NB
O91	H14	Pos	Neg	Neg	Pos	1	NB
O103	H2	Pos	Neg	Pos	Pos	3	B
O128	H2	Pos	Neg	Neg	Neg	1	B
O146	H21	Pos/Neg[c]	Pos	Neg	Pos	1	NB
O157	H7	Neg	Pos	Pos	Pos	4	B
O157	H$^-$	Pos	Pos	Pos	Pos	1	B
O174	H2	Pos	Pos	Neg	Pos	1	B
O174	H8	Pos	Pos	Neg	Neg	1	B
O-nt[d]	H25	Pos	Pos	Pos	Pos	1	B
O-nt[d]	H16	Neg	Pos	Neg	Neg	1	NB
O-nt[d]	H25	Pos	Neg	Pos	Pos	1	B

[a] B, bloody diarrhea; NB, non-bloody diarrhea.
[b] one case mixed infection with *Campylobacter*.
[c] two different stx profiles in stool of the patient.
[d] O-nt: O-non typable with available antisera.

STEC with *stx*$_{2f}$ variant.

In the two laboratories that screened 211 stools with primers and probes adapted to include the *stx*$_{2f}$ variant sequence, seven (3.3%) STEC were

found, three (1.4%) of which containing the *stx*$_{2f}$ variant gene. These were from a 3-year old boy with uncomplicated diarrhea and two adults with a history of bloody diarrhea, aged 47 (male) and 66 (female) years. One of the adult stool samples was also culture-positive for *Campylobacter.* In the other adult, an adenocarcinoma of the rectum was diagnosed several months later. For the child, no stool sample was left for isolation, but for both adults an STEC O63:H6, *eae*-positive was isolated.

Other enteric bacterial pathogens.

Between 51% and 69% of the stools were cultured for *Campylobacter*, *Salmonella, Shigella* or *Yersinia* as part of the routine workup. Of these, 8.4%, 4.8%, 0.2% and 0.1%, respectively, yielded a positive result (Table 7.3). Overall and for each inclusion criterion separately, the STEC prevalence was clearly below the prevalence for *Salmonella* and *Campylobacter*, but was higher than the prevalence for *Shigella* and *Yersinia* (Tables 7.1 and 7.3).

TABLE 7.3. Result of cultures for other bacterial pathogens related to inclusion criterion of stools for the STEC study, The Netherlands, 24 October 2005 to 13 November 2006[a]

	Organism (number of specimens tested)							
	Campylobacter (n = 2,936)		*Salmonella* (n = 2,923)		*Shigella* (n = 2,894)		*Yersinia* (n = 2,228)	
Criterion for inclusion in study	Pos (n)	(%)	Pos (n)	(%)	Pos (n)	(%)	Pos (n)	(%)
Macroscopic blood	68	(15.6)	44	(10.1)	3	(0.7)	0	(0)
History of bloody diarrhea	123	(17.6)	58	(8.5)	2	(0.3)	0	(0)
Either macroscopic blood or history of bloody diarrhea	174	(16.7)	87	(8.5)	5	(0.5)	0	(0)
Children aged below six years	53	(4.5)	59	(5.1)	0	(0)	2	(0.2)
Children aged below four years	44	(4.9)	43	(4.8)	0	(0)	2	(0.3)
Children aged below two years	19	(3.8)	14	(2.9)	0	(0)	0	(0)
HUS patients	39	(4.7)	15	(1.8)	1	(0.1)	0	(0)
Random diarrheal stool sample	246	(8.4)	140	(4.8)	6	(0.2)	2	(0.1)

[a] see table 7.1 for STEC results in these subcategories for comparison.

DISCUSSION.

In this large-scale, nationwide study, the overall STEC prevalence was 1.7%, ranging from 0.9% for young children to 3.5% for patients with macroscopic blood in stool. These results correspond well with the limited number of similar studies performed previously [2, 7, 41, 52]. A prevalence of 1.6% was observed in a study testing faecal samples submitted to the

diagnostic laboratories of two hospitals in Melbourne using direct plating, a cytotoxicity assay, and both an enzyme immunoassay (EIA) and PCR for Shiga toxin [2]. Similar to our observations, they did not find a difference in the prevalence of young children and adults, and found the highest prevalence for samples containing blood. In a Spanish study, testing fecal samples submitted from inpatients and outpatients of one hospital by PCR, yielded an overall prevalence of 2.5% [7]. In this study, the prevalence increased from 0% in the first study year to 4.4% in the last study year. In one clinical laboratory in Belgium, during 16 years of screening with PCR, 0.9% of samples were STEC-positive, without any trend in time [41]. In the United States, an overall prevalence of 0.3% and 1.4% among samples containing occult blood was found by testing all stool samples submitted to two hospitals in Michigan with an EIA [34]. Screening bloody or suspect samples with the same EIA by 20 other laboratories in Michigan yielded a 10% prevalence [34], clearly higher than found in aforementioned studies.

Among the isolated STEC, the non-O157 strains clearly predominated over the O157 serogroup. This was also observed by others, where the proportion of non-O157 STEC was 64%, 78% and 82%, respectively [2, 7, 41]. The serogroups O26, O103 and, to a lesser extent, O91 observed in our study are well-recognized non-O157 STEC in many other countries as well [2, 3, 7, 10, 16, 19, 30, 41]. Remarkably, O145 and especially O111, which are also among the most common non-O157 serogroups worldwide [2, 3, 7, 10, 15, 17, 19, 23, 30, 34, 40, 41], were not observed in this study. However, in the past both types were found in some Dutch HUS and gastroenteritis cases [12, 49], and the relative importance of O111 appears to have decreased in recent years [14]. On the contrary, serogroups O8 and O174 are observed only sporadically in other countries [2, 3, 7, 10, 16, 19, 20, 41, 42, 52]. The almost exclusive high occurrence of STEC O45 in the United States and O117 in Denmark, mainly related to travel to developing countries, suggests that some circulating types might be continent- or country-specific [10, 19, 34, 40]. Future data should inform us whether the observed discrepancies were a result of the small number of non-O157 isolates in our study or that the distribution of circulating types should indeed be considered country-specific.

One limitation of rt-PCR screening of stool specimens for STEC is that it remains susceptible to PCR inhibition. The amount of inhibition (5.2%)

observed in this study can be regarded as excellent, although it might be of concern that inhibition was observed nearly twice as often (9.6%) in the patient group with the highest STEC prevalence. Although these specimens were regarded as inhibited by our criteria, this does not mean that their PCR data are not interpretable. Using the quantitative nature of rt-PCR, the amount of inhibition, and subsequently its effect on the analytical sensitivity, can be deduced from the Ct/Cp values of the PhHV internal control. As the value for the PhHV of an inhibited specimen shows an increase of n cycles compared to the mean of the PhHV internal control values of non-inhibited specimens, the analytical sensitivity would be reduced by a factor 2^n (factor 10 for each increase of 3.32 cycles). When the PhHV Ct/Cp values for the inhibited specimens in this study are considered, the large majority (~60%) could be interpreted at 10-fold reduced sensitivity, whereas another 25% could be assessed at 100-fold reduced sensitivity. Taking into account the high sensitivity of both published rt-PCRs, a small reduction in sensitivity seems acceptable. For the remaining 15%, the sensitivity would drop with 1000-fold or more, making their rt-PCR data of limited use. These inhibited specimens could be analyzed by other detection methods, such as (rt-)PCR detection of (colony) sweeps of primary cultures or antigen detection.

The relatively low success rate of 38% for isolation of STEC by culture compared to others (75%-97%) [20, 34, 41] was possibly caused by our method targeting feces by PCR directly instead of using enrichment cultures of the (fresh) stool as starting point. This direct method was chosen to limit costs for material and labor in the clinical diagnostic laboratories, with a future extensive testing strategy in mind. Alternatively, the isolation of the STEC, based on typing of individual colonies found positive for *stx*-genes by conventional PCR, might be hampered by the loss of *stx*-genes over time [6, 36]. Storage duration, ranging from one up to more than eight weeks, and the change in storage temperature was not found to affect the STEC isolation rate. To improve the isolation rate of STEC, this study should be followed up by a study comparing the direct feces rt-PCR with simultaneous culture with detection of the causative strain by rt-PCR of the sweep and individual colonies on the plate.

Validation of the two assays used in this multi-centre study showed that the LightCycler assay was between 1 $\log_{10}$ to 2 $\log_{10}$ more sensitive than the

traditional SMAC culture, but between 0.35 $\log_{10}$ and 1.11 $\log_{10}$ less sensitive than the TaqMan assay [46]. The slightly lower sensitivity for the LightCycler assay was supported by the discrepant results of re-testing positive samples by the alternate rt-PCR platform: the TaqMan assay confirmed all but one positive results originally obtained by the LightCycler, while 14% of the TaqMan positive results could not be reproduced by the LightCycler. Although, except for one, we could not prove by culture that these were true STEC-positive samples, it is not likely that these were all false-positive results, as the specificity of both assays was excellent with regard to a large panel of bacterial and fungal strains [46]. Consequently, the STEC prevalence might be slightly underestimated, especially as the prevalence seemed relatively high in the areas where the LightCycler was used (2.1% versus 1.5% in the laboratory-areas where the TaqMan assay was used).

The occurrence of STEC with the shiga toxin variant *stx*$_{2f}$ was clearly higher than expected from the literature: an *stx*$_{2f}$ STEC strain was found only once in 948 STEC patients with diarrhea or HUS in 1996-2004 from Germany [32] and only one of 62 English STEC patients in 1983-2000 was *stx*$_{2f}$-positive [30]. Although we tested for this variant during the seasonal peak, it remains a remarkable observation. So far, serotypes associated with *stx*$_{2f}$ in humans have been mainly O128ab:H$^-$ [28, 30] and sporadically O145:H34 and O119:H$^-$ (personal communication Flemming Scheutz, Statens Serum Institute, Denmark). So the O63:H6 serotype we observed is rather uncommon, even with the classic *stx*-genes [10, 29]. In pigeons, from which these STEC were first isolated and who are considered to be the natural reservoir for these particular strains, also other serotypes have been observed, such as O15, O18ab, O25, O45, O75 and O152 [38]. Usually, *stx*$_{2f}$ (as *stx*$_{1c}$, *stx*$_{1d}$, and *stx*$_{2d}$) in humans are isolated from cases with uncomplicated diarrhea [11, 28, 30, 32]. The occurrence of bloody diarrhea in our two adult cases could be explained by other causes, i.e. a co-infection with *Campylobacter* and an adenocarcinoma. Many traditional PCR assays, as well as several EIA's are not able to detect all different variants of *stx*$_1$ and *stx*$_2$, which will lead to an underestimate of the true occurrence of these variants [5, 39, 45]. As was done with our rt-PCR assays, protocols should be modified to provide comprehensive detection of *stx*-variant genes for future

application. Recently, an EIA capable of detecting all known *stx*-variants was described, but the performance of this assay for use directly on feces was not assessed [4].

The observation of 4.4% co-infections among the STEC-patients is within the range of 2.3% and 8% mixed infections with enteropathogens, we observed in previous large-scale studies among gastroenteritis patients [12, 13]. However, the occurrence of severe co-morbidity in at least 11% of the cases was remarkable, but was not commented on in other similar studies to assess whether this observation is truly unexpected.

The seasonality of STEC in this one-year study seemed similar, although less pronounced, to the known seasonality of STEC O157, with most cases being identified during summer and fall. Such moderate seasonal variation was also observed by others [7, 10]. Comparing the age distribution of STEC O157 cases from the routine surveillance with the age of the STEC cases from this study, showed that young children aged 0-4 years prevailed (Table 7.4). However, STEC O157 patients were more often aged 5 to 19 years and less often were adults of 30-59 years. For the STEC patients in the study symptoms of diarrhea, abdominal pain, abdominal cramps, blood in stool and fever were less often reported and they were less often hospitalized than STEC O157 cases in the surveillance (Table 7.4).

TABLE 7.4. Comparison of clinical characteristics of STEC from this study (October 2005-November 2006) with STEC O157 included in the enhanced surveillance (1999-2006).

	Reported incidence (%) in respondents in	
	Current STEC study	STEC O157 surveillance
Age (years)	(n = 68)	(n = 355)
0-4	23.5	25.1
5-19	11.8	27.0
20-59	47.1	27.6
60-69	7.4	10.4
> 69	10.3	9.9
Reported symptoms[a]	(n = 62)	(n = 331)
Diarrhea	85	98
Abdominal pain	55	83
Abdominal cramps	50	88
Blood in stool	50	87
Nausea	21	57
Fever	19	41
Hospitalization	19	43

[a] In this study symptoms were reported by the physician requesting laboratory investigation, in the STEC O157 surveillance information is provided by patients.

This might partially be explained by a different mode of collection of the data; in the O157 surveillance, patients are questioned directly, while in this study questions were targeted at physicians, A less severe clinical course for non-O157 STEC patients overall has been described by others [20, 40]. However, some non-O157 STEC, among others O26, O103, O111 and O145, cause illness as severe as STEC O157 [19, 31]. Therefore, appropriate diagnostics are required for these patients with severe disease, including HUS cases, and for those with serious co-morbidity. Besides, from a public health perspective, conclusive diagnostics are the crucial foundation for laboratory-based surveillance in order to follow trends and detect outbreaks.

For public health surveillance, identification of the toxins alone is inadequate and should not be used to replace culture and serotyping entirely. The importance of obtaining STEC isolates from each Shiga toxin-positive stool by culture and forwarding these to a public health laboratory for further characterization of virulence genes and serotyping, can not be overemphasized. Only then, the full spectrum, and possible emergence, of pathogenic non-O157 serogroups and the illness they cause can be elucidated. Moreover, this microbiological characterization is vital for the detection of STEC non-O157 outbreaks.

In conclusion, STEC were found in about 1 in 70 uncomplicated gastroenteritis patients, and were more common in patients with (a history of) bloody diarrhea (1 in 35), but not in young children. Compared to other enteropathogens commonly tested in many laboratories, STEC were detected more often than *Shigella* and *Yersinia*, but less often than *Salmonella* and *Campylobacter*. It is therefore advised to clinical laboratories to test all specimens from persons with diarrhea for STEC. However, if this is not considered cost-effective or feasible, as a minimum all stools from diarrheal patients with either macroscopic blood in the stool, a history of bloody diarrhea or HUS should be evaluated. As serogroup O157 represented only a fifth of the STEC isolates, laboratories in The Netherlands should be encouraged to use techniques, such as the developed rt-PCR, enabling them to detect the non-O157 serogroups, in tandem with culture of Shiga toxin-positive stools for isolation and subsequent characterization of the STEC strain for public health surveillance.

ACKNOWLEDGMENTS.

Cecile Deisz, Arjen van de Giessen, Xander Huijsdens and Wim Wannet for their advice and support in the design and preparation of the study. Willeke Theune and Richard de Boer for their work in validating the assays. Carolien de Jager for her help in the data collection and transports. Martijn van Luit for his help in the isolation of STEC strains and Sjoerd Kuiling for serotyping of the STEC strains. All laboratory technicians in the participating laboratories for performing the STEC rt-PCR: Nicole Jans-Beken, Marloes vd Biezen-Brinkman, Debby Vastert-Koop, Suzanne van de Werken, Winawai Adelaar, Arianne te Koppele-Vije, Yvette Hondijk, Cock Koomen, and L. Broers.

This work was financially supported by The Ministry of Public Health, Welfare and Sports, projectnumber V/216852/01/VD. BioMérieux, Boxtel, The Netherlands, kindly provided all participating laboratories with the NucliSENS miniMAG system free of charge.

REFERENCES.

1. **Allerberger, F., A. W. Friedrich, K. Grif, M. P. Dierich, H.-J. Dornbusch, C. J. Mache, E. Nachbaur, M. Freilinger, P. Rieck, M. Wagner, A. Caprioli, H. Karch, and L. B. Zimmerhackl.** 2003. Hemolytic-uremic syndrome associated with enterohemorrhagic *Escherichia coli* O26:H- infection and consumption of unpasteurized cow's milk. Int. J. Infect. Dis. **7:**42-45.
2. **Bennett-Wood, V. R., J. Russell, A.-M. Bordun, P. D. R. Johnson, and R. M. Robins-Browne.** 2004. Detection of enterohaemorrhagic *Escherichia coli* in patients attending hospital in Melbourne, Australia. Pathol. **36:**345-351.
3. **Beutin, L., G. Krause, S. Zimmermann, S. Kaulfuss, and K. Gleier.** 2004. Characterization of Shiga Toxin-Producing *Escherichia coli* Strains Isolated from Human Patients in Germany over a 3-Year Period. J. Clin. Microbiol. **42:**1099-1108.
4. **Beutin, L., H. Steinrück, G. Krause, K. Steege, S. Haby, G. Hultsch, and B. Appel.** 2007. Comparative evaluation of the Ridascreen® verotoxin enzyme immunoassay for detection of Shiga toxin-producing strains of *Escherichia coli* (STEC) from food and other sources. J. Appl. Microbiol. **102:**630-639.
5. **Beutin, L., S. Zimmermann, and K. Gleier.** 2002. Evaluation of the VTEC-Screen "Seiken" test for detection of different types of Shiga toxin (verotoxin)-producinmg *Escherichia coli* (STEC) in human stool samples. Diag. Microbiol. Infect. Dis. **42:**1-8.
6. **Bielaszewska, M., R. Prager, R. Köck, A. Mellmann, W. Zhang, H. Tschäpe, P. I. Tarr, and H. Karch.** 2007. Shiga toxin gene loss and transfer in vivo and in vitro during enterohemorrhagic *Escherichia coli* O26 infections in human. Appl. Environ. Microbiol. **73:**3144-3150.
7. **Blanco, J. E., M. Blanco, M. P. Alonso, A. Mora, G. Dahbi, M. A. Coira, and J. Blanco.** 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)- Producing *Escherichia coli* isolates form human patients: prevalence in Lugo, Spain, from 1992 through 1999. J. Clin. Microbiol. **42:**311-319.
8. **Blom, M., A. Meyer, P. Gerner-Smidt, K. Gaarslev, and F. Espersen.** 1999. Evaluation of Statens Serum Institut Enteric Medium for detection of enteric pathogens. *J Clin Microbiol* **37:**2312-2316.
9. **Brooks, J. T., D. Bergmire-Sweat, M. Kennedy, K. Hendricks, M. Garcia, L. Marengo, J. Wells, M. Ying, W. Bibb, P. M. Griffin, R. M. Hoekstra, and C. R. Friedman.** 2004. Outbreak of Shiga toxin-producing *Escherichia coli* O111:H8 infections among attendees of a high school cheerleading camp. Clin. Infect. Dis. **38:**190-198.
10. **Brooks, J. T., E. G. Sowers, J. G. Wells, K. D. Greene, P. M. Griffin, R. M. Hoekstra, and N. A. Strockbine.** 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. J. Infect. Dis. **192:**1422-1429.
11. **Caprioli, A., S. Morabito, H. Brugère, and E. Oswald.** 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Vet. Res. **36:**289-311.
12. **de Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, W. J. van Leeuwen, A. I. M. Bartelds, Y. T. H. P. van Duynhoven.** 2001. Gastroenteritis in general practices in the Netherlands. Emerg. Infect. Dis. **7:**82-91.
13. **de Wit, M. A. S., M. G. P. Koopmans, L. M. Kortbeek, W. J. B. Wannet, J. Vinjé, F. van Leusden, A. I. M. Bartelds, Y. T. P. H. van Duynhoven YTHP.** 2001. Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and aetiology. Am. J. Epidemiol. **154:**666-674.
14. **EFSA.** 2006. The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2005. The EFSA Journal **94:**122-136.
15. **Eklund, M., J. P. Nuorti, P. Ruutu, and A. Siitonen.** 2005. Shigatoxigenic *Escherichia coli* (STEC) infections in Finland during 1998-2002: a population-based surveillance study. Epidemiol. Infect. **133:**845-52.

16. **Eklund, M., F. Scheutz, and A. Siitonen.** 2001. Clinical isolates of non-O157 Shiga toxin-producing *Escherichia coli*: serotypes, virulence characteristics, and molecular profiles of strains of the same serotype. J. Clin. Microbiol. **39:**2829-2834.
17. **Elliott, E. J., R. M. Robins-Browne, E. V. O'Loughlin, V. Bennett-Wood, J. Bourke, P. Henning, G. G. Hogg, J. Knight, H. Powell, and D. Redmond.** 2001. Nationwide study of haemolytic uraemic syndrome: clinical, microbiological and epidemiological features. Arch. Dis. Childhood. **85:**125-131.
18. **Espie, E., F. Grimont, V. Vaillant, M. P. Montet, I. Carle, H. de Valk, and C. Vernozy-Rozand.** 2006. O148 Shiga toxin-producing *Escherichia coli* outbreak: microbiological investigation as a useful complement to epidemiological investigation. Clin. Microbiol. Infect. **12:**992-998.
19. **Ethelberg, S., K. E. P. Olsen, F. Scheutz, C. Jensen, P. Schiellerup, J. Engberg, A. M. Petersen, B. Olesen, P. Gerner-Smidt, and K. Molbak.** 2004. Virulence factors for haemolytic uremic syndrome, Denmark. Emerg. Infect. Dis. **10:**842-847.
20. **Friedrich, A. W, M. Bielaszewska, W.-L. Zhang, M. Pulz, T. Kuczius, A. Ammon, and H. Karch.** 2002. *Escherichia coli* harbouring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J. Infect. Dis. **185:**74-84.
21. **Fruth, A., R. Prager, A. Friedrich, T. Kuczius, P. Roggentin, H. Karch, A. Ammon, J. Bockemühl, and H. Tschäpe.** 2002. Infektionen des menschen durch enterohämorrhagische *Escherichia coli* (EHEC) in der Bundesrepublik Deutschland von 1998 bis 2001. Bundesgesundheitsbl Gesundheitsforsch Gesundheitsschutz. **45:**715-21.
22. **Garg, A. X., R. S. Suri, N. Barrowman, F. Rehman, D. Matsell, P. M. Rosas-Arellano, M. Salvadori, R. B. Haynes, and W. F. Clark.** 2003. Long-term renal prognosis of diarrhea-associated hemolytic uremic syndrome. JAMA. **290:**1360-70.
23. **Gerber, A., H. Karch, F. Allerberger, H. M. Verweyen, and L. B. Zimmerhackl.** 2002. Clinical course and the role of shiga toxin-producing *Escherichia coli* infection in the hemolytic-uremic syndrome in pediatric patients, 1997-2000, in Germany and Austria: a prospective study. J. Infect. Dis. **186:**493-500.
24. **Havelaar, A. H., Y. T. H. P. van Duynhoven, M. J. Nauta, M. Bouwknegt, A. E. Heuvelink, G. A. de Wit, M. G. M. Niewenhuizen, and N. C. A. J. van de Kar.** 2004. Disease burden in the Netherlands due to infections with shiga toxin-producing *Escherichia coli* O157. Epidemiol. Infect. **132:**467-84.
25. **Heuvelink, A. E., N. C. A. J. van de Kar, J. F. G. M. Meis, L. A. H. Monnens, and W. J. G. Melchers.** 1995. Characterization of verocytotoxin-producing *Escherichia coli* O157 isolates from patients with haemolytic uraemic syndrome in Western Europe. Epidemiol. Infect. **115:**1-14.
26. **Hussein, H.-S.** 2007. Prevalence and pathogenecity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. J. Anim. Sci. **85:**E63-72.
27. **Hussein, H.-S., and T. Sakuma.** 2005. Prevalence of Shiga toxin-producing *Escherichia coli* in dairy cattle and their products. J. Anim. Sci. **88:**450-65.
28. **Isobe, J., K. Kimata, M. Shimojima, S. Hosorogi, D. Tanaka, and Y. Gyobu.** 2004. Isolation of *Escherichia coli* O128:HNM harboring *stx2f* gene from diarrhea patients. Kansenshogaku Zasshi **78:**1000-1005.
29. **Ito, K., M. Iida, M. Yamazaki, K. Moriya, S. Moroishi, J. Yatsuyanagi, T. Kurazono, N. Hiruta, and O.-A. Ratchtrachenchai.** 2007. Intimin types determined by heteroduplex mobility assay of intimin gene (*eae*-)positive *Escherichia coli* strains. J. Clin. Microbiol. **45:**1038-1041.
30. **Jenkins, C., G. A. Willshaw, J. Evans, T. Cheasty, H. Chart, D. J. Shaw, G. Dougan, G. Frankel, and H. R. Smith.** 2003. Subtyping of virulence genes in verocytotoxin-producing *Escherichia coli* (VTEC) other than serogroup O157 associated with disease in the United Kingdom. J. Med. Microbiol. **52:**941-947.
31. **Johnson, K.E., C. M. Thorpe, and C. L. Sears.** 2006. The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. Clin. Infect. Dis. **43:**1587-1595.
32. **Karch, H., A. W. Friedrich, A. Gerber, L. B. Zimmerhackl, A. Schmidt, and M. Bielaszewska.** 2006. New aspects in the pathogenesis of enteropathic hemolytic uremic syndrome. Semin. Thromb. Hemost. **32:**105-111.

33. **Lynn, R. M., S. J. O'Brien, M. Taylor, G. K. Adak, H. Chart, T. Cheasty, J. E. Coia, I. A. Gillespie, M. E. Locking, W. J. Reilly, H. R. Smith, A. Waters, and G. A. Willshaw.** 2005. Childhood hemolytic uremic syndrome, United Kingdom and Ireland. Emerg. Infect. Dis. **11:**590-596.
34. **Manning, S. D., R. T. Madera, W. Schneider, S. E. Dietrich, W. Khalife, W. Brown, T. S. Whittam, P. Somsel, and J. T. Rudrik.** 2007. Surveillance for Shiga toxin-producing *Escherichia coli*, Michigan, 2001-2005. Emerg. Infect. Dis. **13:**318-321.
35. **McCarthy, T. A., N. L. Barrett, J. L. Hadler, B. Salsbury, R. T. Howard, D. W. Dingman, C. Brinkman, W. F. Bibb, and M. L. Cartter.** 2001. Hemolytic-Uremic Syndrome and *Escherichia coli* O121 at a Lake in Connecticut, 1999. Pediatrics **108:**59.
36. **Mellmann, A., M. Bielaszewska, L. B. Zimmerhackl, R. Prager, D. Harmsen, H. Tschäpe, and H. Karch.** 2005. Enterohemorrhagic *Escherichia coli* in human infection: in vivo evolution of a bacterial pathogen. Clin. Infect. Dis. **41**:785-792.
37. **Misselwitz, J., H. Karch, M. Bielazewska, U. John, F. Ringelmann, G. Rönnefarth, and L. Patzer.** 2003 Cluster of hemolytic-uremic syndrome caused by Shiga toxin-producing *Escherichia coli* O26:H11. Pediatr. J. Infect. Dis. **22:**349-354.
38. **Morabito, S., G. Dell'Omo, U. Agrimi, H. Schmidt, H. Karch, T. Cheasty, and A. Caprioli.** 2001. Detection and characterization of Shiga toxin-producing *Escherichia coli* in feral pigeons. Vet. Microbiol. **83:**275-283.
39. **Paton, A.W., and J. C. Paton.** 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1, stx2, eaeA*, Enterohemorrhagic *E. coli hlyA*, rfb_{O111} and rfb_{O157}. J. Clin. Microbiol. **36:**598-602.
40. **Phan, Q., P. Mshar, T. Rabatsky-Her, C. Welles, R. Howard, J. Hadler, S. Hurd, P. Clogher, R. Marcus, and L. Demma.** 2007. Laboratory-confirmed non-O157 Shiga toxin-producing *Escherichia coli*- Connecticut, 2000-2005. MMWR **56:**29-31.
41. **Piérard, D., O. Soetens, J. Breynaert, D. Stevens, and S. Lauwers.** 2006. Sixteen years of screening for verocytotoxin-producing *Escherichia coli* in stool culture samples. Program and abstract book 6th international symposium on shiga toxin (verocytotoxin)- producing *Escherichia coli* infections, VTEC 2006, Melbourne, Australia: **136:**p14.1.16.
42. **Rivas, M., E. Miliwebsky, I. Chinen, C. D. Roldan, L. Balbi, B. Garcia, G. Fiorilli, S. Sosa-Estani, J. Kincaid, J. Rangel, and P. M. Griffin.** 2006. Characterization and epidemiologic subtyping of Shiga toxin-producing *Escherichia coli* strains isolated form hemolytic uremic syndrome and diarrhea cases in Argentina. Foodborne Path. Dis. **3:**88-96.
43. **Scheutz, F., K. E. P. Olsen, and G. Hoy Kock-Hansen.** 2003. Verocytotoxin-producing *E. coli* 2001-2002. Epi-News **2003:**20/21,1-2.
44. **Scheutz, F., J. Lassen, and K. Nygard.** 2006. VTEC O103 outbreak in Norway. EPI-News **2006:**14,1.
45. **Schmidt, H., J. Scheef, S. Morabito, A. Caprioli, L. H. Wieler, and H. Karch.** 2000. A new Shiga toxin 2 variant *(Stx2f)* from *Escherichia coli* isolated from pigeons. Appl. Environ. Microbiol. **66:**1205-1208.
46. **Schuurman, T., A. Roovers, W. K. van der Zwaluw, A. A. van Zwet, L. J. M. Sabbe, A. M. D. Kooistra-Smid, Y. T. H. P. van Duynhoven.** 2007. Evaluation of 5'-Nuclease and Hybridization Probe Assays for the Detection of Shiga Toxin-Producing *Escherichia coli* in Human Stools. J. Microbiol. Meth. **70**:406-415.
47. **Suri, R. S., W. F. Clark, N. Barrowman, J. L. Mahon, H. R. Thiessen-Philbrook, M. P. Rosas-Arellano, K. Zarnke, J. S. Garland, and A. X. Garg.** 2005. Diabetes during diarrhea-associated hemolytic uremic syndrome. Diabetes Care **28:**2556-2562.
48. **Tarr, P. I., C. A. Gordon, and W. L. Chandler.** 2005. Shiga toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet **365:**1073-1086.
49. **te Loo, D. M., A. E. Heuvelink, E. de Boer, J. Nauta, J. van der Walle, C. Schröder, V. W. M. van Hinsbergh, H. Chart, N. C. A. J. van de Kar, and L. P. W. J. Heuvel.** 2001. Verocytotoxin binding to polymorphonuclear leukocytes among households with children with hemolytic uremic syndrome. J. Infect. Dis. **184:**446-450.

50. **Tozzi, A. E., A. Caprioli, F. Minelli, S. Morabito, A. Gianviti, L. De Petris, A. Edefonti, G. Montini, A. Ferretti, T. De Palo, M. Gaido, and G. Rizooni.** 2003. Shiga toxin-producing *E. coli* infections associated with hemolytic uremic syndrome, Italy, 1988-2000. Emerg. Infect. Dis. **9:**106-108.
51. **van Duynhoven, Y. T. H. P., C. M. de Jager, A. E. Heuvelink, W. K. van der Zwaluw, H. M. E. Maas, W. van Pelt, and W. J. B. Wannet.** 2002. Enhanced laboratory-based surveillance of Shiga toxin-producing *Escherichia coli* O157 in the Netherlands. Eur. J. Clin. Microbiol. Infect. Dis. **21:**513-522.
52. **Welinder-Olsson, C., M. Badenfors, T. Cheasty, E. Kjellin, and B. Kaijser.** 2002. Genetic profiling of enterohemorrhagic *Escherichia coli* strains in relation to clonality and clinical signs of infection. J. Clin. Microbiol. **40:**959-964.
53. **Werber, D., A. Fruth, A. Lieegang, M. Littman, U. Buchholz, R. Prager, H. Karch, T. Breuer, H. Tschäpe, and A. Ammon.** 2002. A multistate outbreak of Shiga toxin-producing *Escherichia coli* O26H11 infections in Germany, detected by molecular subtyping surveillance. J. Infect. Dis. **186:**419-422.

CHAPTER 8

Comparison of Microscopy, Real-Time PCR and a Rapid Immunoassay for the Detection of *Giardia lamblia* in Human Stool Specimens

T. Schuurman [1]
P. Lankamp [1]
A. van Belkum [2]
M. Kooistra-Smid [1]
A. van Zwet [1]

[1] Laboratory for Infectious Diseases, Department of Research & Development, Groningen
[2] Erasmus Medical Center, Department of Medical Microbiology and Infection Control, Rotterdam
The Netherlands

Clinical Microbiology and Infection. 2007. **13:**1186-1191

ABSTRACT.

Giardia lamblia is one of the most common intestinal parasites worldwide, with microscopy being the diagnostic reference standard for use with human stools. However, microscopy is time-consuming, labour-intensive and lacks sensitivity when single stools are examined. In the present study, microscopy, real-time PCR and a rapid immunoassay were compared for the detection of *G. lamblia* in human stools. All three methods were highly sensitive, with values of 99%, 100% and 98%, respectively. Specificity and positive and negative predictive values were ≥ 97%, except when using real-time PCR, for which the specificity and positive predictive value were 92% and 93%, respectively. The lower specificity of real-time PCR was associated mostly with failure to detect specimens regarded as true positives for *G. lamblia* DNA, although cross-contamination was suspected in a minority of cases because of the large amount of *G. lamblia* DNA present in most positive specimens. It was concluded that microscopy should remain the primary diagnostic tool for identifying *G. lamblia* in human stools, mainly because of its ability to simultaneously detect other gastrointestinal parasites. However, the simple and rapid immunoassay is a valuable tool to decrease turn-around time. Real-time PCR provides additional sensitivity, although there is a risk of cross-contamination. Based on this observation, and the need for other real-time assays to be developed to detect other intestinal parasites, real-time PCR is currently useful only as an additional test supplementary to microscopy.

INTRODUCTION.

Infectious gastroenteritis is still a major public health burden in developed countries, although the mortality rate is low [12]. In The Netherlands, the total number of cases of gastroenteritis in the population was estimated at 4.5 million in 1999 [24]. The intestinal protozoan *Giardia lamblia* (syn. *intestinalis* or *duodenalis*) is the most frequent pathogenic parasite involved in infectious gastroenteritis in The Netherlands [8]. Older children (aged 5–14 years) seem to be the predominant age group infected [8].

Classically, diagnosis of giardiasis is conducted by microscopical analysis of multiple stool specimens for the presence of *G. lamblia* cysts or trophozoites. Analysis of single stool specimens has been shown to be effective in only 70% of cases [5, 6, 7]. However, the sensitivity of microscopy is largely dependent on the skill of the microscopist [14, 20, 21, 22], and this makes the technique time-consuming and expensive. In recent years, direct fluorescent-antibody staining tests and enzyme immunoassays have been shown to be sensitive and cost-effective alternatives to microscopical examination of stools [16], but these assays still require numerous reagent additions, washing procedures and incubation steps. Several immunochromatographic lateral flow immunoassays provide a rapid means of detecting *G. lamblia* and *Cryptosporidium parvum*, but are slightly less sensitive than the direct fluorescent-antibody staining test [10, 11, 14, 15]. Nevertheless, rapid immunoassays might be useful for smaller hospital laboratories, as they do not require the use of specialized equipment or the skills of experienced microscopists. Real-time PCR methods for direct detection of *G. lamblia* in stools have also been described, with similar or improved sensitivity, when compared to microscopy and antigen detection, when used with single fecal specimens [13, 18, 25, 26].

The present study describes the first direct comparison of the use of microscopy, a lateral flow immunoassay and real-time PCR for the detection of *G. lamblia* in human stool specimens.

MATERIALS AND METHODS.

Bacteria and fungi.

In total, 39 bacterial and fungal strains were used as negative controls, representing gastrointestinal pathogens and normal human gastrointestinal flora (Table 8.1). All organisms were grown using appropriate media and growth conditions for 16–48 h. After harvesting (approxiamtely 10^9 CFU) into 1 mL of physiological saline, chromosomal DNA was extracted from the strains by heat lysis for 10 min at 95°C.

TABLE 8.1. Bacterial and fungal strains used in this study.

Species	Origin[a]	Species	Origin[a]
Bacteroides fragilis	ATCC 25285	*Klebsiella oxytoca*	ATCC 43863
Bacteroides melaninogenicus	ATCC 25845	*Klebsiella pneumoniae*	ATCC 13883
Campylobacter coli	Lvl 176	*Listeria monocytogenes*	ATCC 19117
Campylobacter jejuni	ATCC 29428	*Peptostreptococcus anaerobicus*	ATCC 27337
Candida albicans	ATCC 10231	*Proteus mirabilis*	ATCC 12453
Candida kruzei	ATCC 14243	*Proteus vulgaris*	ATCC 13315
Citrobacter diversus	Lvl 197	*Pseudomonas aeruginosa*	ATCC 27853
Citrobacter freundii	ATCC 8090	*Salmonella enterica* serovar Enteritidis	ATCC 13076
Clostridium difficile	ATCC 9689	*Salmonella enterica* serovar Typhimurium	ATCC 14028
Clostridium perfringens	ATCC 13124	*Serratia liquefaciens*	ATCC 27592
Corynebacterium diphteriticum	ATCC 11913	*Serratia marcescens*	ATCC 13880
Corynebacterium pseudodiphteriticum	ATCC 10700	*Shigella boydii*	ATCC 9207
Enterobacter aerogenes	ATCC 13048	*Shigella dysenteriae* serotype 2	Lvl 168
Enterobacter agglomerans	Lvl 74	*Shigella flexneri*	ATCC 12022
Enterobacter amnigenus	Lvl 137	*Shigella sonnei*	ATCC 25931
Enterobacter cloacae	ATCC 13047	*Staphylococcus aureus*	ATCC 25923
Enterococcus faecalis	ATCC 51299	*Staphylococcus epidermidis*	ATCC 155
Escherichia coli	ATCC 25922	*Streptococcus pneumoniae*	ATCC 49619
Escherichia coli O157:H7	ATCC 35150	*Yersinia enterocolitica*	ATCC 23715
Hafnia alvei	Lvl 112		

[a] ATCC, American Type Culture Collection; Lvl, Laboratorium voor Infectieziekten (Laboratory for Infectious Diseases) culture collection.

Fecal specimens.

Unpreserved (non-fixed) stool samples positive for *G. lamblia* (n = 103), and 97 stools negative for *G. lamblia* according to microscopy, were collected between August and October 2004. Specimens were labeled blindly, and stored at −20°C until further processing by real-time PCR and rapid immunoassay. All stools originated from patients with a suspicion of gastroenteritis, and were sent to the Laboratory for Infectious Diseases, Groningen, The Netherlands, for bacterial and parasitological examination.

Among the 103 specimens positive for *G. lamblia* by microscopy, 23 also contained other (possibly pathogenic) parasites, pathogenic bacteria or yeast species that were detected by microscopy and culture methods, including: *Entamoeba coli* (n = 9), *Blastocystis hominis* (n = 8), *Candida albicans* (n = 1), yeast species (n = 1), *Endolimax nana* + *Ent. coli* + *B. hominis* (n = 1), *Dientamoeba fragilis* (n = 1), *B. hominis* + *Campylobacter jejuni* (n = 1), and *D. fragilis* + *B. hominis* (n = 1). Among the 97 specimens negative for *G. lamblia*, 17 contained other parasites, bacteria or yeast species, including *B. hominis* (n = 6), *Ent. coli* (n = 2), *Ent. coli* + *B. hominis* (n = 2), *D. fragilis* + *B. hominis* (n = 2), *D. fragilis* + *End. nana* (n = 1), *Campylobacter coli* + *B. hominis* (n = 1), *Camp. jejuni* (n = 1), *End. nana* (n = 1) and an unidentified yeast species (n = 1).

Microscopical examination for *G. lamblia*.

Fifty-nine of the 103 specimens positive by microscopy were sent to the Laboratory for Infectious Diseases as single, unpreserved fecal specimens. The remaining 44 were sent as a triple feces test set, containing one unpreserved specimen and two specimens fixed in sodium acetate–acetic acid–formalin [23]. Three of the 97 microscopy-negative fecal specimens were unpreserved single samples, while the remaining 94 were part of a triple feces test set. Microscopy was conducted within 1 day of arrival at the initial diagnostic laboratory. Unpreserved fecal specimens were first concentrated using the method of Ridley et al. [19], and an iodine-stained wet-mount preparation was examined at 400 × magnification. Sodium acetate–acetic acid–formalin-preserved fecal specimens were initially examined using an iodine-stained wet-mount preparation, and this was followed by a chlorazol-black stain when a suspicion of vegetative-stage protozoa was raised by the iodine-stain examination.

Rapid immunoassay.

The rapid immunoassay (ImmunoCard STAT! *Cryptosporidium*/*Giardia* Rapid Assay; Meridian Bioscience, Boxtel, The Netherlands) was used according to the manufacturer's instructions. In brief, fecal samples were thawed and diluted by adding three volumes of distilled water. Two drops of sample treatment buffer (contained in the assay kit) were added to the specimen dilution tube. Next, approximately 60 µL of diluted fecal specimen was transferred to the specimen dilution tube and two drops of conjugate

reagent A were added, followed by two drops of conjugate reagent B. After gentle mixing by manual swirling, the sample was transferred to the test device and the results were read after 10 min. A specimen was regarded as positive if both the 'organism' and 'control' lines showed a band, as negative if only the 'control' line showed a band, and as invalid if no band was visible at the 'control' line.

Fecal sample preparation and real-time PCR.

Nucleic acid was extracted from fecal suspensions using the method described by Boom *et al.* [3, 4] and Beld *et al.* [2] with minor modifications. In brief, 100 µL of fecal suspension (33–50% w/v) was added to a mixture containing 50 µL silica particles (SC-F, prepared as described previously [2], but with 2400 µL of HCl 32% v/v, rather than 600 µL as described by Beld *et al.* [2]) and 900 µL of lysis buffer L6, mixed and left at ambient temperature for 10 min. After centrifugation, the silica–nucleic acid complexes were washed as described previously [3]. The nucleic acids were eluted in 100 µL of 1 × TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and either processed for PCR immediately or stored at −20°C.

Real-time PCR was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) using the TaqMan Universal PCR Master Mix (Applied Biosystems) and the *G. lamblia* small-subunit rRNA gene-specific primers and probe described by Verweij *et al.* [26]. The reaction mixture (25 µL) consisted of 1 × TaqMan Universal PCR Master Mix, 300 nM each of primers Giardia F (Applied Biosystems) (5'-GAC GGC TCA GGA CAA CGG TT) and Giardia R (Applied Biosystems) (5'-TTG CCA GCG GTG TCC G), 200 nM TaqMan probe Giardia T (Applied Biosystems) (5'-FAM-CCC GCG GCG GTC CCT GCT AG-TAMRA-3'), 2.5 µg of bovine serum albumin (Roche Diagnostics, Almere, The Netherlands) and 5 µL of template DNA. Amplification comprised 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR inhibition was monitored by running a duplicate reaction spiked with 50 pg of purified *G. lamblia* cyst DNA, equivalent to approximately 160 *G. lamblia* cysts. Amplification data were analyzed using Sequence Detector software v. 1.9.1 (Applied Biosystems).

RESULTS.

Sensitivity and specificity of *G. lamblia* small-subunit rDNA real-time PCR.

To assess the sensitivity of the *G. lamblia* real-time PCR, ten-fold serial dilutions of purified *G. lamblia* cyst DNA (250 ng to 250 fg) were tested in duplicate. All dilutions from 250 ng to 2.5 pg were detected in both tests. The dilution containing 250 fg was detected only once, and no signal was detected in the controls without any template. A *G. lamblia* cyst contains approximately 313 fg of chromosomal DNA, and a trophozoite contains approximately 144 fg [9]. Thus, in principle, the *G. lamblia* real-time PCR assay was able to detect DNA originating from a single *G. lamblia* cyst.

A stool specimen, negative for *G. lamblia* according to microscopy and real-time PCR, was spiked with ten-fold serial dilutions of purified *G. lamblia* cyst DNA (33 ng to 33 fg/fecal extraction) and DNA was isolated from these specimens. Real-time PCR was performed in duplicate on the fecal DNA extracts. In addition, *G. lamblia* cyst DNA serially diluted ten-fold was amplified in parallel in duplicate, using the amount that would be expected if recovery of DNA from the feces was 100%. The purified *G. lamblia* cyst DNA and the fecal DNA extracts both showed nearly identical Ct-values (the number of PCR cycles required to detect a positive reaction) for the specimens containing 1.7 ng to 1.7 pg of DNA/real-time PCR. Assuming 100% recovery, this corresponds to between 5000 and five *G. lamblia* cysts/real-time PCR. Further dilutions failed to yield positive results with either purified *G. lamblia* cyst DNA or the fecal DNA extracts. Thus, in a fecal background, the real-time PCR could detect the equivalent of five *G. lamblia* cysts, or 2000–3000 *G. lamblia* cysts/g of feces, depending on the fecal suspension (i.e. 33–50% w/v), assuming 100% extraction and detection efficiency.

The specificity of the *G. lamblia*-specific real-time PCR has been documented previously for other intestinal parasites, including *Entamoeba histolytica*, *Entamoeba dispar*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Cyclospora cayetanensis* and *C. parvum* [25, 26]. No cross-reactions were observed with the panel of 39 bacterial and fungal strains (Table 8.1) representing gastrointestinal pathogens and normal human flora.

Detection of *G. lamblia* in clinical samples by rapid immunoassay and real-time PCR.

Ninety-eight of the 103 microscopy-positive fecal specimens were also found to be positive according to both rapid immunoassay and real-time PCR. Ct-values for these specimens in real-time PCR averaged 21.90 (range 14.53–31.16). Real-time PCR also detected *G. lamblia* DNA in two microscopy-positive fecal specimens (Ct-values of 16.16 and 17.73) that were negative according to the rapid immunoassay. The three remaining microscopy-positive samples were detected neither by rapid immunoassay nor by real-time PCR. Of the 97 specimens negative for *G. lamblia* according to microscopy, one sample was positive for *G. lamblia* according to both rapid immunoassay and real-time PCR (Ct 33.30). Eight additional fecal specimens were also positive according to real-time PCR (Ct range 32.74–38.69), but were negative according to both rapid immunoassay and microscopy. When these samples were retested in quadruplicate using two new DNA preparations, three were negative and were regarded as false-positives caused by specimen cross-contamination. For the remaining five specimens, between one and eight of the replicates were positive.

The distribution of Ct-values among all the real-time PCR-positive stool specimens is shown in Figure 8.1, and indicates that most specimens contained large amounts of *G. lamblia* DNA. For 34.9% of the specimens, the Ct-values were < 20, and for 63.3% and 89.9% of the specimens, the Ct-values were < 25 and < 30, respectively.

None of the 200 fecal specimens showed evidence of PCR inhibition. However, two microscopy-negative samples gave invalid results with the rapid immunoassay; both samples showed valid negative results upon retesting, and these results were used for the comparative analysis.

Independent analysis of the performance of all methods.

To independently compare all three methods for the detection of *G. lamblia*, an extended reference standard was used for true-positive stool specimens. A true-positive result was defined as a specimen with at least two positive results among the three available tests. Table 8.2 shows the results of all three methods in comparison with the extended reference standard. The sensitivity, specificity, positive predictive value and negative predictive value were 99%, 97%, 97% and 99% for microscopy, 100%, 92%,

93% and 100% for real-time PCR, and 98%, 100%, 100% and 98% for rapid immunoassay, respectively.

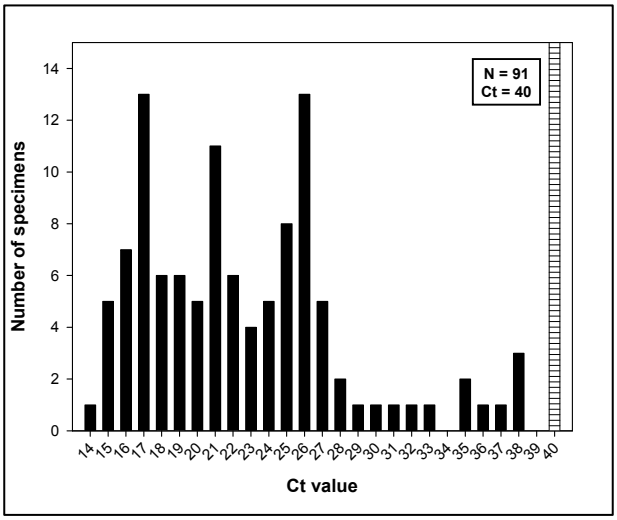


Figure 8.1. Distribution of Ct-values in the *G. lamblia* real-time PCR.

Closed bars represent the number of fecal specimens for a given Ct range. Dashed bar represents fecal specimens with no detectable amplification signal (Ct = 40.00). Ct-value groups cover the following ranges: Ct-value group 14 represents Ct-values from 14.00 to 14.99; Ct-value group 15 represents Ct-values from 15.00 to 15.99; and so on.

TABLE 8.2. Results of microscopy, real-time PCR and rapid immunoassay compared with the extended "gold standard".

Test result for			Total number	Extended "gold standard"
Microscopy	Real-time PCR	Rapid Immunoassay	of specimens	interpretation[a]
Pos	Pos	Pos	98	True positive
Pos	Pos	Neg	2	True positive
Pos	Neg	Pos	0	True positive
Neg	Pos	Pos	1	True positive
Pos	Neg	Neg	3	True negative
Neg	Pos	Neg	8	True negative
Neg	Neg	Pos	0	True negative
Neg	Neg	Neg	88	True negative
			+	
			200	

[a] A true positive result was defined as a specimen with at least 2 positive results out of the 3 available tests; a true negative result was defined as a specimen with at least 2 negative results out the 3 available tests.

Detection of *Cryptosporidium parvum* by rapid immunoassay.

Although the present study was aimed at detecting *G. lamblia*, the rapid immunoassay was also capable of detecting *C. parvum* in stool specimens. Of the 200 specimens tested, three samples showed a *Cryptosporidium*-specific band in the rapid immunoassay. This resulted in the identification of two probable mixed infections with *G. lamblia* and *C. parvum*.

DISCUSSION.

Real-time PCR, rapid immunoassay and microscopy were all highly sensitive (98–100%) when compared with an extended reference standard for the detection of *G. lamblia* in human stools. As recommended previously, a composite standard was used in the absence of a good reference standard test [1, 17]. Specificity and positive and negative predictive values were also ≥ 97%, except when using real-time PCR, where the specificity and positive predictive value were 92% and 93%, respectively.

Fourteen fecal specimens showed discrepant results with one or two of the methods. However, both the real-time PCR and the rapid immunoassay might generate false-negative results because of intermittent shedding of *G. lamblia* cysts in stools. Also, the amount of specimen analyzed may have influenced the results, as the amount of feces analyzed by microscopy is approximately 100–200 mg, which is then concentrated further using the method of Ridley and Hawgood [19] for unpreserved specimens. For real-time PCR and rapid immunoassay, 1.67–2.5 mg and 15 mg of feces are analyzed, respectively, assuming that 1 mg equals approximately 1 µL.

The two false-negative results obtained with the rapid immunoassay may have been caused by an overload of the rapid immunoassay with *G. lamblia* antigen, leading to the prozone effect. Both of these specimens showed high *G. lamblia* DNA levels according to real-time PCR (Ct-values of 16.16 and 17.73, respectively), and it is therefore likely that high antigen levels were also present in the stools.

For the eight specimens that were positive only according to real-time PCR, there was a strong suspicion of cross-contamination, based on the large number of strong positive specimens (Fig. 8.1). The source of contamination could be related to the extremely large amount of *G. lamblia* DNA present in most positive specimens, with contamination occurring during DNA extraction or during the addition of template DNA to the real-time PCR assays. The latter seems most likely to be the case in the present study, as additional amplification of the original DNA extracts for the three specimens regarded as false-positive did not yield any positive real-time PCR results. These results emphasize the need for great care when performing PCR, and weak positive results should always be interpreted in

relation to the clinical data. It is not clear whether the presence of small amounts of DNA in a patient's stool correlates with disease, or indicates asymptomatic infection, which has been described previously for *G. lamblia* in a case-control study in The Netherlands [8]. Further studies are needed to elucidate the relevance of the positive real-time PCR results with high Ct-values.

The performance of the rapid immunoassay was better than has been reported previously [11, 14], and was in line with the performance reported for a similar type of rapid assay [10, 15]. Although the detection of *G. lamblia* in stools was the focus of the present study, the rapid immunoassay also detected *Cryptosporidium* antigen in three specimens. Of these, one was negative for *G. lamblia* according to all three detection methods, but the other two samples were positive for *G. lamblia* according to all three methods. Retesting of these three specimens with a different batch of rapid immunoassay reagents yielded identical results, thereby providing clear evidence of co-infection by both parasites. However, Ziehl–Neelsen staining or PCR analysis is needed to confirm *Cryptosporidium*-positive rapid immunoassay results, especially as mixed infections with *G. lamblia* and *C. parvum* are rare. The three samples in the present study were also positive for *C. parvum* according to a specific real-time PCR assay (A. Bergmans, personal communication).

In conclusion, all three methods investigated were sensitive for the detection of *G. lamblia* in stools. Microscopy remains the primary diagnostic method for detecting gastrointestinal parasites because of its ability to detect parasites other than *G. lamblia*. However, the rapid immunoassay is a valuable tool for the routine clinical microbiology laboratory because of its speed and simplicity, especially when microscopical examinations of stools are not performed in the laboratory. When using the rapid immunoassay, specimens still need to be processed by microscopy in order to detect other parasites and the small number of false-negative *G. lamblia* stools, and for confirmation of rapid immunoassay *Cryptosporidium*-positive stools. Although real-time PCR seems to be the most sensitive method for detection of *G. lamblia* in human stools, it does not, as yet, provide additional advantages when used as a single test, mainly because of the risk of false-positive results and the fact that it detects only one of the clinically relevant

gastrointestinal parasites. However, because of its higher sensitivity, it may eventually become the method of choice once it has been developed to detect all gastrointestinal parasites in one or a few reactions.

ACKNOWLEDGMENTS.

This study was presented, in part, at the meeting of the Dutch Society for Medical Microbiology (NVMM), Papendal, The Netherlands (2005) and the 4th European Meeting on Molecular Diagnostics, Scheveningen, The Netherlands (2005).

We thank J. H. Venekamp for collecting the faecal specimens and skillfully performing the microscopical examinations of the stool specimens, J. J. Verweij for providing the purified *G. lamblia* DNA to serve as an inhibition control for the real-time PCR, and A. M. C. Bergmans for performing the real-time PCR confirmation of the *C. parvum*-positive stools.

Meridian Bioscience Europe supported this study by donating the ImmunoCard STAT! *Cryptosporidium*/*Giardia* kits.

REFERENCES.

1. **Banoo, S., D. Bell, P. Bossuyt, A. Herring, D. Mabey, F. Poole, P. G. Smith, N. Sriram, C. Wongsrichanalai, R. Linke, R. O'Brien, M. Perkins, J. Cunningham, P. Matsoso, C. M. Nathanson, P. Olliaro, R. W. Peeling, and A. Ramsay; TDR Diagnostics Evaluation Expert Panel.** 2006. Evaluation of diagnostic tests for infectious diseases: general principles. Nat. Rev. Microbiol. **4:**S20–S32.
2. **Beld, M., R. Sentjes, S. Rebers, J. Weel, P. Wertheim-van Dillen, C. Sol, and R. Boom.** 2000. Detection and quantification of hepatitis C virus RNA in feces of chronically infected individuals. J. Clin. Microbiol. **38:**3442–3444.
3. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
4. **Boom, R., C. Sol, J. Weel, K. Lettinga, Y. Gerrits, A. van Breda, and P. Wertheim-van Dillen.** 2000. Detection and quantitation of human cytomegalovirus DNA in faeces. J. Virol. Methods **84:**1–14.
5. **Burke, J. A.** 1977. The clinical and laboratory diagnosis of giardiasis. Crit. Rev. Clin. Lab. Sci. **7:**373–391.
6. **Cartwright, C.P.** 1999. Utility of multiple stool specimen ova and parasite examinations in a high prevalence setting. J. Clin. Microbiol. **37:**2408–2411.
7. **Danciger, M., and M. Lopez.** 1975. Numbers of *Giardia* in the feces of infected children. Am. J. Trop. Med. Hyg. **24:**237–242.
8. **de Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, N. J. van Leeuwen, J. Vinjé, and Y. T. H. P. van Duynhoven.** 2001. Etiology of gastroenteritis in sentinel general practices in The Netherlands. Clin. Infect. Dis. **33:**280–288.
9. **Erlandsen, S.L., and E. M. Rasch.** 1994. The DNA content of trophozoites and cysts of *Giardia lamblia* by microdensitometric quantitation of Feulgen staining and examination by laser scanning confocal microscopy. J. Histochem. Cytochem. **42:**1413–1416.
10. **Garcia, L. S., and R. Y. Shimizu.** 2000. Detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens using the ColorPAC combination rapid solid-phase qualitative immunochromatographic assay. J. Clin. Microbiol. **38:**1267–1268.
11. **Garcia, L. S., R. Y. Shimizu, S. Novak, M. Carroll, and F. Chan.** 2003. Commercial assay for detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens by rapid solid-phase qualitative immunochromatography. J. Clin. Microbiol. **41:**209–212.
12. **Guarrant, R. L., J. M. Hughes, N. L. Lima, and J. Crane.** 1990. Diarrhea in developed and developing countries: magnitude, special settings, and etiologies. Rev. Infect. Dis. **12:**S41–S50.
13. **Guy, R. A., C. Xiao, and P. A. Horgen.** 2004. Real-time PCR assay for detection and genotype differentiation of *Giardia lamblia* in stool specimens. J. Clin. Microbiol. **42:**3317–3320.
14. **Johnston, S.P., M. M. Ballard, M. J. Beach, L. Causer, and P. P. Wilkins.** Evaluation of three commercial assays for the detection of *Giardia* and *Cryptosporidium* organisms in fecal specimens. J. Clin. Microbiol. **41:**623–626.
15. **Katanik, M.T., S. K. Schneider, J. E. Rosenblatt, G. S. Hall, and G. W. Procop.** 2001. Evaluation of ColorPAC *Giardia/Cryptosporidium* rapid assay and ProSpecT *Giardia/Cryptosporidium* microplate assay for detection of *Giardia* and *Cryptosporidium* in fecal specimens. J. Clin. Microbiol. **39:**4523–4525.
16. **Mank, T. G., J. O. Zaat, A. M. Deelder, J. T. van Eijk, and A. M. Polderman.** 1997. Sensitivity of microscopy versus enzyme immunoassay in the laboratory diagnosis of giardiasis. Eur. J. Clin. Microbiol. Infect. Dis. **16:**615–619.
17. **McAdam, A. J.** 2000. Discrepant analysis: how can we test a test? J. Clin. Microbiol. **38:**2027–2029.
18. **Ng, C.T., C. A. Gilchrist, A. Lane, S. Roy, R. Haque, and E. R. Houpt.** 2005. Multiplex real-time PCR assay using scorpion probes and DNA capture for genotype-

specific detection of *Giardia lamblia* in fecal samples. J. Clin. Microbiol. **43:**1256–1260.

19. **Ridley, D. S., and B. C. Hawgood.** 1956. The value of formol-ether concentration of faecal cysts and ova. J. Clin. Pathol. **9:**74–76.
20. **Rosenblatt, J. E., L. M. Sloan, and S. K. Schneider.** 1993. Evaluation of an enzyme-linked immunosorbent assay for the detection of *Giardia lamblia* in stool specimens. Diagn. Microbiol. Infect. Dis. **16:**337–341.
21. **Rosoff, J. D., C. A. Sanders, S. S. Sonnad, P. R. De Lay, W. K. Hadley, F. F. Vincenzi, D. M. Yajko, and P. D. O'Hanley.** 1989. Stool diagnosis of giardiasis using a commercially available enzyme immunoassay to detect *Giardia*-specific antigen (GSA 65). J. Clin. Microbiol. **27:1**997–2002.
22. **Scheffler, E. H., and L. L. Van Etta.** 1994. Evaluation of rapid commercial enzyme immunoassay for detection of *Giardia lamblia* in formalin-preserved stool specimens. J. Clin. Microbiol. **32:**1807–1808.
23. **van Gool, T., R. Weijts, E. Lommerse, and T.G. Mank.** 2003. Triple Faeces Test: an effective tool for detection of intestinal parasites in routine clinical practice. Eur. J. Clin. Microbiol. Infect. Dis. **22:**284–290.
24. **van Pelt, W., M. A. de Wit, W. J. Wannet, E. J. Ligtvoet, M. A. Widdowson, and Y. T. van Duynhoven.** 2003. Laboratory surveillance of bacterial gastroenteric pathogens in The Netherlands, 1991–2001. Epidemiol. Infect. **130:**431–441.
25. **Verweij, J. J., R. Blangé, K. Templeton, J. Schinkel, E. A. Brienen, M. A. van Rooyen, L. van Lieshout, and A. M. Polderman.** 2004. Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. J. Clin. Microbiol. **42:**1220–1223.
26. **Verweij, J. J., J. Schinkel, D. Laeijendecker, M. A. A. van Rooyen, L. van Lieshout, and A. M. Polderman.** 2003. Real-time PCR for the detection of *Giardia lamblia*. Mol. Cell. Probes **17:**223–225.

CHAPTER 9

Detection of Diarrhea-Causing Protozoa in General Practice Patients in The Netherlands by Multiplex Real-Time PCR

R. ten Hove [1]
T. Schuurman [2]
M. Kooistra [2]
L. Möller [3]
L. van Lieshout [1]
J. J. Verweij [1]

[1] Leiden University Medical Center, Department of Parasitology, Leiden
Laboratory for Infectious Diseases, Departments of [2] Research & Development and [3]
Bacteriology, Groningen
The Netherlands

Clinical Microbiology and Infection. 2007. **13:**1001-1007

ABSTRACT.

The diagnostic value of a multiplex real-time PCR for the detection of *Entamoeba histolytica, Giardia lamblia* and *Cryptosporidium parvum*/*Cryptosporidium hominis* was evaluated by comparing the PCR results obtained with those of routinely performed microscopy of fecal samples from patients consulting their general practitioner (GP) because of gastrointestinal complaints. Analysis of 722 fecal DNA samples revealed that the prevalence of *G. lamblia* was 9.3% according to PCR, as compared to 5.7% by microscopy. The number of infections detected was more than double in children of school age. Furthermore, *G. lamblia* infection was detected in 15 (6.6%) of 228 fecal samples submitted to the laboratory for bacterial culture only. *C. parvum*/*C. hominis* infections were not diagnosed by routine procedures, but DNA from these organisms was detected in 4.3% of 950 DNA samples. A strong association with age was noted, with *Cryptosporidium* being detected in 21.8% of 110 children aged < 5 years. *C. hominis* was the most prevalent species. *E. histolytica* was not detected in this study population. Analysis of microscopy data revealed that the number of additional parasites missed by PCR was small. Overall, the study demonstrated that a multiplex real-time PCR approach is a feasible diagnostic alternative in the clinical laboratory for the detection of parasitic infections in patients consulting GPs because of gastrointestinal symptoms.

INTRODUCTION.

Diarrhea is a major health problem worldwide, killing approximately 3–4 million individuals each year. Those most affected by diarrhea are children and immunocompromised individuals living in developing countries. Although the mortality rate from diarrhea in developed countries has fallen considerably, morbidity remains high [10]. In most cases, the etiologies of diarrhea are related to viruses, bacteria and parasites. In The Netherlands, there are approximately 4.5 million cases of gastroenteritis annually [6]. The intestinal parasite with the highest prevalence is *Giardia lamblia*, followed by *Cryptosporidium parvum*/*Cryptosporidium hominis* [5]. Infection with *Entamoeba histolytica* is rare, but its high morbidity and, in particular, mortality make accurate diagnosis crucial. Classically, diagnosis of *Giardia*, *Cryptosporidium* and *E. histolytica* infections is achieved by microscopical examination of fecal samples. However, microscopy has several important disadvantages: (i) correct identification depends greatly on the experience and skills of the microscopist; (ii) sensitivity is low, and therefore examination of multiple samples is needed; (iii) *E. histolytica* cannot be differentiated from the non-pathogenic *Entamoeba dispar* simply on the basis of the morphology of cysts and small trophozoites; and (iv) in settings with relatively large numbers of negative results, e.g., The Netherlands, microscopy can be tedious, with relatively high costs for each case detected.

Although molecular methods such as PCR have proven to be highly sensitive and specific for the detection of *E. histolytica*/*E. dispar*, *G. lamblia* and *C. parvum*/*C. hominis* infections [2, 8, 13, 19, 21], their use in routine diagnostic laboratories is still very limited. The introduction of molecular methods has been hindered by time-consuming methods for the isolation of DNA from fecal specimens and the presence of inhibitory substances in such samples. Furthermore, amplification of DNA was previously laborious and expensive, and cross-contamination among samples was a notorious problem. However, newly developed methods have greatly reduced these obstacles [3, 7, 18]. Real-time PCR reduces labor time, reagent costs and the risk of cross-contamination, and offers the possibility of detecting multiple targets in a single multiplex reaction. A multiplex real-time PCR has been described for the simultaneous detection of the three most important diarrhea-causing parasites, i.e., *E. histolytica*, *G. lamblia* and *C. parvum*/*C.*

hominis, and has demonstrated high sensitivity and specificity with species-specific DNA controls and a range of well-defined stool samples [20]. However, the role of this assay as a diagnostic tool in a routine clinical laboratory requires further evaluation with respect to large-scale screening and improved patient diagnosis [7, 11, 18, 20].

In the present study, the diagnostic results obtained using a multiplex real-time PCR for the detection of *E. histolytica*, *G. lamblia* and *C. parvum*/*C. hominis* were compared with those obtained by routine microscopy of fecal samples from patients visiting their general practitioner (GP) because of gastrointestinal symptoms.

MATERIALS AND METHODS.

Fecal specimens.

DNA samples (n = 956) were initially obtained as part of a study designed to evaluate the efficacy of molecular diagnosis of *Salmonella enterica* and *Campylobacter jejuni* (T. Schuurman *et al.*, **Chapter 5**), and were selected from approximately 1900 fecal samples submitted between June and September 2005 to the Laboratory for Infectious Diseases, Groningen, The Netherlands, for routine bacterial culture and/or microscopical analysis. Fecal specimens from which DNA was extracted were submitted with a request from the GP for bacterial culture for *S. enterica*, *Shigella* spp. or *C. jejuni*, and contained a sufficient amount of fecal material for all tests. Other specimens received during this period were not subjected to DNA extraction.

All samples originated from patients who had visited their GP during the previous few days and who were suspected of having a gastrointestinal infection. According to the normal practice of the GP concerned, each patient submitted either an unpreserved fecal sample (classical method) or a set of three fecal samples (Triple Feces Test; TFT) collected on consecutive days; one sample was unpreserved and two samples were preserved with sodium acetate acetic acid formalin [17]. Upon arrival, the consistency of and the presence of mucus and/or blood in the unpreserved sample were recorded by a trained technician.

By definition, all 950 unpreserved fecal samples included for PCR analysis (six fecal samples were excluded because of inhibitory substances

in the DNA extracts) were cultured routinely for *Campylobacter* spp., *Salmonella* spp. and/or *Shigella* spp., and in some cases for other pathogens, according to the request made by the GP or the clinical microbiologist. From this group, 722 samples were also examined by microscopy for the presence of parasites, according to the request made by the GP or the clinical microbiologist. For comparison, an additional 913 samples from the same group of 1900 cases who submitted fecal samples for the detection of parasites only were also examined by microscopy.

Microscopy.

Unpreserved samples were investigated for ova and cysts by microscopy of iodine-stained wet-mount preparations of a formalin–ether concentrate [1]. Sodium acetate acetic acid formalin-preserved samples were first screened by iodine-stained direct smears. Parasite-like structures were confirmed by microscopy of a chlorazol black dye permanent stain preparation [17]. Modified Ziehl–Neelsen staining for the detection of *Cryptosporidium* was only performed if cryptosporidiosis was suspected by the GP.

DNA extraction.

DNA was extracted from fecal suspensions (33–50% w/v) using the semi-automated NucliSENS miniMAG instrument (bioMérieux, Boxtel, The Netherlands) in combination with NucliSENS Magnetic Extraction Reagents (bioMérieux), according to the manufacturer's instructions. In brief, 100 µL of fecal suspension was added to 2 mL of lysis buffer and incubated at room temperature for 10 min, after which an internal control (phocin herpes virus-1 (PhHV-1); approximately 6000 copies/sample) and 50 µL of magnetic silica particles were added. The mixture was mixed and incubated for 10 min at room temperature. After centrifugation for 2 min at 1500 x g, the supernatant was removed by aspiration and the pellet of silica–nucleic acid complexes was resuspended and washed in three washing buffers. Each washing step was conducted for 30 s on step 1 of the miniMAG instrument, with the exception of wash buffer 3 (15 s on step 1), after which the fluid was removed by aspiration. DNA was eluted in 100 µL of elution buffer for 5 min at 60°C on a thermoshaker (Eppendorf, Hamburg, Germany) at 1400 rpm. The extracted DNA was stored at −20°C.

PCR amplification and detection.

The sample population was analyzed by real-time PCR without reference to the initial microscopy results. Amplification and detection of *E. histolytica*, *C. parvum*/*C. hominis* and *G. lamblia* DNA, as well as the PhHV-1 internal control DNA, were performed on all samples using a multiplex real-time PCR, essentially as described previously [20], but with minor modifications.

In brief, amplification reactions were performed in 25-µL volumes containing PCR buffer (Hotstar mastermix; Qiagen, Venlo, The Netherlands), 5 mM $MgCl_2$, 2.5 µg of bovine serum albumin (Roche Diagnostics, Almere, The Netherlands), 3.125 pmol each of the *E. histolytica*- and *G. lamblia*-specific primers, 12.5 pmol of the *Cryptosporidium*-specific primers, 1.25 pmol of VIC-labelled MGB-Taqman probe (Applied Biosystems, Warrington, UK) for *E. histolytica*, 2.5 pmol of FAM-labelled double-labelled probe (Biolegio, Nijmegen, The Netherlands) for *G. lamblia*, 2.5 pmol of Texas-red-labelled double-labelled probe for *Cryptosporidium*, and 5 µL of template DNA. The PhHV-1-specific primers and probe set consisted of 3.75 pmol of each PhHV-1-specific primer and 2.5 pmol of Cy5-labelled double-labelled probe.

Amplification comprised 15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 30 s at 60°C. Amplification, detection and data analysis were performed using the I-cycler Real-Time PCR System and v.3.1.7050 software (Bio-Rad, Hercules, CA, USA).

Fecal DNA samples were considered to contain inhibitors if the PhHV-1 internal control was not detected, or if the expected cycle threshold (Ct) value of 32 cycles was increased by more than five cycles. Because of inhibition, six samples were excluded from the parasite analysis.

Ct-values obtained for *G. lamblia*, *E. histolytica* or *Cryptosporidium* amplification are considered less reproducible at > 36 cycles. Therefore, the real-time PCR was repeated for 17 fecal samples with an initial Ct-value of 36.4–47.4 (median 40.7). For 12 samples, amplification was detected with comparable Ct-values (range 33.0–39.1, median 38.0), while five samples showed no DNA amplification and were therefore considered to be negative.

The primers described by Morgan *et al.* [12] were used to differentiate between *C. hominis* and *C. parvum* in DNA samples positive for

Cryptosporidium DNA. PCR was performed in 25-µL volumes containing PCR buffer, 5 mM $MgCl_2$, 2.5 µg of bovine serum albumin, 12.5 pmol of forward primer (021F) annealing to both *C. hominis* and *C. parvum*, 6.25 pmol of *C. hominis*-specific reverse primer (CP-HR) and 6.25 pmol of *C. parvum*-specific reverse primer (CP-CR). Amplification comprised 15 min at 95°C, followed by 45 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, with a final extension for 5 min at 72°C. Amplification products (411 bp for *C. hominis* and 312 bp for *C. parvum*) were detected following electrophoresis in agarose 2% w/v gels stained with ethidium bromide.

Data analysis.

Statistical analyses were performed using SPSS v.11.0.1 (SPSS Inc., Chicago, IL, USA). Proportions were compared among groups, and odds ratios (Ors) and 95% confidence intervals (CIs) were calculated. Continuous variables were described by range and median values, and were compared among groups by the Mann–Whitney rank sum test, with $p < 0.05$ considered to be statistically significant.

RESULTS.

Study group.

Table 9.1 summarizes the patient characteristics of the 950 cases included for detection of intestinal protozoa by real-time PCR, and the 913 cases examined for parasites only by routine microscopy. The age range of the patients was 0–95 years (median 33 years). The group examined for parasites by PCR contained significantly fewer children aged < 15 years as compared to the microscopy group (18.9% vs. 36.8%; OR 0.40, 95% CI 0.33–0.50).

Information concerning travel history was supplied by the GP for 639 (44.3%) patients, including 336 subjects for whom ‘no travel history’ was specifically noted. Eighty-four patients had travelled in areas considered to be low-risk areas for intestinal parasite infections (western Europe, North America and Australia), while 186 had travelled in areas considered to be high-risk areas (Africa, South America and Asia, including Turkey). No increased frequency of travel was reported for the subjects examined for parasites by microscopy only (Table 9.1). For 33 subjects (age 0–6 years, median 1 year), fecal specimens were sent to the laboratory for routine fecal

examination as part of an adoption protocol. The countries of origin of these children were often not reported by the GP.

TABLE 9.1. Characteristics and outcome of faecal examination for parasites in 1863 patients who visited their general practitioner because of gastrointestinal complaints[a].

	Patients analysed for parasites by:		
	PCR and microscopy (n = 722)	PCR without microscopy (n = 228)	Microscopy without PCR (n = 913)
Gender - % male	41.8	41.2	43.4
Age - range (median)	0 - 95 (36.0)	0 - 92 (41.5)	0 - 92 (27.0)
Travel history - number (%)			
not known	578 (80.1)	203 (89.0)	443 (48.5)
no travel	7 (1.0)	3 (1.3)	326 (35.7)
low risk areas	49 (6.8)	6 (2.6)	29 (3.2)
high risk areas	88 (12.2)	5 (2.2)	93 (10.2)
adoption child	0 (0.0)	11 (4.8)	22 (2.4)
Microscopy - number (%)			
TFT procedure	247 (34.2)	ND	637 (69.8)
classical procedure	475 (65.8)	ND	276 (30.2)
Giardia lamblia	41 (5.7)	ND	44 (4.8)
Cryptosporidium spp.	0	ND	1 (0.1)
Entamoeba histolytica/E. dispar	0	ND	1 (0.1)
Blastocystis hominis	21 (2.9)	ND	70 (7.7)
Dientamoeba fragilis	8 (1.1)	ND	17 (1.9)
Endolimax nana	4 (0.6)	ND	14 (1.5)
Entamoeba coli	3 (0.4)	ND	20 (2.2)
Iodamoeba butschlii	0	ND	3 (0.3)
Hymenolepis nana	0	ND	1 (0.1)
Real-time PCR - number (%)			
Giardia lamblia	67 (9.3)	15 (6.6)	ND
Cryptosporidium parvum/C. hominis	36 (5.0)	5 (2.2)	ND
Entamoeba histolytica	0 (0.0)	0 (0.0)	ND

[a] ND, not done; TFT, Triple Faeces Test.

Information concerning the gastrointestinal symptoms of the patients was supplied by the GP for 65.8% of 950 cases tested by PCR. In most (88%) cases, diarrhea was mentioned; other symptoms mentioned were watery (11.0%), bloody (5.9%) or slimy (4.8%) feces. According to the laboratory description, most (90.4%) unpreserved fecal samples were unformed, and the number of watery (3.9%), bloody (1.7%) or slimy (0.8%) samples was limited. Examination for *Cryptosporidium* was specifically requested by the GP for seven patients.

Microbiology.

In 127 (13.4%) of 950 cases, a non-parasite fecal pathogen was demonstrated. Routine testing revealed *Campylobacter* spp. (n = 90), *Salmonella* spp. (n = 29), *Shigella* spp. (n = 2), *Yersinia* spp. (n = 3) and *Clostridium difficile* (n = 3). Table 9.1 summarizes the results of routine microscopy for fecal parasites. In total, 1635 subjects were examined, either by classical microscopy (n = 751) or by TFT (n = 884). Although significantly fewer TFT procedures were performed in the group analyzed for parasites by PCR (34.2% vs. 69.8%; OR 0.23, 95% CI 0.18–0.28), the frequencies of detection of parasites by microscopy in both groups were comparable, with the exception of *Blastocystis hominis* (2.9% vs. 7.7%; OR 2.77, 95% CI 1.68–4.56) and *Entamoeba coli* (0.4% vs. 2.2%; OR 5.36, 95% CI 1.60–18.14). *G. lamblia* was the most common parasite detected (n = 85; 5.2%), either by TFT (n = 48, 5.4%) or by classical microscopy (n = 37, 4.9%; OR 0.90, 95% CI 0.58–1.40). TFT also revealed 25 (2.8%) *Dientamoeba fragilis* infections. Except for one sample with *Hymenolepis nana*, no helminths were seen in any of the fecal samples examined, and only one patient infected with *Cryptosporidium* spp. was detected.

PCR results.

Amplification of the PhHV-1 internal control was, by definition, detected within the correct Ct range for all 950 DNA samples (Ct 29.1–36.9, median 31.7). No *E. histolytica*-specific amplification products were detected.

A *G. lamblia*-specific amplification product was seen for 82 (8.6%) of 950 samples (Ct 21.1–39.1, median 28.6). In the subgroup analyzed by both microscopy and PCR, the prevalence of *G. lamblia* increased from 5.7% to 9.3% (Table 9.1). All but one of 41 samples in which *G. lamblia* was detected by microscopy showed a *G. lamblia*-specific amplification product (Ct 21.1–32.8, median 27.5). The discrepant sample (from a male aged 61 years with watery diarrhea) contained cysts of both *G. lamblia* and *Endolimax nana* when investigated by classical microscopy, with no other pathogens being detected following culture. A *G. lamblia*-specific amplification product was obtained for 16 (3.6%) and 11 (4.8%) of the samples in which *G. lamblia* was not found by classical microscopy or the TFT procedure, respectively. Ct-values were significantly higher for those samples in which *G. lamblia* was not detected by microscopy (Ct median

33.0, range 23.2–39.1; $p < 0.001$). Furthermore, 15 (6.5%) samples yielded a *G. lamblia*-specific amplification product, despite the fact that no microscopical examination for intestinal parasites was requested (Table 9.1). No significant differences in the Ct-values were noted for those samples that were examined microscopically and those that were not.

Compared with microscopy, an increased prevalence of *G. lamblia* infection was revealed by PCR for all age groups, with the exception of patients aged 15–30 years (Fig. 9.1A). The same pattern was seen when the microscopy and PCR data for *G. lamblia* were compared for the 720 subjects analyzed by both procedures (data not shown). No associations were seen between *G. lamblia* infection and stool consistency or travel history, with the exception of 11 children who were screened according to the adoption protocol, five (45.5%) of whom yielded a *G. lamblia*-specific amplification product. In comparison, *G. lamblia* infection was indicated in five (22.7%) of 22 children examined by microscopy only.

A *Cryptosporidium*-specific amplification product was obtained from 41 (4.3%) of 950 DNA samples (Ct 24.5–37.9, median 31.0), including two samples in which *G. lamblia* and *Cryptosporidium* were detected simultaneously. *Cryptosporidium* was also detected in five (2.2%) cases for which no microscopical examination was requested by the GP (Table 9.1). Infections with *Cryptosporidium* were associated strongly with the age of the patients, with detectable *Cryptosporidium* DNA in 21.8% of children aged < 5 years (Fig. 9.1B). No association was seen between *Cryptosporidium* infection and travel history. *Cryptosporidium* was detected more often in patients complaining of watery diarrhea (7 of 72; 9.7%), but this trend was not significant.

Additional PCRs for differentiation of *C. parvum* and *C. hominis* identified 29 samples that contained *C. hominis* and nine samples that contained *C. parvum*; no amplification product was obtained from three samples. *C. parvum* and *C. hominis* were distributed almost equally among the positive samples collected in July, while *C. hominis* was detected exclusively in the samples collected during September (data not shown).

Double infections with parasites and non-parasitic pathogens involved *G. lamblia* and *Campylobacter* spp. (n = 3), and *Cryptosporidium* and *Campylobacter* spp. (n = 1).

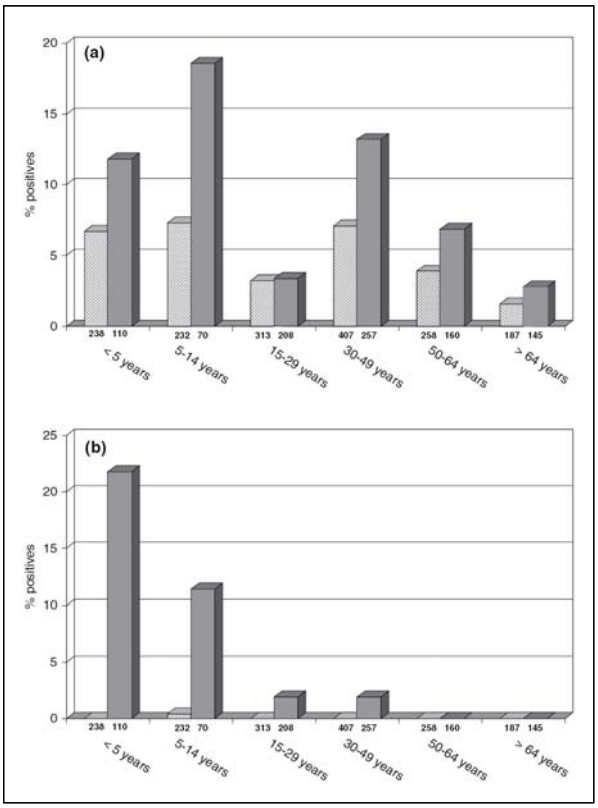


Figure. 9.1. Prevalence of (a) *Giardia lamblia* and (b) *Cryptosporidiumparvum/Cryptosporidium homis* in different age groups, detected either by microscopy (hatched bars; n = 1635) or real-time PCR (solid bars; n = 950). The number of cases examined in each category is indicated below each bar.

DISCUSSION.

A multiplex real-time PCR has been described previously for the simultaneous detection of *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp. DNA in fecal samples [20]. In the present study, the results obtained using this multiplex real-time PCR assay were compared retrospectively with the results obtained by routine microscopy in clinical laboratory practice for patients with diarrhea who consulted their GP. Fecal DNA samples examined in the present study were isolated initially from a selected number of patients, based on the results of bacterial culture. To evaluate the possibility of selection bias, the microscopy data were compared with those for 913 additional subjects, also suffering from gastrointestinal problems, but with suspected parasitic infections. As expected, the latter group included more children. However, no significant differences in travel history or the

number of parasitic infections detected by microscopy were revealed, with the exception of two non-pathogens (*B. hominis* and *E. coli*). In general, pathogenic parasites were detected during routine microscopy in a low number of cases, and with the exception of a single case involving *H. nana*, no helminths were seen. With a detection rate of 5.2% (n = 1635), *G. lamblia* was the most common pathogenic enteric parasite found in patients diagnosed using microscopy. This finding agrees with other studies of *G. lamblia* infection in The Netherlands, including data from a large Dutch case-control study in which *G. lamblia* was detected in 5.4% of patients who consulted their GP for gastroenteritis [5, 9]. Using real-time PCR (n = 950 cases), the rate of detection of *G. lamblia* increased to 8.6%, and the number of infected cases was more than double in children of school age. In addition, very high rates of *G. lamblia* infection were found in adopted foreign children, who had presumably been exposed in their country of origin [14].

Although no specific request for the diagnosis of *Cryptosporidium* infection was made by the GP, an overall prevalence of 4.3% was detected by PCR, with one in five children aged < 5 years being *Cryptosporidium*-positive. Among the infected children, no predisposition towards *C. parvum* or *C. hominis* was noticed. However, a sudden increase in cases positive for *C. hominis* was detected in September 2005, whereas *C. parvum* was detected only in July and August. Seasonal variation in the incidence of cryptosporidiosis related to travel or environmental factors has been suggested [4, 15, 16], but further investigation is needed to unravel the specific underlying factors explaining this complex epidemiology.

E. histolytica was not detected by real-time PCR in the present study. However, cases of amoebiasis in The Netherlands are very limited, and are always directly or indirectly related to travel in high-risk areas. Nevertheless, accurate diagnosis of *E. histolytica* infection is vital in order to interrupt transmission of the parasite and to avoid any progression into invasive disease. Furthermore, PCR enables specific detection of *E. histolytica*, thereby differentiating this species from the non-pathogenic species *E. dispar*, which is more common and is morphologically identical to *E. histolytica*.

No relationship was found between specific pathogens and the patient's symptoms, as the latter tended to be non-specific and vague, making appropriate diagnostic requests difficult. In fecal samples for which only bacterial culture was requested, real-time PCR revealed 20 parasitic infections. Screening all fecal specimens for parasites in a routine diagnostic laboratory would therefore be appropriate.

The specificity of PCR obviously restricts the number of different pathogens that can be detected, in contrast to the broad range of different parasites that can be detected using microscopy. Nevertheless, microscopy showed limited additional value in this patient population, even with the use of the TFT procedure, as most of the additional parasites detected were non-pathogenic. These included *D. fragilis* and *B. hominis*, two organisms whose pathogenicity is still unresolved. *D. fragilis* is one of the priority candidates for inclusion in an expanded multiplex real-time PCR, as such detection would also provide a valuable tool to further elaborate its possible pathogenicity.

The present study focused on the detection of *E. histolytica*, *G. lamblia* and *C. parvum*/*C. hominis* in subjects with community-acquired diarrhea in a developed country. The diagnostic value of this multiplex real-time PCR should also be evaluated in additional patient groups, particularly travelers returning from the tropics and immunocompromised individuals in whom additional intestinal parasites might be expected. The possibility of combining alternative parasite targets or panels within a multiplex real-time PCR should be investigated, e.g., the detection of *Cyclospora cayetanensis*, *E. histolytica* and *G. lamblia* in travelers, or the detection of microsporidia, *Cryptosporidium* and *Isospora belli* in immunocompromised patients. Used in combination with a similar approach for the detection of diarrhea-causing viruses and bacteria, this would give a completely new alternative for the laboratory diagnosis of diarrheal disease. However, until these approaches have been further evaluated and validated, conventional diagnostic techniques, e.g., culture, microscopy and antigen/antibody detection, will still have a prominent role in the diagnosis of diarrheal disease.

In conclusion, the present study revealed that significant numbers of *G. lamblia* and *Cryptosporidium* infections remain undetected by microscopy in patients with gastrointestinal symptoms who consult their GP. Furthermore,

the number of additional parasites detected with microscopy was shown to be limited in this population. Therefore, the introduction of real-time PCR for the routine detection of diarrhea-causing protozoa would improve the diagnostic efficiency of laboratories dealing with fecal samples from this patient group.

REFERENCES.

1. **Allen, A. V., and D. Ridley.** 1970. Further observations on the formol-ether concentration technique for faecal parasites. J. Clin. Pathol. **23:**545–546.
2. **Blessmann, J., H. Buss, P. A. T. Nu, B. T. Dinh, Q. T. Ngo, A. L. Van, M. D. Alla, T. F. Jackson, J. I. Ravdin, and E. Tannich.** 2002. Real-time PCR for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples. J. Clin. Microbiol. **40:**4413–4417.
3. **Boom, R., C. Sol, J. Weel, K. Lettinga, Y. Gerrits, A. van Breda, and P. Wertheim-van Dillen.** 2000. Detection and quantitation of human cytomegalovirus DNA in faeces. J. Virol. Methods **84:**1–14.
4. **Caccio, S. M., R. C. Thompson, J. McLauchlin, and H. V. Smith.** 2005. Unravelling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol. **21:**430–437.
5. **de Wit, M. A., M. P. Koopmans, L. M. Kortbeek, N. J. van Leeuwen, J. Vinjé, and Y. T. van Duynhoven.** 2001. Etiology of gastroenteritis in sentinel general practices in the Netherlands. Clin. Infect. Dis. **33:**280–288.
6. **de Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, W. J. Wannet, J. Vinjé, F. van Leusden, A. I. Bartelds, and Y. T. van Duynhoven.** 2001. Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. Am. J. Epidemiol. **154:**666–674.
7. **Espy, M. J., J. R. Uhl, L. M. Sloan, S. P. Buckwalter, M. F. Jones, E. A. Vetter, J. D. Yao, N. L. Wengenack, J. E. Rosenblatt, F. R. Cockerill 3rd, and T. F. Smith.** 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin. Microbiol. Rev. **19:**165–256.
8. **Ghosh, S., A. Debnath, A. S. De Sil, D. J. Chattopadhyay, and P. Das.** 2000. PCR detection of *Giardia lamblia* in stool: targeting intergenic spacer region of multicopy rRNA gene. Mol. Cell. Probes **14:**181–189.
9. **Homan, W. L., and T. G. Mank.** 2001. Human giardiasis: genotype linked differences in clinical symptomatology. Int. J. Parasitol. **31:**822–826.
10. **Kosek, M., C. Bern, and R. L. Guerrant.** 2003. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull. WHO **81:**97-204.
11. **Lewthwaite, P., G. V. Gill, C. A. Hart, and N. J. Beeching.** 2005. Gastrointestinal parasites in the immunocompromised. Curr. Opin. Infect. Dis. **18:**427–435.
12. **Morgan, U. M., C. C. Constantine, D. A. Forbes, and R. C. Thompson.** 1997. Differentiation between human and animal isolates of *Cryptosporidium parvum* using rDNA sequencing and direct PCR analysis. J. Parasitol. **83:**825–830.
13. **Morgan, U. M., and R. C. A. Thompson.** 1998. PCR detection of *Cryptosporidium*: the way forward? Parasitol. Today **14:**241–245.
14. **Saiman, L., J. Aronson, J. Zhou, C. Gomez-Duarte, P. S. Gabriel, M. Alonso, S. Maloney, and J. Schulte.** 2001. Prevalence of infectious diseases among internationally adopted children. Pediatrics **108:**608–612.
15. **Steffen, R.** 2005. Epidemiology of traveler's diarrhea. Clin. Infect. Dis. **41:**S536–S540.
16. **van Asperen, I. A., T. Mank, G. J. Medema, C. Stijnen, A. S. de Boer, J. F. Groot, P. TenHam, J. F. Sluiters, and M. W. Borgdorff.** 1996. An outbreak of cryptosporidiosis in the Netherlands. Euro Surveill. **1:**11–12.
17. **van Gool, T., R. Weijts, E. Lommerse, and T. G. Mank.** 2003. Triple Faeces Test: an effective tool for detection of intestinal parasites in routine clinical practice. Eur. J. Clin. Microbiol. Infect. Dis. **22:**284–290.
18. **Verweij, J. J., J. Blotkamp, E. A. T. Brienen, A. Aguirre, and A. M. Polderman AM.** 2000. Polymerase chain reaction for differentiation of *Entamoeba histolytica* and *Entamoeba dispar* cysts on DNA isolated from faeces with spin columns. Eur. J. Clin. Microbiol. Infect. Dis. **5:**358–361.
19. **Verweij, J.J., F. Oostvogel, E. A. Brienen, A. Nang-Beifubah, J. Ziem, and A. M. Polderman.** 2003. Short communication: prevalence of *Entamoeba histolytica* and *Entamoeba dispar* in northern Ghana. Trop. Med. Int. Health **8:**1153–1156.

20. **Verweij, J. J., A. Roy, K. Templeton, J. Schinkel, E. A. Brienen, M. A. van Rooyen, L. van Lieshout, and A. M. Polderman.** 2004. Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. J. Clin. Microbiol. **42:**1220–1223.
21. **Verweij, J. J., J. Schinkel, D. Laeijendecker, M. A. A. van Rooyen, L. van Lieshout, and A. M. Polderman.** 2003. Real-time PCR for the detection of *Giardia lamblia*. Mol. Cell. Probes **17:**223–225.

CHAPTER 10

Summarizing Discussion

Over the past twenty-five years, molecular methods have evolved from basic research to an appreciated diagnostic tool within the field of clinical microbiology. In this respect, clinical virology has benefited the strongest from these technological developments. In fact, molecular methods have become the diagnostic standard in clinical virology testing, whereas with regard to bacteriology, parasitology, and mycology, their use has been limited to fastidious, non-cultivable organisms or those pathogens for which conventional diagnostic tests lack sensitivity. In this thesis, new possibilities of molecular diagnostic testing were explored in applications where conventional culture- and microscopy-based methodologies have long been considered the "gold standard". The following paragraphs will address the implementation of these new methods within a routine, community-based clinical microbiology laboratory.

BROAD-RANGE rDNA-BASED DETECTION OF BACTERIAL PATHOGENS.

Within the field of clinical bacteriology, culture will probably remain the "cornerstone" in the confirmation of bacterial infections for some time to come. The main reason for this is that for most bacterial pathogens specific culture media are available, and furthermore, culturing on more general media allows for broad-spectrum detection of bacteria, when multiple pathogens are considered within the differential diagnosis. In addition, for non-fastidious bacterial pathogens culture is relatively fast (i.e. primary identifications are available within 1-2 days) and fairly inexpensive. However, if anti-microbial therapy has already started, the sensitivity of culture drops substantially.

With the method developed in **Chapter 2**, it is theoretically possible to detect DNA from all bacteria in clinical specimens originating from normally-sterile human body sites. The broad-range character of 16S rDNA amplification is not only its strongest asset; it also constitutes its greatest challenge, as it is quite vulnerable to contamination, significantly more than species-specific amplification. Besides the potential risk for specimen to specimen contamination in both the specimen preparation and amplicon detection stage, which also poses a risk for species-specific PCR, the reagents themselves become a serious source of contamination when

applying universal PCR. Therefore, all stages in the diagnostic process represent a potential risk for introduction of contaminating nucleic acids (NA), and contaminants have been reported in decontamination fluids (Betadine iodine and chlorhexidine solutions) [100], blood sample containers [63], NA extraction reagents [24, 65, 75, 102], and amplification reagents [8, 17, 64]. Several approaches have been employed to remove the contaminating DNA from both amplification and extraction reagents. These methods include UV-irradiation, psoralen (8-MOP) + UV-irradiation, DNase I treatment, restriction endonuclease digestion, and ultra-filtration. However, to date, the effectiveness of many of these methods is controversial or needs further evaluation [7, 17]. This is confirmed by our own experience with some of the more successful contamination removing methodologies, restriction endonuclease digestion and ultrafiltration for cleaning of the PCR mastermix [64, 108], and GenElute filtration to clean-up the extraction reagents [65]. Although at first a reduction of the amount of background DNA in the reagents was observed beyond detectable levels with all three methods, none of them proved to be fully reproducible [Tim Schuurman and Richard de Boer, Laboratory for Infectious Diseases (Lvl), unpublished data]. Therefore, at present, the most reliable way to avoid false-positives in broad-range PCR remains careful management of the working environment and reagents [63], combined with optimizing the number of amplification cycles [37, 67]. Unfortunately, the contamination issue still renders rDNA targeted broad-range PCR more prone to false-positives compared to species specific PCRs.

Recently, a novel decontamination method has been described which is applicable to both conventional and real-time PCR [38, 85]. In this method, the reagent derived DNA is treated with the photo-reactive compounds ethidium monoazide (EMA) or propidium monoazide which are covalently cross-linked to DNA by long wavelength light exposure, rendering the DNA non-amplifiable by PCR. Although the first reports are promising, this method also needs further evaluation, as method-associated problems will likely be unveiled with increased use, as was also the case for other contamination reducing methods in broad-range PCR. In addition, the EMA treatment may also provide effective removal of contaminating DNA in NA extraction reagents, although this has not been attempted at this time.

For the assay developed in **Chapter 2**, an additional strategy was included to minimize the potential for contamination of the amplification reaction. This was done by amplifying nearly the entire 16S rRNA gene (1500 bp) as opposed to a smaller amplicon (i.e. 500 bp or smaller). This strategy proved successful and showed much less vulnerability to contamination [Tim Schuurman and Richard de Boer, LvI, unpublished data]. Similar results were also reported by others using real-time PCR when comparing a 467 bp amplicon to 167 and 159 bp amplicons [106], or a 796 bp amplicon compared to 466 and 346 bp amplicons [110].

This assay has been routinely used at the LvI since 2003, and has proven its clinical utility in numerous cases [103]. Since the introduction almost 500 clinical specimens have been screened, and in fact, nearly 25% of the specimens tested by the broad-range 16S rDNA PCR were regarded as positive. For the majority (87%) of these positive results, sequencing provided the identification of a single bacterial species, whereas for the reminder mixed sequences were retrieved. The results of the routine use of broad-range PCR in our laboratory are in line with reports from two other groups [37, 79], although the comparison between culture and PCR was only available for a subset of the data from 2003-2004 (i.e. 138 out of 179 specimens) (Table 10.1).

With these 138 specimens, culture and PCR showed concordant results for 110 specimens (102 negative, 8 positive). Only a single specimen yielded weak growth of a mixed anaerobic flora, which was missed by PCR. However, in 20 specimens negative by culture the presence of a bacterial species was detected by PCR only. Sequencing successfully identified the bacterium in 19 of these specimens, whereas the final specimen showed evidence of a mixed infection rendering sequencing unsuccessful. In addition, for 8 growth-positive blood cultures the organism was identified by PCR, whereas sub-cultures remained negative.

For specimens without culture information (mostly from external referring laboratories), as well as for the specimens received in 2005 to 2006, similar detection rates were obtained as compared to the specimens from 2003-2004 (approximately 25%). This high detection rate illustrates the added value of introducing a broad-range bacterial PCR into the routine work-flow. Part of this, however, may also be linked to the selective procedure by which

the clinical microbiologists request 16S rDNA PCR for a given specimen. In general, 16S rDNA PCR is indicated only when antibiotics have already been administrated prior to specimen collection, or when cultures remain negative despite a strong suspicion of bacterial infection.

TABLE 10.1. Comparison of culture and broad-range PCR at Lvl during 2003-2004[a].

Number of samples	16S rRNA gene method		Culture	Specimen type(s)
	PCR	Identification		
102	Neg	NA	Neg	Various
1	Neg	NA	Mixed anaerobic flora	Cavum
1	Pos	*Corynebacterium tuberculostearicum*	Neg	Spondylodiscitis biopsy
1	Pos	*Fusobacterium nucleatum*	Neg	Pus
1	Pos	*Helicobacter heilmannii*	Neg	Antrum biopsy
1	Pos	*Neisseria meningitidis*	Neg	CSF
1	Pos	*Propionibacterium acnes*[b]	Neg	Neck lesion swab
1	Pos	*Shigella dysenteriae*	Neg	Liver abscess
1	Pos	*Streptococcus dysgalactiae*	Neg	Synovial fluid
1	Pos	*Streptococcus mitis*	Neg	Aortic valve tissue
8	Pos	*Streptococcus pneumoniae*	Neg	Synovial fluid (1) CSF (1) Pleural fluid (4) Aneurism (2)
1	Pos	*Corynebacterium* sp.	Neg	Bone tissue
1	Pos	*Fusobacterium* sp.	Neg	Pleural fluid
1	Pos	*Peptostreptococcus* sp.	Neg	Liver abscess
1	Pos	Mixed sequence	Neg	Pleural fluid
1	Pos	*Bacteroides thetaiotaomicron*	Pos/Neg	Blood culture
1	Pos	*Fusobacterium necrophorum*	Pos/Neg	Blood culture
4	Pos	*Streptococcus pneumoniae*	Pos/Neg	Blood culture
1	Pos	*Streptococcus pyogenes*	Pos/Neg	Blood culture
1	Pos	Unknown organism[b]	Pos/Neg	Blood culture
3	Pos	*Bacteroides fragilis*	*B. fragilis*	Blood culture
1	Pos	*Eubacterium aerofaciens*	*E. aerofaciens*	Blood culture
1	Pos	*Neisseria lactamica*[c]	*N. meningitidis*	CSF
1	Pos	*Porphyromonas asaccharolytica*	*P. asaccharolytica*	Abscess abdomen
1	Pos	*Pseudomonas aeruginosa*	*P. aeruginosa*	Spinal column washing
1	Pos	*Streptococcus dysgalactiae*	*S. dysgalactiae*	Pus

[a] Neg, negative; Pos, positive; Pos/Neg, positive blood culture with negative subculture; NA, not applicable; CSF, cerebrospinal fluid.
[b] Clinical relevance doubtful.
[c] Sequence was identified as *N. lactamica* with *N. meningitidis* as second best match.

Although all specimens considered to be sterile in healthy subjects, can be analyzed by broad-range PCR, there is one application in which sensitivity is of greater concern than in others, namely blood stream infections (BSI). The major issue here is the large difference in the amount of specimen analyzed by each method (up to approximately 200 µl for PCR

vs 50 times that amount for blood culture). Although some reports have shown adequate sensitivity of both broad-range and species specific PCR assays in detecting bacterial BSI in selected patient groups, the consensus is that bacterial numbers in BSI are generally low (i.e. 1-10 CFU/ml) [16, 35, 36, 39, 46, 76]. To cope with such low numbers, current technological developments are aimed at increasing the amount of blood that can be processed for NA extraction. However, with the increase of processed blood volume, potentially so does the amount of inhibitory substances which need to be overcome [1, 2, 15]. A major inhibitory substance and one which is hard to get rid off, is human DNA. It is co-purified with the pathogen DNA [15], and its depletion from a specimen would offer great diagnostic potential. Based on separate principles, both MolYsis® (Lysis of human cells, followed by MolDNase degradation of human DNA [Molzym, Bremen, Germany]) and PUREPROVE® (Selective binding of bacterial and fungal DNA to protein p181, followed by removal of human DNA by a wash procedure [LOOXTER® universal, SIRS-lab, Jena, Germany]) significantly reduce the amount of human DNA and thereby enriches bacterial/fungal DNA [42]. However, further clinical studies are required to assess the effectiveness and applicability of these methods in routine diagnostics.

Septi*Fast*, a species-specific real-time PCR-based assay for detection of BSI recently introduced by Roche (Roche Diagnostics GmbH, Penzberg, Germany) may also improve the molecular detection of BSI [57]. This kit is capable of detecting 25 different bacteria and fungi that are amongst the most frequently isolated species in blood cultures. Reported analytical sensitivities are in the range of 3 - 30 colony-forming units/ml for 23 out of the 25 species that are detected. This relatively high sensitivity is related to the amount of blood that is processed, which in the current format is 3 ml. However, as results of clinical testing have not yet been reported, the utility of this assay in the routine diagnostic laboratory remains to be established.

In most cases, organisms identified by direct 16S rDNA sequencing are clinically relevant, although occasionally environmental bacterial species will also be identified. This illustrates the need to interpret the results of the 16S rDNA PCR alongside all other clinical and laboratory parameters available to the clinician. Furthermore, it has also recently been demonstrated that there may be (transient) bacterial DNA present in the blood of healthy volunteers,

suggesting that some anatomical sites previously considered to be sterile using cultivation, and therefore suitable for broad-range bacterial DNA detection, may in fact harbor significant amounts of bacterial DNA [69].

To further improve the routine use of the assay developed in **Chapter 2**, it has recently been converted to automated sample preparation (NucliSENS easyMAG) and real-time PCR. With these adaptations, the assay is implemented within the routine (molecular) diagnostic laboratory with greater ease, as the conventional assay format still requires highly specialized laboratory personnel. Furthermore, it has also resulted in enhancement of several performance characteristics compared to the conventional assay [Tim Schuurman and Richard de Boer, Lvl, unpublished data].

Introduction of a universal 16S rDNA probe in the real-time PCR format enhanced the specificity of the assay, as its clearly distinguishes between specific amplification product and non-specific bands, which may originate from human DNA, of similar size visible on gel in the conventional assay. Others have reported similar increases in specificity by introducing a broad-range TaqMan probe [106, 108, 110]. This reduces the number of specimens that need further processing without yielding a positive result. Furthermore, the real-time assay proofed to be far less vulnerable to inhibition (10% vs 49%), which again reduces the need for additional testing, as specimens do not need to be tested diluted. Also, the quantity of real-time PCR product obtained from weakly positive specimens appeared to be sufficient for direct sequencing more often as with the conventional assay, thereby reducing the number of specimens that need reamplification. And finally, automation resulted in a decrease in hands-on time of about 50%. In addition, by using real-time PCR, the difference in Ct-value (ΔCt) between a specimen and the no-template control can be used to discriminate background signals from positive signals, as has also been suggested by others [46, 82, 110].

Overall, general detection of bacterial DNA by broad-range PCR clearly supports the clinical microbiology laboratory.

(SEMI-)AUTOMATED NUCLEIC ACID EXTRACTION.

With the increasing numbers of specimens for which molecular testing is requested, sample preparation has become a major bottleneck in NA testing. Although the manual extraction method developed by René Boom [6] is

acknowledged for its performance, the continued use of this laborious method in today's high-throughput molecular laboratory is no longer feasible. Therefore manual sample preparation needs to be replaced by automated procedures. Besides reducing the hands-on time, automation will result in increased standardization and precision, as a robot will perform the different stages with less experimental variation compared to manual processing [22, 48, 59]. In addition, automation reduces the potential for human error, and decreases the potential for contamination, as this has been shown to be related to the number of hands-on manipulations involved in sample processing [14, 55].

Several automated NA extraction systems are available and have been evaluated with various specimen and application types. Their performance has been shown to be acceptable and most likely superior to manual methods. However, the composition of a clinical specimen will vary depending on the infecting organism. The typical microbial dose, and the physical specimen type, together with reliability of sampling and speed of sample transport are important variables. For that reason, extrapolation of previous studies on certain sample types to non-evaluated specimen types should be done with great care [95, 107]. Therefore, thorough evaluations of NA extraction methods in combination with specific molecular tests remain mandatory to elucidate both the strengths and limitations of the available methods.

In **Chapter 3**, we showed that during application of the Total Nucleic Acid Isolation Kit (TNA) a substantial portion of the total amount of DNA present in a clinical specimen is simply lost. Although this may not pose a serious problem for many applications due to the relatively high numbers of organisms present in most clinical specimens, it may pose a serious problem with specimens that contain only limited numbers of pathogens (e.g. bacterial meningitis and BSI). We therefore concluded that the performance of the TNA kit was unsatisfactory and could not replace the manual method of Boom *et al.* in the assays developed in this thesis.

For the extraction of NAs from stool specimens however, automated sample preparation is mandatory due to the complexity of the sample and the high numbers of fecal specimens that need to be processed on a daily basis. We therefore investigated the performance of manual, semi- and

automated NA extraction systems with regard to their performance in stools. In **Chapter 4** we showed that both the MagNA Pure LC (DNA isolation Kit III Bacteria/Fungi) (Roche) and the NucliSENS miniMAG (bioMérieux) are suitable instruments for processing stool specimens. Based on the lower inhibition rate and resemblance of the miniMAG to our manual in-house NA extraction method, we opted to further evaluate this system in molecular detection of gastrointestinal disease. In addition, a NA extraction method that simultaneously isolates both DNA and RNA from stool is preferred, as gastroenteritis is caused in majority by RNA viruses. The generic isolation kit for the MagNA Pure (TNA) was shown to suffer from reduced DNA recovery in **Chapter 3**. Evidence from the literature suggests that the same is true for RNA in several specimen types including stool [5, 31, 47, 50, 56, 77].

Due to the semi-automated nature of miniMAG, processing stool specimens would still require considerable hands-on time. Therefore, the NucliSENS easyMAG, a fully automated extractor using the exact same chemistry as miniMAG, was evaluated for processing stool specimens. During this evaluation, it was again shown that results from previous evaluations can only be extrapolated with care, even when there are only minute differences between methods, as is the case with miniMAG/easyMAG. Our results showed that the easyMAG system was less efficient than miniMAG in approximately 30% of the specimens [89].

When studied in more detail, the difference in performance proved to be mainly associated with the elution of DNA from the magnetic silica particles. Therefore, a new protocol (Specific A 1.0.2) was developed in collaboration with the manufacturer, and evaluated with stool specimens. This evaluation indicated that not only the problems associated with the elution of DNA were resolved by Specific A, but also that the performance with specimens that contain a competitor for NA binding was improved. This is important, as we showed in **Chapter 4** that this problem is also encountered by miniMAG in 16% of the specimens. Figure 10.1 summarizes both the problems associated with the standard easyMAG protocol as well as the results from the evaluation of Specific A.

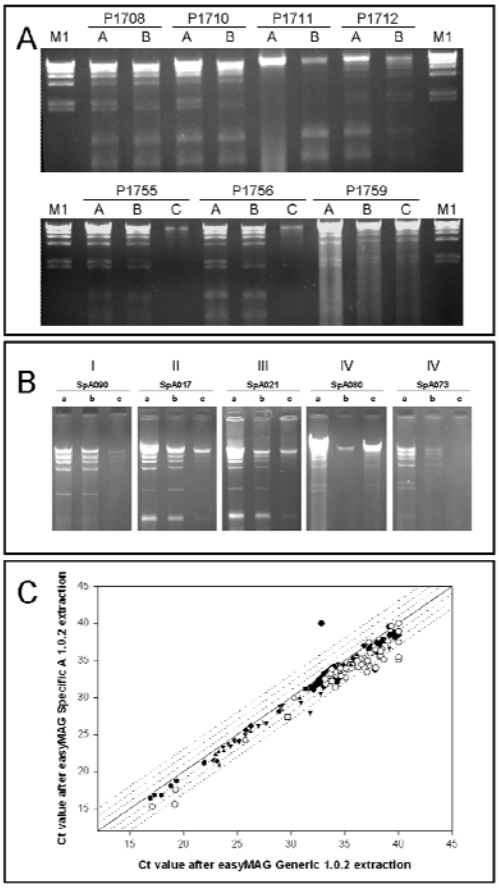


Figure 10.1. Problems associated with fecal DNA extraction by standard NucliSENS easyMAG extraction protocol (Generic 1.0.2) and evaluation of the Specific A 1.0.2 protocol.

A) Comparison of DNA extracted from stool specimens by NucliSENS miniMAG (A) and easyMAG Generic 1.0.2. Lanes designated C show DNA not eluted from the magnetic silica particles during elution with protocol Generic 1.0.2. Impaired recovery of DNA from feces compared to miniMAG, as is shown for specimens P1711, P1712 and P1759, was present in 30% of the stool specimens.

B) Different types of DNA recovery observed after easyMAG extraction with Specific A 1.0.2 (a) or Generic 1.0.2 (b) protocols. (c) DNA retrieved from magnetic silica particles after Generic 1.0.2 extraction. Recovery type (RT) I, nearly identical recovery (65% of specimens); RTs II to IV, increasing impaired DNA recovery with Generic 1.0.2 (15% of the specimens); RT V, Impaired recovery of DNA due to competition for binding to magnetic silica (20% of specimens).

C) Comparison of real-time PCR Ct-values after easyMAG Generic 1.0.2 (x-axis) or Specific A 1.0.2 (y-axis). ■ *Salmonella enterica*, ▲ *Campylobacter jejuni*, ▼ *Giardia lamblia*, ◆ Shiga toxin-producing *Escherichia coli*, ⬢ *Shigella* spp./entero-invasive *E. coli*, ● phocin herpes virus (internal control). Closed symbols represent RT I, open symbols represent RTs II to V.

In conclusion, automated methods have rapidly become the standard in NA extraction within the clinical microbiology laboratory. However, thorough evaluations of new applications remain mandatory to ensure reliable use of these extractors.

MOLECULAR SCREENING OF GASTROINTESTINAL PATHOGENS BY REAL-TIME PCR.

Routine screening of stool specimens for gastrointestinal pathogens is still largely conducted by conventional procedures such as culture and microscopy. Antigen detection has been shown to be a fast and effective alternative for several pathogens, including adenovirus, rotavirus, astrovirus, *Clostridium difficile*, Shiga toxin-producing *Escherichia coli* (STEC), *Giardia lamblia*, and *Cryptosporidium parvum/hominis*. However, with antigen detection, multiple separate tests needs to be performed for each targeted pathogen as the availability of combined tests is not widespread. Therefore, conventional methods will remain in use, as overall replacement by antigen detection will not be cost-effective. Molecular detection of gastrointestinal viruses by real-time PCR has been receiving increasing attention, as is also illustrated by the recent reports in the literature. A pubmed search (31-12-2007) using the following criteria (pathogen [adenovirus; astrovirus; *Campylobacte*r; *Clostridium difficile*; *Cryptosporidium*; *Entamoeba histolytica*; enterovirus; *Giardia*; norovirus; parechovirus; rotavirus; *Salmonella*; sapovirus; Shiga toxin; *Yersinia enterocolitica*], stool/feces, and real-time PCR/5'-nuclease/taqman/lightcycler) revealed 35 articles regarding real-time PCR detection of diarrheal viruses, indicating that molecular detection is becoming an accepted standard in the diagnosis of viral gastroenteritis. Although for the prokaryotic pathogens involved in bacterial gastroenteritis 31 papers were retrieved (excluding work from this thesis), the interest remains more limited. Furthermore, direct detection from human stools has only been evaluated for small numbers of specimens in most of these reports. For the detection of diarrheic parasites by real-time PCR another 21 publications (excluding work from this thesis) were retrieved indicating that molecular detection provides a sensitive alternative to microscopy and antigen detection. However, due to the assumption that microscopy is a catch-all methodology, the simplicity of antigen detection by commercial kits, and the fact that molecular methods may not be operational in community-based laboratories, the applicability of real-time PCR as a diagnostic tool for diarrheal parasites has remained limited to date.

The major problem with conventional detection of gastrointestinal pathogens is its laborious and time-consuming nature, especially at the

seasonal peaks during summer, autumn and winter [94, 105]. We have therefore developed and validated real-time PCR-based methods for the detection gastrointestinal pathogenic bacteria and protozoa in **Chapters 4** to **9**. All these methods are clearly of great value in the diagnosis of gastroenteritis, as they proved to be rapid and more sensitive compared to conventional diagnostic procedures. However, before these methods can be implemented in the routine diagnostic laboratory several items still need to be addressed.

First of all, implementation of molecular screening methods (MSMs) for single organisms does not seem feasible as this would result in a limitation in the testing capacity with most of the current real-time PCR systems (96-well formats). In addition, the use of single target assays will make the cost-effectiveness highly questionable. Therefore, the single target MSMs have to be converted into multiplex real-time PCRs. However, it has been an issue of concern that multiplexing may result in a loss of sensitivity due to interaction of the different primer/probe sets [33, 81]

Second, the MSMs described in this thesis should be further expanded to accommodate the laboratory's strategy for testing stool specimens in order to replace bacterial culture as the primary screening tool. In our Dutch setting this means including *Shigella* species, as they are also routinely screened for using the culture-based approach for detecting *S. enterica*. In addition, other diarrhea-causing viruses and parasites could be included in an attempt to reduce the overall workload. In the community-based setting, where the majority of specimens originate from general practice patients, it would likely be sufficient to include enterovirus, parechovirus, adenovirus, rotavirus, norovirus, and astrovirus. With regard to parasites, it may be preferential to also include *Dientamoeba fragilis* and to a lesser extend *Blastocystis hominis*, although their role in diarrheal disease is still a matter of controversy. Additional targets could also be included when dealing with specific patient groups, such as nosocomial diarrhea in hospitalized patients (*C. difficile*), immuno-compromised patients (*Entamoeba histolytica*, *Isospora belli*, *Microsporidium* spp., *Mycobacterium avium* complex, and *Strongyloides stercoralis*) [97], and travelers (*Aeromonas* spp., *Cyclospora cayetanensis*, *Entamoeba histolytica*, enteroaggregative *E. coli* [EAggEC], enteroinvasive *E.coli* [EIEC], enterotoxigenic *E. coli* [ETEC], *Plesiomonas*

shigelloides, and *Vibrio parahaemolyticus*) [20, 73]. Furthermore, when MSMs are in place, it is only a small step to also include other causative agents of community acquired diarrhea for which the current diagnostic methods are just not feasible for routine use due to high expenses. These include, ETEC, enteropathogenic *E. coli*, EAggEC, and diffusely adherent *E. coli*, for which no selective culture media and phenotypic identification test are available [68]. Screening for these diarrheagenic *E. coli* is based on toxin detection (ELISA/cell culture), adhesion/invasiveness of HEp-2 cells in cell culture or molecular detection of virulence genes in primary stool cultures by PCR or colony blotting.

At present, PCR is believed to be more expensive than conventional culture with reported direct costs of PCR reagents, equipment, dedicated space, personnel training, and labor as high as US$ 125 per reaction [109]. However, refinement in PCR technology and automation facilitate a reduction in the costs to competitive levels, as is shown for *Neisseria gonorrhoea* and *Chlamydia trachomatis* PCR testing at approximately US$ 9 per reaction [109]. Therefore, early in the development of the MSMs for *S. enterica* and *C. jejuni*, the cost-effectiveness of replacing conventional culture by MSM was assessed on the basis of historical data from the LvI. This indicated that MSMs could be performed for *S. enterica* and *C. jejuni* at nearly identical costs for materials and personnel (€ 8.60 MSM; € 8.70 culture) [104]. However, for implementation of MSMs into routine practice, some of the molecular methods used in the cost-assessment study should be adapted to manage with the potential high number of specimens. With these adaptations (i.e. automated NA extraction and multiplex MSM), the costs per specimen for MSMs with an assumed run size of 22 specimens and 2 controls would range from approximately € 12 (3 targets) to € 15 (12 targets), including all chemicals, disposables and staff costs, but excluding costs associated with subsequent MSM-guided cultures. There are other costs which need to be taken into account, including equipment acquisition and maintenance, amortization, laboratory space, staff training, and developmental costs. However, these costs are associated with the molecular laboratory as a whole, and not specifically for the gastrointestinal MSMs, and have therefore not been included in any of the cost assessments. The MSM costs are in line with the reported costs for the

detection of 17 respiratory pathogens by multiplex real-time PCR (€ 36.65), and compare favorably with a report for the detection of 14 respiratory pathogens by real-time PCR (€ 330) [96].

In order to implement the MSMs at our laboratory, we have converted them into two multiplex real-time PCRs, one combining *S. enterica*, *C. jejuni*, *G. lamblia* and PhHV (internal control), and one combining STEC, *Shigella* species/enteroinvase *E. coli* (EIEC) and PhHV, as is also depicted in Figure 10.2.

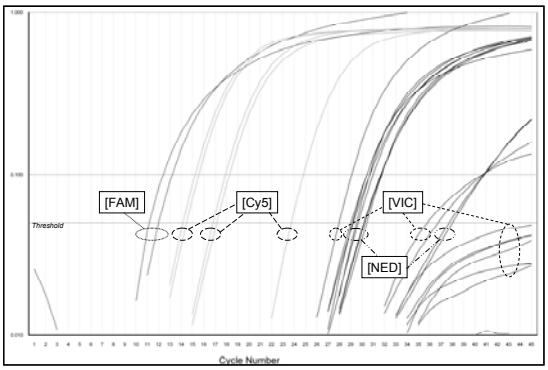


Figure 10.2. Multiplex MSM for detection of 3 gastrointestinal pathogens implemented at the Lvl.

MSM targeting *Salmonella enterica* [FAM]), *Campylobacter jejuni* [VIC], and *Giardia lamblia* [Cy5]. [NED] represent the detection of the phocin herpes virus, which serves as an internal control for each specimen. The horizontal line indicates the cut-off (Delta Rn = 0.04) for determining the cycle threshold value, which flags a specimen as positive.

A reduction in sensitivity has been an issue of concern in multiplexing. Assessment of the analytical sensitivity with spiked stool specimens for both multiplex MSMs however, showed no detrimental effect for the used combination of primer/probe sets, resulting in identical detection limits for the multiplexed and singleplex MSMs [88]. When challenged with panel of 851 clinical stool specimens, the multiplex MSMs showed complete concordance for 98.2% of the specimens positive by routine diagnostic methods (n = 281). Furthermore, in the 570 specimens negative by routine diagnostic procedures, 83 additional positive specimens were identified, again illustrating the higher sensitivity of the MSMs.

As the MSMs detect DNA rather than the living organism, this does raise the question of the clinical relevance of detecting pathogen DNA in the absence of a positive MSM-guided culture or microscopy result. Contamination does not seem a likely explanation, as was also shown using confirmatory real-time PCR testing for *S. enterica* and *C. jejuni* in **Chapter 5**,

and STEC in **Chapter 7**, although it can not be ruled out completely as was shown for *G. lamblia* in **Chapter 8**.

For *S. enterica* and *C. jejuni* MSM-only positives were confirmed in 64.3% (21.4% culture; 42.9% confirmatory PCR) and 88.0% (32.0% culture; 56.0% confirmatory PCR) of these specimens, respectively. With regard to STEC, it was anticipated in advance that the MSM would yield significantly more positives, as the conventional approach only targets the O157 STEC serotype. During the developmental evaluation of the STEC MSMs (**Chapter 6**) all 9 specimens positive only by MSM were detected by both MSMs. During the multicenter evaluation (**Chapter 7**), the STEC MSM-positives (n = 68) could be confirmed by a confirmatory MSM in 89.1% (57 of 64) of the specimens available, whereas culture yielded a STEC in only 37.9% (25 of 66). Although we did not attempt to confirm positive specimens for *G. lamblia* by MSM only in **Chapter 9**, typing of *C. hominis/parvum* MSM positive specimens confirmed the presence of *C. hominis/parvum* DNA in 92.7% (38 of 41) of these specimens. Furthermore, there is circumstantial evidence that supports the *G. lamblia* and *C. hominis/parvum* MSM-only positives as true positives. For *G. lamblia*, the age distribution of MSM-positives was identical to the distribution of microscopy positives (**Fig. 9.1**), whereas for *C. hominis/parvum*, a high prevalence was detected in children of < 5 years of age (~22%), and children aged 5–14 years (~11%), compared to ages 15–29 (1.9%), 30–49 (1.9%), 50–64 (0%), and aged 65 and older (0%). When compared to a large case-control study the distribution of the incidence with age is in line, although the observed prevalence is higher when analyzed by MSM [19]. The high incidence in children of < 5 years of age can be suggestive of asymptomatic carriage. However, when this age group is divided into two sub groups (< 1 years and 1–4 years), and compared with data from the case-control study of de Wit *et al.* [19], it suggests that for children aged 1–4 years the MSM would most likely be detecting symptomatic *C. hominis/parvum* infection, rather than asymptomatic carriage, which was associated with the age-group < 1 years in the case-control study. This association was also recently reported from a large case-control study from the United Kingdom which also compared molecular detection with conventional detection [3]. Therefore, in most cases, the additional MSM-positives probably reflect the true causative agent of the

patient's episode of diarrhea. Finally, conventional diagnostic methods such as culture and microscopy also lack discrimination between active infection and asymptomatic carriage. In this respect, the (semi-) quantitative nature of real-time PCR may prove useful, as asymptomatic carriers will probably shed lower numbers of organisms compared to patients with diarrhea.

Should it be necessary to distinguish between viable and dead organisms using the highly sensitive MSMs described in this thesis, there are currently two methodologies available to do so. First of all, the MSMs could be converted to target mRNA rather than DNA, as mRNAs are highly instable outside the viable organism. However, this would require a large effort to transform the real-time PCRs to mRNA targeting reverse transcriptase real-time PCRs. A much simpler approach would be to incorporate a treatment of the stool specimen with EMA. This photo-reactive compound can not pass through the intact cell wall of viable microorganisms, and therefore only enters damaged cells, where it is covalently cross-linked to the DNA in the damaged cells, as well as any free DNA in the specimen, by UV light exposure. Subsequent processing of the EMA-treated specimen by MSM will then in theory only yield positive results when viable microorganisms are present. Successful EMA-PCR-mediated live/dead discrimination has recently been described for several bacterial species [34, 70-72, 83, 84, 86, 92], although another report questions the ability of EMA/real-time PCR as a discriminator of viability [28]. Using EMA-treatment seems the more favorable method, as it is already compatible with the MSMs described in this thesis, therefore eliminating the need to develop new assays. However, as EMA-treatment of stool specimens has yet to be evaluated, it remains to be seen whether this strategy is effective for discriminating between viable and dead microorganisms in feces.

At the LvI the transition has recently been made from conventional culture for *S. enterica*, *C. jejuni*, *Shigella* species and STEC to the multiplex MSMs. On the short term, this panel will be further expanded with other intestinal pathogens, including *C. difficile*, *C. hominis/parvum*, EPEC, EAggEC, *G. lamblia*, and *Yersinia enterocolitica*. In addition, MSMs can be followed by direct molecular typing methods to rapidly further characterize the virulence and epidemiological properties of pathogens identified by the MSM. In principle, these typing assays can be performed on the same NA

extract as the primary MSM and can therefore provide results within 2-3 hours of the primary identification of the pathogen when real-time molecular methods are in place. Confirmation of these direct typing results can subsequently be obtained when the organism is retrieved from the MSM-guided culture. The extra speed provided by direct typing is especially important when clinical management is dependent on the outcome of the typing results. With respect to gastrointestinal pathogens, there are several applications where direct typing may prove to be of great value.

For instance in case of *Salmonella* enteritis, differentiation between non-Typhiod *Salmonellae* and *S. enterica* serovariants Typhi, Paratyphi, and Sendai is important as the latter give rise to typhoid fever. A recent study has identified new genetic loci (STY4220 and STY4221) that seem suitable for direct identification of these pathogens in clinical specimens [98]. Other potential targets for serovar-specific identification of *Salmonellae* are the Vi-antigen, O-antigen and flagelin encoding genes [25, 27, 98].

In Guillain-Barré syndrome (GBS), molecular typing of the *C. jejuni* lipo-oligosaccharides may facilitate rapid identification of patients at risk for developing GBS during their episode of diarrhea. When this is combined with an assay that characterizes the host-susceptibility for progression to GBS, pre-emptive treatment strategies can be initiated for those patients identified, using plasma exchange, high-dose intravenous immunoglobulin or alternative future treatments [12, 54]. However, the (cost-) effectiveness of such a pre-emptive treatment strategy would need to be elucidated first.

For the detection of *Shigella* spp. and EIEC it is also important to distinguish *Shigella* spp. from EIEC, as *Shigella* infection is a reportable disease in The Netherlands. In addition, as *Shigella dysenteriae* is a more virulent species, associated with large epidemics, early identification is also of great clinical importance. In the current format, identification of *Shigella* spp. is based on the outcome of the MSM-guided culture. Direct molecular differentiation of *Shigella* spp. and EIEC is difficult, as both groups of organisms are considered a single genomovar of *E. coli*. However, the *rfc* gene, which encodes for O-antigen polymerase that polymerizes the O-antigen subunit into lipo-polysaccharide chains, may facilitate molecular differentiation [43]. Others have described a more complex typing scheme,

involving the virulence genes *iuc* and *ipaH*, and restriction fragment length polymorphisms of the *ial* gene located on the large plasmid [49].

In STEC infection, typing of the toxin genes may prove helpful to assess whether a patient has an increased risk to acquire the haemolytic uremic syndrome (HUS). There is a strong association between HUS, the type of Shiga toxin (Stx) and additional virulence factors; the combined presence of the *E. coli* attaching and effacing (*eae*) and especially Stx2 or Stx2c seem important predictors of HUS [9, 23, 29, 45, 87]. Other types of Stx (Stx1c, Stx2d, Stx2e, and Stx2f) show a strong association with mild, uncomplicated diarrhea, or asymptomatic carriage. Typing of the *stx* and putative virulence genes may identify patients at high(er) risk for HUS development in the future. Preventive treatment can then be started, such as early isotonic volume expansion, or administration of humanized monoclonal antibodies (i.e., passive immunity) against Stx. As all of these measures have to be started during the 3rd to 5th day of the window between diarrhea and onset of HUS, the need for rapid detection and typing is nicely illustrated.

Finally, with the emergence of the highly virulent ribotype (RT) 027 of *C. difficile*, rapid detection and identification of this strain is also important [53, 74]. Detection of RT 027 specific deletions in the *tcdC* gene by real-time PCR may facilitate the rapid presumptive identification of RT 027 from a specimen with a positive MSM result for *C. difficile*, without the need to isolate the strain first [18].

FUTURE PERSPECTIVES.

The previous chapters and paragraphs have shown some of the current molecular developments within the diagnostic microbiology laboratory. This final paragraph provides a brief perspective of the anticipated developments within diagnostic molecular microbiology for the near and more distant future.

On the short term molecular detection of specific pathogens will be further expanded. Multiplexed MSMs will replace conventional testing for the primary detection and identification of many pathogens in clinical specimens. This MSM-testing will be performed according to syndrome-based detection panels, rather than by a specified physicians' request. The MSMs developed for gastrointestinal pathogens in this thesis are an excellent example of such

an approach. However, gastroenteritis is not the only clinical condition where MSM-based screening is appropriate. Other examples of clinical manifestations, where multiple pathogens can be involved with overlapping clinical presentations, are respiratory tract infections, central nervous system infections, sexually transmitted diseases, microbiological monitoring in both solid organ and stem cell transplant recipients, and BSI.

Although an MSM-based approach does not provide antimicrobial susceptibility, and/or epidemiology data, MSM-guided cultures can be inoculated as soon as the primary screening results are available without significant loss of sensitivity. Thereby maximizing the advantages of molecular detection (i.e. sensitivity and speed), without compromising the benefits of obtaining a clinical isolate. With this, the primary role of culture has moved from detection/identification to confirmation and epidemiology. However, as more and more resistance mechanisms are resolved to their genetic basis, genotypic resistance profiling will complement the phenotypic methods, and may eventually replace them in a similar fashion as with *mecA* gene detection, which has become the standard in the assessment of methicillin-resistant *Staphylococcus aureus* (MRSA).

The developments on the long run, will be largely technology driven, as further automation, integration and miniaturization of molecular diagnostic equipment will eventually lead to “real-time point of care” diagnostics (POC), providing results within an hour after obtaining a patient’s specimen. The first integrated molecular systems have already become available and are finding their way into the laboratory.

A nice example of such an integrated sample preparation, real-time amplification/detection system is the recently introduced GeneXpert® (Cepheid, Sunnyvale, CA, USA) [30, 51, 101]. Unfortunately, the GeneXpert is a closed system which, at present, can only be used in conjunction with a limited number of proprietary real-time PCR applications. These applications include MRSA screening, rapid intrapartum Group B Streptococcus testing, and enteroviral meningitis detection (www.cepheid.com, [30, 51]). Other examples include PCR-light® (Enigma Diagnostic Ltd., Wiltshire, England), Opti GENE molecular diagnostics instrument with HyBeacon® detection technology (Osmetech, Atlanta, GA, USA, and LGC, Middlesex, England), the laboratory-in-a-tube (Liat™ analyzer, IQ^{uum} Inc., Allston, MA, USA),

Verigene AutoLab™ and Verigene Mobile™ (Nanosphere Inc., Northbrook, IL, USA), bioluminescent assay in real-time (BART, Lumora Ltd., Cambridge, England), Nanochip (Nanogen Inc., San Diego, CA, USA), laboratory-on-a-chip (HandyLab™ Inc. Ann Arbor, MI, USA), and MODULAB (Institut für Mikrotechnik Mainz GmbH Mainz, Germany, and Evotec Technologies, Düsseldorf, Germany) [40, 66]. These systems use a variety of methodologies including real-time PCR (GeneXpert®, Opti GENE/HyBeacon®, Liat™), probe-based detection (Verigene AutoLab™, Verigene Mobile™), bioluminescence real-time amplification (BART), microarray (Nanochip), and micropump technology (laboratory-on-a-chip, and MODULAB).

Other technological developments with great promise for molecular diagnostic testing are microarray detection, vibrational and mass spectrometry, and electronic noses. Microarray technology provides unprecedented multiplexing capacity in a single analysis. Although exiting applications of microarrays for infectious disease testing have already been described [10, 13, 52, 58, 60, 93], from a practical standpoint, simply more data are generated from a single, large array than are necessary in clinical medicine, and the cost of the array is prohibitive for routine use [78]. The use of low density microarrays currently seems more appropriate for clinical use. When combined with multiplexed or broad-range amplification, syndrome or disease specific arrays can be constructed for MSM-based detection. With respect to gastroenteritis, a prototype assay (Check-Points BV, Wageningen, The Netherlands) is currently under development that uses a ligase-dependent multiplex PCR in combination with colorimetric detection using a microarray in a tube format (ArrayTube, ClonDiag, Jena, Germany). In this assay, multiplexed amplification is conducted by universal primers after ligation of species-specific probes that also contain the universal primer sites. Preliminary results have been encouraging and further evaluation of this prototype assay in clinical practice is underway [44] (Fig. 10.3).

Phenotypic identification of microorganisms using vibrational spectrometry also provides a rapid means for diagnosing infections. With the use of Raman or Fournier transformed infrared spectroscopy, and without the need for extraction, amplification, or labeling, Maquelin and co-workers correctly identified 92% and 98% of pathogens in growth-positive blood cultures by their overall molecular composition [61, 62]. However, although the

methodology is simple and straightforward, the necessary equipment is rather expensive, and not commonly available to routine diagnostic laboratories.

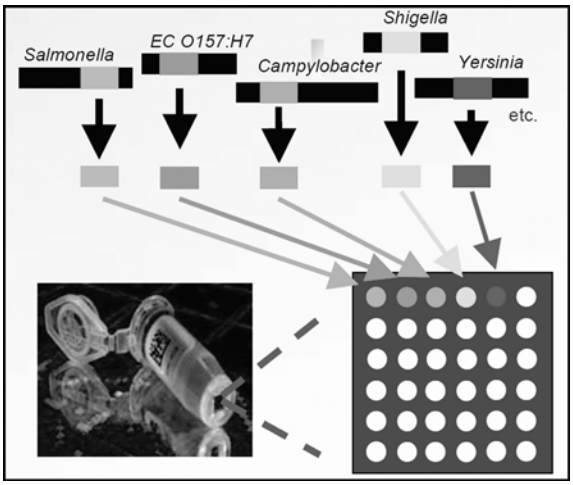


Figure 10.3. Schematic representation of the ligase-dependent multiplex PCR microarray for detection of gastrointestinal pathogens.

Each pathogen is targeted by specific oligonucleotide probes, which hybridize adjacent to each other. These probes are ligated and introduce generic primer sites, which are subsequently amplified with a single primer pair in a multiplex PCR. Amplification products are detected by pathogen specific probes on a microarray in a tube with colorimetric detection. Courtesy of Check-Points BV, Wageningen, The Netherlands

MALDI-TOF mass spectrometry analysis of amplification products has recently been shown effective in diagnosing human herpesvirus infections and for high-throughput screening of oncogenic human papiloma virus genotypes with overall concordances compared to reference methods of > 95% [90, 91]. Another application of MALDI-TOF mass spectrometry is the use on the protein contents of body fluids. Gravett en colleagues recently identified protein signatures and specific biomarkers in the proteosome of cervical vaginal fluid using a non-human primate intra-amniotic infection (IAI) model. These markers may be used in a non-invasive method for the diagnosis of IAI, which is a major cause preterm birth and perinatal mortality [32].

With the advance in sensor technology, recently devices have become available that can detect and discriminate the production profiles of volatile compounds from microbial infections. These devices, the so called electronic noses, have already been successful in identifying bacteria, yeasts and filamentous fungi from cultures, as well as in detecting bacterial infections in contact dressings from leg ulcers, vaginal swabs, urine, sputum, and breath samples [26, 99].

A final development which will have a major impact on infectious disease testing will be the introduction of a systems biology approach [41]. Systems biology is the integrated study of one or multiple organisms, where this (those) organism(s) is (are) considered as an integrated network of genes, proteins, and biochemical reaction, that when combined support live. In this approach not only a pathogen is identified and characterized, but also the interactions with its host and the host's susceptibility to the pathogen are characterized. Some prominent candidates for host-monitoring during infection are interferon responses, cytokines, stress responses, protein synthesis, and cell cycle and apoptosis. For some of these factors pathogen-specific expression profiles have already been described, including human immunodeficiency virus, varicella-zoster virus, ocular adenovirus, measles, reovirus, human papillomavirus type II, herpes simplex virus, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, and EBV [11]. The natural flora will be profiled and provide an individual fingerprint from which a subject's health status can be deducted. Together with the host's susceptibility that is derived from its genetic make-up, an infectious disease risk assessment can be made. The resulting "in toto" analysis of the disease system will in turn provide predictive, preventative and personalized medicine.

CONCLUDING REMARKS.

Only an educated guess can be made on what timeline these exciting new developments will make their way into the clinical microbiology laboratory, as not only the speed at which the technological developments will continue to take place is important. Other factors such as practicality and economics will have great influence in the speed of these developments. And finally, these new methods and approaches need to be accepted by the clinical microbiology community first, before they can be implemented. This is an aspect we have experienced at first hand with developed methods from this thesis.

Within the next five years we will almost certainly see broad application of syndrome-based MSM detection of pathogens, with MSM-guided cultures to obtain bacterial, fungal and viral isolates for resistance profiling and epidemiological surveillance. The currently used automated NA extractors and real-time PCR systems will be further refined, miniaturized and

integrated into devices that are more suitable for point of care testing. The pace of the subsequent developments is highly subject to speculation. The view that clinical microbiology testing will (partially) move towards the bedside and the general practitioner was recently expressed in several reviews [4, 21, 80]. Clinical microbiology laboratories would act as reference centres for confirmation and elucidation of discrepant POC testing results. In addition, these reference laboratories will continuously monitor emerging pathogens and resistances not included in the POC tests. To be able to cope with this task, expertise in both state of the art molecular methods, as well as “old-school” conventional methods need to be maintained. Regardless of the pace at which clinical microbiology will evolve during the coming decade, we are entering an exiting era during the forthcoming years.

REFERENCES

1. **Al-Soud, W. A., L. J. Jonsson, and P. Radstrom**. 2000. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. J.Clin Microbiol. **38**:345-350.
2. **Al-Soud, W. A. and P. Radstrom**. 2001. Purification and characterization of PCR-inhibitory components in blood cells. J.Clin Microbiol. **39**:485-493.
3. **Amar, C. F., C. L. East, J. Gray, M. Iturriza-Gomara, E. A. Maclure, and J. McLauchlin**. 2007. Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993-1996). Eur.J.Clin Microbiol.Infect.Dis. **26**:311-323.
4. **Baron, E. J.** 2002. Speculations on the microbiology laboratory of the future. Clin Infect.Dis. **35**:S84-S87.
5. **Beld, M., M. R. Habibuw, S. P. Rebers, R. Boom, and H. W. Reesink**. 2000. Evaluation of automated RNA-extraction technology and a qualitative HCV assay for sensitivity and detection of HCV RNA in pool-screening systems. Transfusion **40**:575-579.
6. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-Van Dillen, and N. J. van der**. 1990. Rapid and simple method for purification of nucleic acids. J.Clin Microbiol. **28**:495-503.
7. **Borst, A., A. T. Box, and A. C. Fluit**. 2004. False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy. Eur.J.Clin Microbiol.Infect.Dis. **23**:289-299.
8. **Bottger, E. C.** 1990. Frequent contamination of *Taq* polymerase with DNA. Clinical Chemistry **36**:1258c-1259.
9. **Brooks, J. T., E. G. Sowers, J. G. Wells, K. D. Greene, P. M. Griffin, R. M. Hoekstra, and N. A. Strockbine**. 2005. Non-O157 Shiga toxin-producing Escherichia coli infections in the United States, 1983-2002. J.Infect.Dis. **192**:1422-1429.
10. **Call, D. R.** 2005. Challenges and opportunities for pathogen detection using DNA microarrays. Crit Rev.Microbiol. **31**:91-99.
11. **Campbell, C. J. and P. Ghazal**. 2004. Molecular signatures for diagnosis of infection: application of microarray technology. J.Appl.Microbiol. **96**:18-23.
12. **Chowdhury, D. and A. Arora**. 2001. Axonal Guillain-Barre syndrome: a critical review. Acta Neurol.Scand. **103**:267-277.
13. **Clewley, J. P.** 2004. A role for arrays in clinical virology: fact or fiction? J.Clin Virol. **29**:2-12.
14. **Clewley, J. P.** 1989. The polymerase chain reaction, a review of the practical limitations for human immunodeficiency virus diagnosis. J.Virol.Methods **25**:179-187.
15. **Cogswell, F. B., C. E. Bantar, T. G. Hughes, Y. Gu, and M. T. Philipp**. 1996. Host DNA can interfere with detection of Borrelia burgdorferi in skin biopsy specimens by PCR. J.Clin Microbiol. **34**:980-982.
16. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, A. J. Fox, and E. B. Kaczmarski**. 2001. Simultaneous detection of Neisseria meningitidis, Haemophilus influenzae, and Streptococcus pneumoniae in suspected cases of meningitis and septicemia using real-time PCR. J.Clin Microbiol. **39**:1553-1558.
17. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, E. B. Kaczmarski, and A. J. Fox**. 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J.Clin Microbiol. **38**:1747-1752.
18. **Curry, S. R., J. W. Marsh, C. A. Muto, M. M. O'Leary, A. W. Pasculle, and L. H. Harrison**. 2007. tcdC genotypes associated with severe TcdC truncation in an epidemic clone and other strains of Clostridium difficile. J.Clin Microbiol. **45**:215-221.
19. **de Wit, M. A., M. P. Koopmans, L. M. Kortbeek, N. J. van Leeuwen, J. Vinje, and Y. T. van Duynhoven**. 2001. Etiology of gastroenteritis in sentinel general practices in the netherlands. Clin Infect.Dis. **33**:280-288.

20. **Diemert, D. J.** 2006. Prevention and self-treatment of traveler's diarrhea. Clin Microbiol.Rev. **19**:583-594.
21. **Dunne, W. M., Jr., J. K. Pinckard, and L. V. Hooper**. 2002. Clinical microbiology in the year 2025. J.Clin Microbiol. **40**:3889-3893.
22. **Espy, M. J., P. N. Rys, A. D. Wold, J. R. Uhl, L. M. Sloan, G. D. Jenkins, D. M. Ilstrup, F. R. Cockerill, III, R. Patel, J. E. Rosenblatt, and T. F. Smith**. 2001. Detection of herpes simplex virus DNA in genital and dermal specimens by LightCycler PCR after extraction using the IsoQuick, MagNA Pure, and BioRobot 9604 methods. J.Clin Microbiol. **39**:2233-2236.
23. **Ethelberg, S., K. E. Olsen, F. Scheutz, C. Jensen, P. Schiellerup, J. Enberg, A. M. Petersen, B. Olesen, P. Gerner-Smidt, and K. Molbak**. 2004. Virulence factors for hemolytic uremic syndrome, Denmark. Emerg.Infect.Dis. **10**:842-847.
24. **Evans, G. E., D. R. Murdoch, T. P. Anderson, H. C. Potter, P. M. George, and S. T. Chambers**. 2003. Contamination of Qiagen DNA extraction kits with Legionella DNA. J.Clin.Microbiol. **41**:3452-3453.
25. **Farrell, J. J., L. J. Doyle, R. M. Addison, L. B. Reller, G. S. Hall, and G. W. Procop**. 2005. Broad-range (pan) Salmonella and Salmonella serotype typhi-specific real-time PCR assays: potential tools for the clinical microbiologist. Am.J.Clin Pathol. **123**:339-345.
26. **Fend, R., A. H. Kolk, C. Bessant, P. Buijtels, P. R. Klatser, and A. C. Woodman**. 2006. Prospects for clinical application of electronic-nose technology to early detection of Mycobacterium tuberculosis in culture and sputum. J.Clin Microbiol. **44**:2039-2045.
27. **Fitzgerald, C., M. Collins, D. S. van, M. Mikoleit, T. Brown, and P. Fields**. 2007. Multiplex, bead-based suspension array for molecular determination of common Salmonella serogroups. J.Clin Microbiol. **45**:3323-3334.
28. **Flekna, G., P. Stefanic, M. Wagner, F. J. Smulders, S. S. Mozina, and I. Hein**. 2007. Insufficient differentiation of live and dead Campylobacter jejuni and Listeria monocytogenes cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. Res.Microbiol. **158**:405-412.
29. **Friedrich, A. W., M. Bielaszewska, W. L. Zhang, M. Pulz, T. Kuczius, A. Ammon, and H. Karch**. 2002. Escherichia coli harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J.Infect.Dis. **185**:74-84.
30. **Gavino, M. and E. Wang**. 2007. A comparison of a new rapid real-time polymerase chain reaction system to traditional culture in determining group B streptococcus colonization. Am.J.Obstet.Gynecol. **197**:388-4.
31. **Germer, J. J., M. M. Lins, M. E. Jensen, W. S. Harmsen, D. M. Ilstrup, P. S. Mitchell, F. R. Cockerill, III, and R. Patel**. 2003. Evaluation of the MagNA pure LC instrument for extraction of hepatitis C virus RNA for the COBAS AMPLICOR Hepatitis C Virus Test, version 2.0. J.Clin Microbiol. **41**:3503-3508.
32. **Gravett, M. G., A. Thomas, K. A. Schneider, A. P. Reddy, S. Dasari, T. Jacob, X. Lu, M. Rodland, L. Pereira, D. W. Sadowsky, C. T. Roberts, Jr., M. J. Novy, and S. R. Nagalla**. 2007. Proteomic analysis of cervical-vaginal fluid: identification of novel biomarkers for detection of intra-amniotic infection. J.Proteome.Res. **6**:89-96.
33. **Grondahl, B., W. Puppe, A. Hoppe, I. Kuhne, J. A. Weigl, and H. J. Schmitt**. 1999. Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: feasibility study. J.Clin Microbiol. **37**:1-7.
34. **Guy, R. A., A. Kapoor, J. Holicka, D. Shepherd, and P. A. Horgen**. 2006. A rapid molecular-based assay for direct quantification of viable bacteria in slaughterhouses. J.Food Prot. **69**:1265-1272.
35. **Hackett, S. J., E. D. Carrol, M. Guiver, J. Marsh, J. A. Sills, A. P. Thomson, E. B. Kaczmarski, and C. A. Hart**. 2002. Improved case confirmation in meningococcal disease with whole blood Taqman PCR. Arch.Dis.Child **86**:449-452.
36. **Hackett, S. J., M. Guiver, J. Marsh, J. A. Sills, A. P. Thomson, E. B. Kaczmarski, and C. A. Hart**. 2002. Meningococcal bacterial DNA load at presentation correlates with disease severity. Arch.Dis.Child **86**:44-46.

37. **Harris, K. A. and J. C. Hartley**. 2003. Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. J.Med.Microbiol. **52**:685-691.
38. **Hein, I., W. Schneeweiss, C. Stanek, and M. Wagner**. 2007. Ethidium monoazide and propidium monoazide for elimination of unspecific DNA background in quantitative universal real-time PCR. Journal of Microbiological Methods **doi: 10.1016/j.mimet.2007.09.005**.
39. **Henry, N. K., C. A. McLimans, A. J. Wright, R. L. Thompson, W. R. Wilson, and J. A. Washington**. 1983. Microbiological and clinical evaluation of the isolator lysis-centrifugation blood culture tube. J.Clin Microbiol. **17**:864-869.
40. **Holland, C. A. and F. L. Kiechle**. 2005. Point-of-care molecular diagnostic systems--past, present and future. Curr.Opin.Microbiol. **8**:504-509.
41. **Hood, L., J. R. Heath, M. E. Phelps, and B. Lin**. 2004. Systems biology and new technologies enable predictive and preventative medicine. Science **306**:640-643.
42. **Horz, H. P., S. Scheer, F. Huenger, M. E. Vianna, and G. Conrads**. 2007. Selective isolation of bacterial DNA from human clinical specimens. J.Microbiol.Methods .
43. **Houng, H. S., O. Sethabutr, and P. Echeverria**. 1997. A simple polymerase chain reaction technique to detect and differentiate Shigella and enteroinvasive Escherichia coli in human feces. Diagn.Microbiol.Infect.Dis. **28**:19-25.
44. **Jans-Beken, N., de Boer, R. F., Nijhuis, R., van Zwet, A. A., and Kooistra-Smid, A. M. D.** Simultaneous detection of a panel of bacterial pathogens in faeces using a Microarray PCR. 5th European Meeting on Molecular Diagnostics . 17-10-2007.
45. **Jenkins, C., G. A. Willshaw, J. Evans, T. Cheasty, H. Chart, D. J. Shaw, G. Dougan, G. Frankel, and H. R. Smith**. 2003. Subtyping of virulence genes in verocytotoxin-producing Escherichia coli (VTEC) other than serogroup O157 associated with disease in the United Kingdom. J.Med.Microbiol. **52**:941-947.
46. **Jordan, J. A. and M. B. Durso**. 2005. Real-time polymerase chain reaction for detecting bacterial DNA directly from blood of neonates being evaluated for sepsis. J.Mol.Diagn. **7**:575-581.
47. **Kessler, H. H., A. M. Clarici, E. Stelzl, G. Muhlbauer, E. Daghofer, B. I. Santner, E. Marth, and R. E. Stauber**. 2002. Fully automated detection of hepatitis C virus RNA in serum and whole-blood samples. Clin Diagn.Lab Immunol. **9**:1385-1388.
48. **Kessler, H. H., G. Muhlbauer, E. Stelzl, E. Daghofer, B. I. Santner, and E. Marth**. 2001. Fully automated nucleic acid extraction: MagNA Pure LC. Clin Chem **47**:1124-1126.
49. **Kingombe, C. I., M. L. Cerqueira-Campos, and J. M. Farber**. 2005. Molecular strategies for the detection, identification, and differentiation between enteroinvasive Escherichia coli and Shigella spp. J.Food Prot. **68**:239-245.
50. **Knepp, J. H., M. A. Geahr, M. S. Forman, and A. Valsamakis**. 2003. Comparison of automated and manual nucleic acid extraction methods for detection of enterovirus RNA. J.Clin Microbiol. **41**:3532-3536.
51. **Kost, C. B., B. Rogers, M. S. Oberste, C. Robinson, B. L. Eaves, K. Leos, S. Danielson, M. Satya, F. Weir, and F. S. Nolte**. 2007. Multicenter beta trial of the GeneXpert enterovirus assay. J.Clin Microbiol. **45**:1081-1086.
52. **Kostrzynska, M. and A. Bachand**. 2006. Application of DNA microarray technology for detection, identification, and characterization of food-borne pathogens. Can.J.Microbiol. **52**:1-8.
53. **Kuijper, E. J., J. T. van Dissel, and M. H. Wilcox**. 2007. Clostridium difficile: changing epidemiology and new treatment options. Curr.Opin.Infect.Dis. **20**:376-383.
54. **Kuwabara, S.** 2004. Guillain-Barre syndrome: epidemiology, pathophysiology and management. Drugs **64**:597-610.
55. **Kwok, S. and R. Higuchi**. 1989. Avoiding false positives with PCR. Nature **339**:237-238.
56. **Lee, S. C., A. Antony, N. Lee, J. Leibow, J. Q. Yang, S. Soviero, K. Gutekunst, and M. Rosenstraus**. 2000. Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. J.Clin Microbiol. **38**:4171-4179.

57. **Lehmann, L. E., K. P. Hunfeld, T. Emrich, G. Haberhausen, H. Wissing, A. Hoeft, and F. Stuber**. 2007. A multiplex real-time PCR assay for rapid detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples. Med.Microbiol.Immunol.
58. **Lin, B., A. P. Malanoski, Z. Wang, K. M. Blaney, A. G. Ligler, R. K. Rowley, E. H. Hanson, R. E. von, F. S. Ligler, A. W. Kusterbeck, D. Metzgar, C. P. Barrozo, K. L. Russell, C. Tibbetts, J. M. Schnur, and D. A. Stenger**. 2007. Application of broad-spectrum, sequence-based pathogen identification in an urban population. PLoS.ONE. **2**:e419.
59. **Loeffler, J., N. Henke, H. Hebart, D. Schmidt, L. Hagmeyer, U. Schumacher, and H. Einsele**. 2000. Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. J.Clin Microbiol. **38**:586-590.
60. **Loy, A. and L. Bodrossy**. 2006. Highly parallel microbial diagnostics using oligonucleotide microarrays. Clin Chim.Acta **363**:106-119.
61. **Maquelin, K., C. Kirschner, L. P. Choo-Smith, N. A. Ngo-Thi, V. T. van, M. Stammler, H. P. Endtz, H. A. Bruining, D. Naumann, and G. J. Puppels**. 2003. Prospective study of the performance of vibrational spectroscopies for rapid identification of bacterial and fungal pathogens recovered from blood cultures. J.Clin Microbiol. **41**:324-329.
62. **Maquelin, K., C. Kirschner, L. P. Choo-Smith, B. N. van den, H. P. Endtz, D. Naumann, and G. J. Puppels**. 2002. Identification of medically relevant microorganisms by vibrational spectroscopy. J.Microbiol.Methods **51**:255-271.
63. **Millar, B. C., J. Xu, and J. E. Moore**. 2002. Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. J.Clin.Microbiol. **40**:1575-1580.
64. **Mohammadi, T., H. W. Reesink, C. M. Vandenbroucke-Grauls, and P. H. Savelkoul**. 2003. Optimization of real-time PCR assay for rapid and sensitive detection of eubacterial 16S ribosomal DNA in platelet concentrates. J.Clin.Microbiol. **41**:4796-4798.
65. **Mohammadi, T., H. W. Reesink, C. M. Vandenbroucke-Grauls, and P. H. Savelkoul**. 2005. Removal of contaminating DNA from commercial nucleic acid extraction kit reagents. J.Microbiol.Methods **61**:285-288.
66. **Mothershed, E. A. and A. M. Whitney**. 2006. Nucleic acid-based methods for the detection of bacterial pathogens: present and future considerations for the clinical laboratory. Clin Chim.Acta **363**:206-220.
67. **Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter**. 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology **148**:257-266.
68. **Nataro, J. P. and J. B. Kaper**. 1998. Diarrheagenic Escherichia coli. Clin Microbiol.Rev. **11**:142-201.
69. **Nikkari, S., I. J. McLaughlin, W. Bi, D. E. Dodge, and D. A. Relman**. 2001. Does blood of healthy subjects contain bacterial ribosomal DNA? J.Clin Microbiol. **39**:1956-1959.
70. **Nocker, A. and A. K. Camper**. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl.Environ.Microbiol. **72**:1997-2004.
71. **Nocker, A., C. Y. Cheung, and A. K. Camper**. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J.Microbiol.Methods **67**:310-320.
72. **Nogva, H. K., S. M. Dromtorp, H. Nissen, and K. Rudi**. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. Biotechniques **34**:804-3.
73. **Okhuysen, P. C.** 2001. Traveler's diarrhea due to intestinal protozoa. Clin Infect.Dis. **33**:110-114.
74. **Paltansing, S., R. J. van den Berg, R. A. Guseinova, C. E. Visser, d. van, V, and E. J. Kuijper**. 2007. Characteristics and incidence of Clostridium difficile-associated disease in The Netherlands, 2005. Clin Microbiol.Infect. **13**:1058-1064.

75. **Peters, R. P., T. Mohammadi, C. M. Vandenbroucke-Grauls, S. A. Danner, M. A. van Agtmael, and P. H. Savelkoul**. 2004. Detection of bacterial DNA in blood samples from febrile patients: underestimated infection or emerging contamination? FEMS Immunol.Med.Microbiol. **42**:249-253.
76. **Peters, R. P., M. A. van Agtmael, S. A. Danner, P. H. Savelkoul, and C. M. Vandenbroucke-Grauls**. 2004. New developments in the diagnosis of bloodstream infections. Lancet Infect.Dis. **4**:751-760.
77. **Petrich, A., J. Mahony, S. Chong, G. Broukhanski, F. Gharabaghi, G. Johnson, L. Louie, K. Luinstra, B. Willey, P. Akhaven, L. Chui, F. Jamieson, M. Louie, T. Mazzulli, R. Tellier, M. Smieja, W. Cai, M. Chernesky, and S. E. Richardson**. 2006. Multicenter comparison of nucleic acid extraction methods for detection of severe acute respiratory syndrome coronavirus RNA in stool specimens. J.Clin Microbiol. **44**:2681-2688.
78. **Procop, G. W.** 2007. Molecular diagnostics for the detection and characterization of microbial pathogens. Clin Infect.Dis. **45 Suppl 2**:S99-S111.
79. **Rantakokko-Jalava, K., S. Nikkari, J. Jalava, E. Eerola, M. Skurnik, O. Meurman, O. Ruuskanen, A. Alanen, E. Kotilainen, P. Toivanen, and P. Kotilainen**. 2000. Direct amplification of rRNA genes in diagnosis of bacterial infections. J.Clin.Microbiol. **38**:32-39.
80. **Raoult, D., P. E. Fournier, and M. Drancourt**. 2004. What does the future hold for clinical microbiology? Nat.Rev.Microbiol. **2**:151-159.
81. **Reischl, U., H. J. Linde, N. Lehn, O. Landt, K. Barratt, and N. Wellinghausen**. 2002. Direct detection and differentiation of Legionella spp. and Legionella pneumophila in clinical specimens by dual-color real-time PCR and melting curve analysis. J.Clin Microbiol. **40**:3814-3817.
82. **Rosey, A. L., E. Abachin, G. Quesnes, C. Cadilhac, Z. Pejin, C. Glorion, P. Berche, and A. Ferroni**. 2007. Development of a broad-range 16S rDNA real-time PCR for the diagnosis of septic arthritis in children. J.Microbiol.Methods **68**:88-93.
83. **Rudi, K., B. Moen, S. M. Dromtorp, and A. L. Holck**. 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl.Environ.Microbiol. **71**:1018-1024.
84. **Rudi, K., K. Naterstad, S. M. Dromtorp, and H. Holo**. 2005. Detection of viable and dead Listeria monocytogenes on gouda-like cheeses by real-time PCR. Lett.Appl.Microbiol. **40**:301-306.
85. **Rueckert, A. and H. W. Morgan**. 2007. Removal of contaminating DNA from polymerase chain reaction using ethidium monoazide. J.Microbiol.Methods **68**:596-600.
86. **Rueckert, A., R. S. Ronimus, and H. W. Morgan**. 2005. Rapid differentiation and enumeration of the total, viable vegetative cell and spore content of thermophilic bacilli in milk powders with reference to Anoxybacillus flavithermus. J.Appl.Microbiol. **99**:1246-1255.
87. **Scheutz, F, Lassen, J., and Nygard, K.** VTEC O103 outbreak in Norway. EPI-News 2006 14, 1. 5-4-2007.
88. **Schuurman, T., de Boer, R. F., and Kooistra-Smid, A. M. D.** Rapid and sensitive detection of 5 gastro-intestinal pathogens using 2 internally controlled Multiplex real-time PCRs (P583). Clin Microbiol.Infect. 12[S4], S134. 1-4-2007.
89. **Schuurman, T., de Boer, R. F., van Deursen, P. B. H., de Bie, P., and Kooistra-Smid, A. M. D.** Highly efficient extraction of pathogen DNA from stool using the NucliSENS easyMAG specific A 1.0.2 protocol (P582). Clin Microbiol.Infect. 12[S4], S134. 1-4-2007.
90. **Sjoholm, M. I., J. Dillner, and J. Carlson**. 2007. Multiplex detection of human herpesviruses from archival specimens using MALDI-TOF mass spectrometry. J.Clin Microbiol.
91. **Soderlund-Strand, A., J. Dillner, and J. Carlson**. 2008. High-throughput genotyping of oncogenic human papilloma viruses with maldi-tof mass spectrometry. Clin Chem **54**:86-92.

92. **Soejima, T., K. Iida, T. Qin, H. Taniai, M. Seki, A. Takade, and S. Yoshida**. 2007. Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. Microbiol.Immunol. **51**:763-775.
93. **Striebel, H. M., E. Birch-Hirschfeld, R. Egerer, and Z. Foldes-Papp**. 2003. Virus diagnostics on microarrays. Curr.Pharm.Biotechnol. **4**:401-415.
94. **Svraka, S., E. Duizer, H. Vennema, B. E. de, d. van, V, B. Dorresteijn, and M. Koopmans**. 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. J.Clin Microbiol. **45**:1389-1394.
95. **Tang, Y. W., S. E. Sefers, H. Li, D. J. Kohn, and G. W. Procop**. 2005. Comparative evaluation of three commercial systems for nucleic acid extraction from urine specimens. J.Clin Microbiol. **43**:4830-4833.
96. **Templeton, K. E.** Improved detection of respiratory pathogens by real-time PCR. PhD thesis University of Leiden . 25-10-2005.
97. **Thom, K. and G. Forrest**. 2006. Gastrointestinal infections in immunocompromised hosts. Curr.Opin.Gastroenterol. **22**:18-23.
98. **Tracz, D. M., H. Tabor, M. Jerome, L. K. Ng, and M. W. Gilmour**. 2006. Genetic determinants and polymorphisms specific for human-adapted serovars of Salmonella enterica that cause enteric fever. J.Clin Microbiol. **44**:2007-2018.
99. **Turner, A. P. and N. Magan**. 2004. Electronic noses and disease diagnostics. Nat.Rev.Microbiol. **2**:161-166.
100. **Ugahary, L., W. van de Sande, J. C. van Meurs, and A. van Belkum**. 2004. An Unexpected Experimental Pitfall in the Molecular Diagnosis of Bacterial Endophthalmitis. Journal of Clinical Microbiology **42**:5403-5405.
101. **Ulrich, M. P., D. R. Christensen, S. R. Coyne, P. D. Craw, E. A. Henchal, S. H. Sakai, D. Swenson, J. Tholath, J. Tsai, A. F. Weir, and D. A. Norwood**. 2006. Evaluation of the Cepheid GeneXpert system for detecting Bacillus anthracis. J.Appl.Microbiol. **100**:1011-1016.
102. **van der, Z. A., M. Peeters, J. C. de, H. Verbakel, J. W. Crielaard, E. C. Claas, and K. E. Templeton**. 2002. Qiagen DNA extraction kits for sample preparation for legionella PCR are not suitable for diagnostic purposes. J.Clin Microbiol. **40**:1126.
103. **van Slochteren, K. R., E. van Zanten, G. J. Wisselink, T. Schuurman, and A. M. Kooistra-Smid**. 2007. Direct detection of bacterial DNA in clinical samples; evaluating a 4 1/2 year period of sample collection and analysis. Clin.Microbiol.Infect. **13**:S391.
104. **van Zanten, E., Schuurman, T., Kooistra-Smid, A. M., and van Zwet, A. A.** A Cost-Effectiveness Study Comparing Real-Time PCR with Traditional Culture for Detection of *Salmonella Spp*. and *Campylobacter Jejuni* in Feces. 44 th ICAAC , O1626. 30-9-2004.
105. **van, Pelt, W., M. A. de Wit, W. J. Wannet, E. J. Ligtvoet, M. A. Widdowson, and Y. T. van Duynhoven**. 2003. Laboratory surveillance of bacterial gastroenteric pathogens in The Netherlands, 1991-2001. Epidemiol.Infect. **130**:431-441.
106. **Warwick, S., M. Wilks, E. Hennessy, J. Powell-Tuck, M. Small, J. Sharp, and M. R. Millar**. 2004. Use of quantitative 16S ribosomal DNA detection for diagnosis of central vascular catheter-associated bacterial infection. J.Clin Microbiol. **42**:1402-1408.
107. **Wilson, D., B. Yen-Lieberman, U. Reischl, I. Warshawsky, and G. W. Procop**. 2004. Comparison of five methods for extraction of Legionella pneumophila from respiratory specimens. J.Clin Microbiol. **42**:5913-5916.
108. **Yang, S., S. Lin, G. D. Kelen, T. C. Quinn, J. D. Dick, C. A. Gaydos, and R. E. Rothman**. 2002. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. J.Clin Microbiol. **40**:3449-3454.
109. **Yang, S. and R. E. Rothman**. 2004. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect.Dis. **4**:337-348.
110. **Zucol, F., R. A. Ammann, C. Berger, C. Aebi, M. Altwegg, F. K. Niggli, and D. Nadal**. 2006. Real-time quantitative broad-range PCR assay for detection of the 16S rRNA gene followed by sequencing for species identification. J.Clin Microbiol. **44**:2750-2759.

CHAPTER 11

Acknowledgments

Het proefschrift is klaar en de promotiedatum nadert! Op zo'n moment denk je dan terug aan wat er in de voorbije jaren allemaal gebeurt is en vooral ook wie daar allemaal een rol in hebben gehad. Voor mij begint dit hele traject eigenlijk al ruim 9 jaar geleden, in november 1998 om precies te zijn. Op dat moment loop ik samen met Jan Weel richting de afdeling klinische virologie van het AMC om mijn eerste werkdag te starten als analist binnen de moleculaire diagnostiek van infectieziekten. Ergens halverwege de route van de hoofdingang naar de afdeling zegt Jan iets van de volgende strekking tegen me: "Tim, het is wel de bedoeling dat je hier met een jaar of zo promotieonderzoek gaat doen." Ik weet nog goed dat ik toen bij mezelf iets dacht van: "Jan, je bent gek." Deze gedachte kwam voort uit het feit dat ik op dat moment nog geen jaar afgestudeerd was als HLO-er (ja, toen was het nog redelijk moeilijk om als HLO-analist aan het werk te komen) en ik er absoluut (nog) niet van overtuigd was dat ik het in mij zou hebben om te promoveren. Nu, bijna 10 jaar na dato, Jan, blijkt dat jouw inschatting destijds de juiste was, al ben ik uiteindelijk niet in het AMC gepromoveerd.

Uiteraard doe je een promotieonderzoek niet alleen en vandaar dat ik hier iedereen die een bijdrage heeft geleverd aan dit totstandkoming van dit boekje wil bedanken. Een aantal mensen wil ik echter in het bijzonder bedanken.

Prof. dr. dr. A.F. van Belkum, beste Alex, mijn promotor, een promotie op afstand, zo mag je de combinatie onderzoek in Groningen, promoveren in Rotterdam wel noemen. Echter is de reisafstand nooit een belemmering geweest in het hele traject. Bedankt voor je enthousiasme en de altijd rappe correcties op de manuscripten die ik je toestuurde.

Dr. A.A. van Zwet, beste Ton, mijn co-promotor en destijds als hoofd R&D ook mede-initiator van de onderzoekslijnen waarop veel van dit boekje gebaseerd is. Jouw klinische inbreng en inzicht als arts-microbioloog, of "de domme dokter" zoals je het zelf weleens verwoordde tijdens menige technische discussie, zorgde ervoor dat we met beide benen op de grond bleven staan en het klinisch belang niet uit het oog verloren.

Ing. A.M.D. Kooistra-Smid, beste Mirjam, naast Ton was ook jij de stuwende kracht achter de onderzoekslijnen van de afdeling R&D. Je visie en managementcapaciteiten hebben je gebracht tot hoofd R&D, ondanks de horden die onderweg soms genomen dienden te worden. Ik wil je bedanken voor de mogelijkheid die je mij geboden hebt om promotieonderzoek doen binnen mijn functie als researchanalist en heb onze samenwerking altijd als zeer prettig ervaren. Ik hoop dat we in de toekomst gezamenlijk weer een leuk project kunnen doen en als laatste wens ik je veel succes bij het afronden van je eigen promotieonderzoek.

De leden van de raad van bestuur van het Laboratorium voor Infectieziekten, Dr. B.P. Overbeek en drs K. Donkervoort. Beste Berry, uiteraard moet ik ook jou als directeur medische zaken bedanken voor de mogelijkheid die mij geboden is om als researchanalist een promotieonderzoek te doen. Ik weet nog goed dat je heel stellig was over wie we als promotor moesten benaderen toen Ton en ik daar voor het eerst

met je over spraken. Uiteindelijk bleek dit een zeer goede keuze te zijn. Beste Kees, ook jou wil ik bedanken dat je destijds als directeur economische zaken het mogelijk hebt gemaakt om binnen het Laboratorium voor Infectiekziekten promotieonderzoek doen. Jij stelde ons vaak de vraag wat het gedane onderzoek uiteindelijk zou opbrengen buiten de presentaties en publicaties. Ik denk dat met de invoering van de moleculaire screening op *S. enterica*, *C. jejuni* en *Shigella* species in de routine diagnostiek die vraag positief beantwoord kan worden, al heb je de invoering niet meer actief meegemaakt door het aantreden in je nieuwe functie als bestuurslid en voorzitter van de raad van bestuur van het Bethesda ziekenhuis in Hoogeveen.

Mijn (voormalige) collega researchanalisten van de afdeling R&D, Richard de Boer, Evert van Zanten, Kees van Slochteren, Guido Wesselink en Rachèl Patty, voor de grote gezelligheid en hulp bij de vele bepalingen die we binnen de projecten hebben uitgevoerd. Richard, mijn paranimf, we waren de afgelopen jaren vaak een 2-eenheid bij de projecten waaraan we gezamenlijk werkten. Jouw eerste artikel is in de maak, wie weet waartoe dat nog leidt. Veel succes in de komende jaren. Evert, mijn 2de paranimf, jouw inbreng vanuit de conventionele bacteriologie was een verrijking voor de afdeling met zijn hoog moleculair biologische gehalte. Kees, bedankt voor de vele handige stukjes software die ons werk toch een stuk aangenamer en eenvoudiger maakten. Daarnaast was je ook onmisbaar in de statistische ondersteuning van de afdeling.

Prof. dr. H.A. Verbrugh, Prof. dr. E.J. Kuipers en Prof. dr. J. Verhoef, bedankt voor zitting nemen in de kleine promotiecommisie en het beoordelen van het manuscript. Ik ben vereerd.

Mijn voormalige collega's uit het AMC, Pauline Wertheim-van Dillen, Jan Weel, Menno de Jong, Marcel Beld en René Boom, voor jullie bijdrage aan mijn vorming van het eerste uur binnen het vakgebied van de moleculaire diagnostiek van infectieziekten. Marcel, ik ben blij dat we onze samenwerking hebben kunnen vormgeven met de studie uit hoofdstuk 3.

De samenwerking met het VUmc, Prof. dr. P.H.M. Savelkoul, Dr. M.A. van Agtmael en Dr. R.P.H. Peters, heb ik altijd als erg prettig en stimulerend ervaren. Beste Remco, de vele gezellige congresbezoeken zullen me nog lang blijven heugen, dat moeten we zeker nog eens over doen als je terug bent uit Afrika.

De deelnemers aan de landelijke STEC werkgroep, Yvonne van Duynhoven, Igrid Vriesema en Kim van der Zwaluw vanuit het RIVM, Luc Sabbe en Alexander Roovers vanuit de Oosterschelde Ziekenhuizen, voor de mooie samenwerking en resultaten van de hoofdstukken 6 en 7. Ik hoop dat we in toekomst opnieuw iets moois kunnen doen.

De parasitologiegroep van het Leids Universitair Medisch Centrum, Jaco Verweij, Robert ten Hove en Lisette van Lieshout, voor de prettige samenwerking tijdens de studie uit hoofdstuk 9.

De medewerkers van de afdeling bacteriologie en de decentrale vestigingen van het Laboratorium voor Infectieziekten voor het verzamelen

van klinisch materiaal voor onze studies en het uitvoeren van de routine bepalingen voor deze studies. Lieke Möller, Henk Schepers en Dinneke Dijk, in het bijzonder bedankt voor jullie bijdrage aan hoofdstuk 5.

De stagières die direct of indirect een bijdrage hebben geleverd, Milli Top, Rianne Scholts, Linda Niks en Petra Lankamp. Milli, jouw project was de eerste stap op weg naar de moleculaire screening van darmpathogenen. Rianne, jij vervolgde het project van Milli en liet zien dat de PCR van *Salmonella* species uit feces veelbelovend was. Ik wens je veel succes als researchanalist op de afdeling R&D. Linda, ondanks dat bij jouw project de real-time PCR resultaten wat tegen vielen door problemen met de probes, heb je toch waardevolle data gegenereerd met betrekking tot het aankweken van *Campylobacter* species. Petra, bedankt voor jouw werk aan de "proof of principle" van de PCR detectie van *Campylobacter jejuni*. Daarnaast heeft de *Giardia lamblia* PCR/sneltest/microscopie-studie geleid tot de mooie publicatie uit hoofdstuk 8.

Erik Sijtsma (Bisytes Art) wil ik bedanken voor het tekenen van de cartoons op de cover. Beste Erik, ik heb altijd vermakelijk moeten lachen om je cartoons in het LvI personeelsblad en ben blij dat ik daar een ook een glimp van kan laten zien op het omslag van dit boekje.

Ruud Karsten, beste Ruud, het gezegde: "beter een goede buur dan een verre vriend" gaat ook in dit geval op. Bedankt voor je wegwijs in photoshop waardoor we in een uurtje of 2 toch het ontwerp van de kaft zo goed als klaar hadden.

Hoogleraar Moleculaire Diagnostiek bij de Sectie Klinische Virologie, afdeling Medische Microbiologie van het Universitair Medisch Centrum Groningen, Prof. dr. H.G.M. Niesters, beste Bert, bedankt voor de ruimte die je me gegeven hebt voor de afronding van mijn proefschrift. Ik kijk er naar uit om onder jouw supervisie te starten met de opleiding tot Medisch Moleculair Microbioloog.

Mijn schoonfamilie, Hans, Lies en Marieke van Sister, bedankt voor jullie intresse, betrokkenheid en steun. Hans en Lies ook bedankt voor de extra oppasochtenden waardoor ik wat extra tijd kon vrij maken voor het schrijven van de verschillende hoofdstukken. Ik weet zeker dat ook Jesper dit erg heeft kunnen waarderen en dat Meike dat in toekomst ook zal doen.

Mijn ouders, Wim en Tiny, lieve pap en mam, voor jullie onvoorwaardelijke steun, interesse, betrokkenheid, liefde, motivatie, ondanks dat jullie af en toe geen idee meer hadden waar het onderzoek nou precies over ging.

Mijn lieve schatten, Jesper en Meike, jullie hebben ons leven compleet gemaakt, ook al zijn de afgelopen maanden tropenmaanden geweest. Lieve Jesper, bedankt dat jij zo'n heerlijke regelmatige middagslaper bent, waardoor pappa op zijn vrije dagen altijd de ruimte kreeg om achter de computer te kruipen. Lieve Meike, doordat jij een veel grotere aanslag op onze nachtrust doet, heeft de voltooiing van dit boekje wel enige vertraging op gelopen. Gelukkig blijf je overdag echter altijd de vrolijkheid zelve en dat is iets dat ik voor geen goud heb willen missen.

Lieve Kirsten, mijn andere schat, het boekje is nu eindelijk klaar. Bedankt voor alle liefde die ik van je mag ontvangen, ik hoop nog lang met jou en onze kids van het leven te kunnen genieten. Nu is het jouw beurt om te gaan studeren.

CHAPTER 12

Curriculum Vitea and Bibliography

CURRICULUM VITAE

Tim Schuurman was born in Wormerveer, on October 31, 1974. He passed his secondary school exam in 1993 at the Bertrand Russell College in Krommenie. From 1993 to 1997 he studied biochemistry at the Hogeschool Alkmaar where he graduated for his bachelor degree (BSc/Ing.) on a project to study the diversity of ammonia-oxidizing bacteria in the Schelde estuary using denaturing gradient gel electrophoresis of PCR-amplified 16S ribosomal RNA genes. In 1998 he worked for 6 months at the Dutch Institute for Pigment Disorders (SNIP) in Amsterdam, where he cultured human melanocytes for transplantation in patients with pigment disorders. Late 1998 he started working in group of René Boom at the Department of Medical Microbiology, Section of Clinical Virology at the Academic Medical Center in Amsterdam. During his nearly 4 years at the AMC he participated in the molecular diagnostic laboratory and the research on human herpesvirus infections. In 2001 he moved to the Department of Research & Development at the Laboratory for Infectious Diseases (formerly Regional Public Health Laboratory for Groningen and Drenthe), where he started his PhD project on “Developments and Clinical Applications in Diagnostic Molecular Microbiology” in 2002. In 2007 he joined the Department of Medical Microbiology, Section of Clinical Virology at the University Medical Center Groningen, where he participates in founding the new clinical virology laboratory. After obtaining his registration for Medical Microbiological Researcher (Medisch Microbiologisch Onderzoeker SMBWO) he will start his training for the Medical Molecular Microbiologist registration under the supervision of Prof.dr. H.G.M. Niesters. He has a long-term relationship with Kirsten van Sister, and they are the proud parents of Jesper and Meike.

BIBLIOGRAPHY.

Y.T.H.P. van Duynhoven, I.H.M. Friesema, T. Schuurman, A. Roovers, A.A. van Zwet, L.J.M. Sabbe, W.K. van der Zwaluw, D.W. Notermans, B.Mulder, E.J. van Hannen, F.G.C. Heilmann, A. Buiting, R. Jansen, and A.M.D. Kooistra-Smid.
Prevalence, characterisation and clinical profiles of Shiga toxin-producing *Escherichia coli* in The Netherlands.
C*lin. Microbiol. Infect.* 2008. **14:**737-745.

T. Schuurman, P. Lankamp, A. van Belkum, M. Kooistra-Smid, and A. van Zwet.
Comparison of microscopy, real-time PCR and a rapid immunoassay for the detection of *Giardia lamblia* in human stool specimens
Clin. Microbiol. Infect. 2007. **13:**1186–1191.

T. Schuurman, R. de Boer, R. Patty, M. Kooistra-Smid, and A. van Zwet.
Comparative evaluation of in-house manual, and commercial semi-automated and automated DNA extraction platforms in the sample preparation of human stool specimens for a *Salmonella enterica* 5'-nuclease assay.
J. Microbiol. Methods. 2007. **71:**238-245.

T. Schuurman, R.F. de Boer, E. van Zanten, K.R. van Slochteren, H.R. Scheper, B.G. Dijk-Alberts, A.V.M. Möller, and A.M.D. Kooistra-Smid.
Feasibility of a molecular screening method for detection of *Salmonella enterica* and *Campylobacter jejuni* in a routine community-based clinical microbiology laboratory.
J. Clin. Microbiol. 2007. **45:**3692-3700.

R. ten Hove, T. Schuurman, M. Kooistra, L. Möller, L. van Lieshout, and J.J. Verweij.
Detection of diarrhoea-causing protozoa in general practice patients in The Netherlands by multiplex real-time PCR.
Clin. Microbiol. Infect. 2007. **13:**1001–1007.

T. Schuurman, A. Roovers, W.K. van der Zwaluw, A.A. van Zwet, L.J.M. Sabbe, A.M.D. Kooistra-Smid, and Y.T.H.P. van Duynhoven.
Evaluation of 5′-nuclease and hybridization probe assays for the detection of shiga toxin-producing *Escherichia coli* in human stools.
J. Microbiol. Methods. 2007. **70:**406-415.

T. Schuurman, A. van Breda, R. de Boer, M. Kooistra-Smid, M. Beld, P. Savelkoul, and R. Boom.
Reduced PCR sensitivity due to impaired DNA recovery with the MagNA Pure LC total nucleic acid isolation kit.
J. Clin. Microbiol. 2005. **43:**4616-4622.

T. Schuurman, R.F. de Boer, A.M.D. Kooistra-Smid, and A.A. van Zwet.
Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for diagnosis of bacterial meningitis in a clinical setting.
J. Clin. Microbiol. 2004. **42:**734-740.

R. Boom, C.J.A. Sol, T. Schuurman, A. van Breda, J.F.L. Weel, M. Beld, I.J.M. ten Berge, P.M.E Wertheim-van Dillen, and M.D. de Jong.
Human cytomegalovirus DNA in plasma and serum specimens of renal transplant recipients is highly fragmented.
J. Clin. Microbiol. 2002. **40:**4105-4113.

M.J.M. de Bie, A.G.C.L. Speksnijder, G.A. Kowalchuk, T. Schuurman, G. Zwart, J.R. Stephen, O.E. Diekmann, H.J. Laanbroek.
Shifts in the dominant populations of ammoniaoxidizing β-subclass Proteobacteria along the eutrophic Schelde estuary.
Aquat. Microb. Ecol. 2001. **23:**225–236.

M.D. de Jong, J.F.L. Weel, T. Schuurman, P.M.E. Wertheim-van Dillen, and R. Boom.
Quantitation of varicella-zoster virus DNA in whole blood, plasma, and serum by PCR and electrochemiluminescence.
J. Clin. Microbiol. 2000. **38:**2568-2573.