CLINICAL MICROBIOLOGICAL DIAGNOSTICS 2.0

RENÉ TE WITT

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Voor Dirk-Jan (20-12-1974 – 22-11-2008)

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Introduction

Chapter 1

General introduction and outline of the thesis

René te Witt

Historical aspects

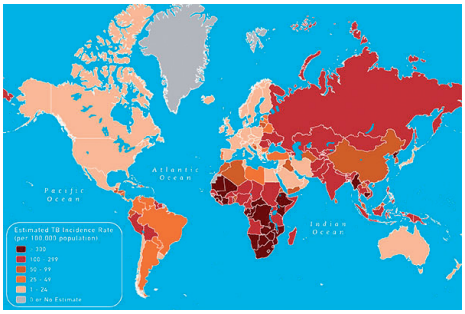
Life has changed since the Dutch botanist Anthonie van Leeuwenhoek (1632-1723) revealed the diversity and ubiquity of the microbial world through the discovery of microscopy. Van Leeuwenhoek can be considered to be the first genuine microbiologist. Microscopic evidence provided support for the emerging germ theory of disease in the 19th century. In the 1880s, Robert Koch defined his postulates for determining whether or not a microorganism is the etiological agens of a disease. Since the 19th century, advances in knowledge have included the discovery of viruses, chlamydiae, mycoplasmata and rickettsiae as new classes of microorganisms that cannot (yet) be grown in pure culture, but require living cells for reproduction. The spectrum of bacterial, fungal and protozoan pathogens has been expanding with improved culture techniques and the development of advanced imaging techniques. However, the most revolutionary advance in biomedical science since Van Leeuwenhoek, is due to the discovery of nucleic acids in 1871 by Miescher, which lead to the discovery of DNA as the source of genetic information and as the basis for characterization of an organism in 1953 by Watson, Crick and Wilkins (1-2).

Introduction to infection disease diagnostics

Today, infectious diseases remain a global burden to human health. Infectious diseases can be classically omnipresent (tuberculosis, cholera, malaria and gastro-enteritis) or display annual epidemics (norovirus, influenza and seasonal colds). They can present as incidental emerging infectious diseases (avian influenza, SARS and hemorrhagic fevers) or they can be caused by emerging antibiotic-resistant pathogens (methicillin-resistant *Staphylococcus aureus* (MRSA), Extended Spectrum Beta-Lactamase (ESBL) producers, carbapenem-resistant bacteria, multidrug-resistant *Acinetobacter* species and other multidrug resistant bacteria). And, finally, infectious diseases can present as pandemic infections (AIDS, the recent H1N1 outbreak and the already mentioned tuberculosis) (For examples, see **Figure 1**).

Current diagnosis of most bacterial infections is still based on conventional microscopy and culture-based methods. Substantial advantages of conventional diagnostics are high-throughput and ease-of-use. Essentially, laboratories can process direct and easy tests on hundreds to thousands of samples on a daily basis, using inexpensive culture media and simple techniques. This strategy also provides bacterial isolates, which can be further characterized if needed, i.e. more detailed identification of the species, analysis of their antibiotic susceptibility profiles and/or epidemiological type for outbreak analysis and surveillance studies.

However, this strategy has limitations. Culture has limited sensitivity and is time-consuming. Many new diagnostic techniques and strategies have been developed during the past two decades, all with the objective to save time and to improve accuracy.



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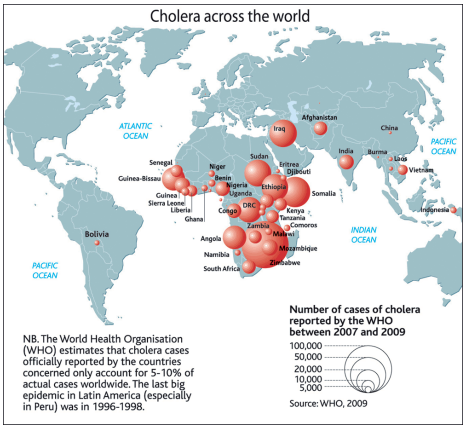

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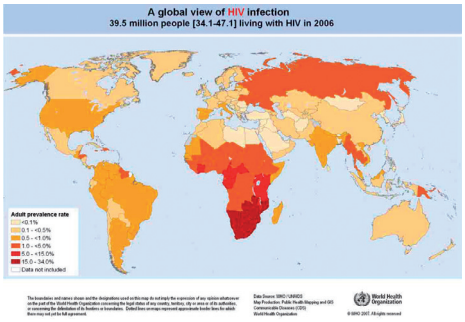

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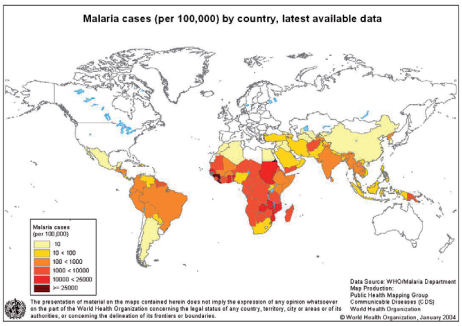

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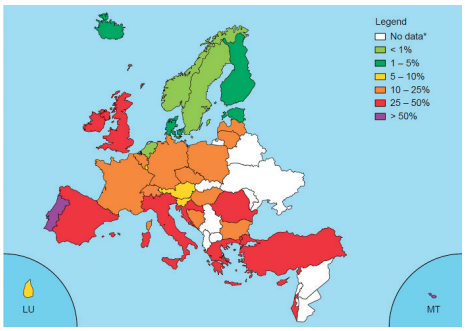

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Figure 1. Incidence rates of examples of infectious diseases.

A. Worldwide TBC incidence rate, 2010 (Data from the World Health Organization (WHO) (www.who.int).
B. Worldwide Cholera incidence rate, 2009 (Data from the World Health Organization (WHO) (www.who.int).
C. Worldwide HIV incidence rate, 2006 (Data from the World Health Organization (WHO) (www.who.int).
D. Worldwide malaria incidence rate, 2004 (Data from the World Health Organization (WHO) (www.who.int).
E. European MRSA incidence rate, 2007 (Data from the European Antimicrobial Resistance Surveillance Network (EARS-NET) (www.ecdc.europa.eu/en/activities/surveillance/EARS-NET).

Biomarkers

While fever in a patient can presage an infection that can be rapidly progressive and fatal (sepsis, meningitis), it may also be a manifestation of a self-limiting, more trivial infection (Salmonellosis) or even sterile inflammation (fever of unknown origin, allergy etc.). Nevertheless, the initial clinical evaluation should focus on infections that can be treated, may be transmissible and that may cause serious sequelae or even death. The clinician must decide in a short time whether the infection is likely to be caused by bacteria, viruses or parasites and whether the patient should be admitted for intensified empirical treatment with antibiotics. Results of routine (microbiological) laboratory tests may provide additional clues for the diagnosis. Traditionally, an elevated number of circulating leukocytes (leukocytosis) is considered to be suggestive of bacterial infection, whereas a decreased number of leukocytes (leukopenia) may suggest viral infection. Unfortunately, a number of bacterial infections, such as uncomplicated typhoid fever, brucellosis and rickettsial infections are associated with a normal to low white blood cell count. As a consequence, the clinician cannot completely rely on these traditional parameters for decision making in the acute care setting. In order to reduce empirical, broad-spectrum antibiotic treatment, a rapid identification of the cause of the clinical symptoms is needed. Concerns about the accuracy of microbiological diagnostic techniques have led to the hypothesis that the use of biologic markers could improve fast recognition of a true infection in patients in an early state, thereby facilitating decisions on patient management. Host-specific biomarkers can provide both diagnostic and prognostic information for individual patient care. Similarly, biomarkers can indicate (ab)normal responses to therapeutic intervention. Biomarkers can also help to advance basic knowledge of pathogenesis.

Several studies have been performed on the relevance of biomarkers in the distinction between bacterial and viral infections prior to or on admission of a patient to the hospital. In certain clinical studies procalcitonin (PCT) demonstrated to be good predictive biomarker for bacterial infection (sepsis and pneumonia) (3-6), whereas neopterin showed to be predictive for viral infection (7). Moreover, PCT was proven to be an accurate monitor for the efficacy of antibiotic treatment (6, 8).

PCT is the propeptide of calcitonin, which plays an important role in the regulation of bone calcium and phosphate metabolism (9). A specific protease cleaves PCT to calcitonin (10). Normally all the procalcitonin is cleaved and none is released into the bloodstream. PCT levels are therefore very low (<0.1 ng/ml) in healthy humans (11). Inducers for PCT synthesis are inflammatory cytokines, Interleukin-1 (IL-1) and Tumour Necrosis Factor-α (TNF-α), but also bacterial membrane structures or bacterial cell wall products such as lipopolysaccharides (LPS) or peptidoglycans (PG) (12). Bacterial infections may induce PCT in measurable concentrations in serum only after triggering an inflammatory response (either local or systemic) and this response should have reached a certain severity or threshold. Consequently, local bacterial infections may not always induce PCT. Therefore, it is not possible to reliably use

PCT measurement to exclude any type of bacterial infection (11, 13). Still, PCT has a number of properties which support its use in clinical routine diagnostics. For example, PCT levels in blood samples are stable. It can be tested in routinely collected blood samples which can be transported to the laboratory in the usual way, without suffering any significant drop in concentration of PCT (14).

The production of neopterin is induced mainly by the inflammatory factor interferon-gamma (INF-γ) and is co-stimulated by TNF-α and bacterial endotoxins (15-16). In this way, neopterin may be of clinical use as a marker of pro-inflammation and bacterial infection (17-18).

Soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) may be another interesting infectious disease biomarker. sTREM-1 is a cell-surface receptor identified on neutrophils and a subset of monocytes (19). sTREM-1 may be an appropriate candidate as an infection biomarker as concentrations were elevated in patients with bacterial sepsis (20-21). Furthermore, sTREM-1 can possibly predict mortality as concentrations significantly declined in survivors as compared to non-survivors of bacteraemia (21). Of course, there are many more biomarkers that may be of clinical use as a marker of inflammation and bacterial, viral or parasite infection. Most studies focus on tuberculosis (22-23), sepsis or bacteraemia (24-25), respiratory tract infections (26-28) or fungal (mainly *Aspergillus* spp.) infections (29).

Microscopy

Detection of human pathogens by direct microscopy of the clinical specimen is the most ancient, but still highly relevant, diagnostic method in medical microbiology. Many different cell wall structure-specific staining procedures are available (Gram, Ziehl-Neelsen etc.), providing an initial classification of a potential pathogen together with morphology (shape and positioning). However, in the majority of cases, final identification still relies on phenotypic (biochemical and serological) properties of the bacterium after culturing on appropriate media.

Culture

A conventional and commonly used diagnostic technique in clinical settings is culture on and in a variety of solid and liquid media. These media can be universal (supporting the growth of most clinically relevant bacterial species e.g. blood agar, chocolate agar, Müller Hinton broth), specific (selective agar or broth specifically for Gram-positive cocci or anaerobic bacteria etc.) or even species-specific (i.e. MRSA enrichment broth, chromogenic agars) via the addition of specific substrates, growth inhibitors and/or certain antibiotics. In the last decades, many

different commercial chromogenic agars have been developed for specific pathogens, such as diagnostic agar for *Candida* (30) and *Staphylococcus aureus* ID agar or CHROMagar MRSA for MRSA identification directly from clinical samples (31-33).

The introduction of automated laboratory systems for culture, e.g. the blood culture systems Bactec (BD Diagnostics, Erembodegem, Belgium) and BacT/Alert (bioMérieux, Marcy l'Etoile, France), has increased the quality and efficiency of clinical diagnostic laboratories.

Culture methods require time to allow bacteria to grow, generally 24-72 hours. This results in a delay in more specified anti-microbial treatment of the patient. For fastidious or slow-growing bacteria, such as *Mycobacterium tuberculosis*, the diagnostic time is lengthened even further to several weeks, while bacteria that cannot be grown on conventional culture media and under conventional conditions, obviously, will remain undetected. Furthermore, logistic factors may influence bacterial growth. Samples need to be transported to the laboratory prior to analysis. During transport, samples may deteriorate or change e.g. due to changes in temperature, enzymatic effects and/or lysis of cells. This may have dramatic effects on bacterial quality (specificity) and quantity (sensitivity) in the clinical sample. Moreover, transport time will further delay the diagnostic procedure.

An example of a bacterial pathogen that is mainly detected by culture is Group B streptococcus (GBS). GBS has been considered as a human pathogen since 1938. During the course of pregnancy and the postpartum period, GBS may cause a variety of serious infections in both mother and neonate (34-35). Neonatal early-onset GBS disease (GBS-EOD) presents in the first week of life. Mortality is high among preterm infants, with case-fatality rates of approximately 20% and as high as 30% among those <33 weeks' gestation, compared with 2-3% among full-term infants (34). In most cases neonates acquire GBS during delivery from mothers colonized with GBS in the rectovaginal tract (up to 35%) (36-38). Approximately 1% of neonates born from colonized mothers develop GBS-EOD and up to 40% of the surviving neonates suffer serious sequelae, such as mental retardation or seizures (34).

For diagnosis of GBS, several culture techniques have been developed. These techniques focus on the sampling site, increasing of the sensitivity, the moment of sampling and the culture technique. However, little attention has been paid to possible consequences of transport conditions with respect to time, temperature and transport medium. Revised CDC guidelines state specifically that GBS isolates remain viable in Amies transport medium for several days at room temperature. However, a decline of up to 30% for the recovery of GBS isolates is observed over a period of 1-4 days, particularly at elevated temperatures (39-40). Even when appropriate transport media are used, the sensitivity of culture is most optimal when the specimen is stored at 4°C before culture and processed within 24 hours after sampling (34, 39-43).

More sensitive methods than the currently available and recommended transport and culture methods would improve the effectiveness of the screening-based approach and would lead to improved patient management. A new transport and enrichment broth, called

Granada Tube (GT) broth (bioMérieux) was introduced recently. An orange pigment is produced in this broth in the presence of GBS (44-45). Means such as this medium may help to further improve diagnostic accuracy and the ultimate prevention of GBS disease in the target population.

Serology

In diagnostic laboratories, microscopy and culture are usually supplemented with serological techniques for (in)direct diagnostics of infections, by detecting specific antibodies or antigens. Both methods can speed up detection and diagnosis of fastidious or non-culturable microorganisms. Serological techniques may be antibody-based, e.g. Enzyme Linked Immuno Sorbent Assay (ELISA) or specifically geared towards the detection of bacterial antigens. However, there are also numerous drawbacks. Serological results can be unreliable due to cross-reactivity. Furthermore, because of the frequent need of a convalescent serum sample after 1 to 4 weeks, results are available too late to understand infection dynamics and to have an impact on immediate patient management.

Optimal serological testing for individual patient care depends on several factors: age of the patient, timing of serum collection, whether paired (acute and convalescent) sera are available for confirmation of seroconversion, and, not in the least, availability of appropriate equipment and experience of the laboratory personnel. Despite these potential pitfalls, commercial antigen detection and serological assays represent a huge market and are globally used for diagnosis of viral infections and fastidious or non-culturable bacterial species, such as *Legionella* spp. and *Borrelia burgdorferi*. Especially since the introduction of automated, high-throughput antigen and/or antibody detection systems such as VIDAS (bioMérieux) and Luminex (Luminex, Austin, Texas, USA) and robotic workstations such as Hamilton STAR (Hamilton, Reno, Nevada, USA), serological testing has become more accessible (cheaper, faster, easier and high-throughput).

Identification and antimicrobial susceptibility testing

After cultivation of a microorganism, the next step comprises of identification. Most clinical laboratories use biochemical and/or immunological identification assays, which are based on species-specific phenotypic characteristics of a microorganism. However, the results can be influenced by in vitro test conditions, such as incubation time, - temperature, - conditions (O_2, anaerobic incubation, CO_2, etc.) and composition of the culture medium. These factors may lead to misinterpretation of the results and subsequent misidentification of the etiological agens (46). Automated bacterial identification and antimicrobial susceptibility testing (AST)

platforms such as the VITEK (bioMérieux), PHOENIX (BD Diagnostics) and Microscan (Siemens Healthcare Diagnostics, Munich, Germany) are well established in diagnostic bacteriology. All these systems are based on a conventional concept, using enzymatic (or biochemical) properties of bacteria for the identification and micro-dilution for AST.

Antibiotic resistance

Penicillin resistance in *S. aureus* appeared very soon after the general clinical use of penicillin in 1943, and the mechanism of resistance was the production of β-lactamase (47). The first case of MRSA was reported in 1962, only 2 years after methicillin was introduced to treat penicillin-resistant *S. aureus*. The original MRSA strains circulated within healthcare settings, acquiring resistance to a range of antibiotics. Infections occurred only in individuals with risk factors such as hospitalizations or residence in long-term care facilities. However, since the 1990's, there has been a rise in community-acquired MRSA (CA-MRSA) infections in previously healthy individuals without healthcare exposure. These CA-MRSA strains did not escape from hospitals, but arose in the community (48-50). The efficiency of person-to-person transmissibility is one of the key components of its successful spread, combined with increased virulence (51).

However, the largest current threat in antimicrobial resistance is the rapid and widespread dissemination of multidrug resistant Gram-negative bacteria. There are frequent reports in the literature of new β-lactamases and other novel resistance mechanisms as well as mobile genetic elements that may spread easily from cell to cell. Laboratory detection is difficult because multiple resistance mechanisms may be present and resistance may be "hidden" – resistance genes may be present without the characteristic phenotype. Reliance on carbapenems for the treatment of extended-spectrum β-lactamases (ESBLs) has led to increasing carbapenem resistance and the detection of various carbapenemases. The newly described New Delhi metallo-β-lactamase 1 (NDM-1) and its subsequent global dissemination is of great concern as there are few effective antimicrobials beyond carbapenems for these highly resistant organisms (52). More recently, carbapenem multiresistance has been described in a number of bacteria (53). Even with lowered Clinical and Laboratory Standards Institute (CLSI) breakpoints, detection of carbapenemases is problematic, with different susceptibility results obtained with different methods and error rates when using the reference broth microdilution method. Nucleic acid amplification assays are essential for detecting these types of resistance mechanisms.

Nucleic acid amplification

Each species harbours a unique nucleic acid signature, which can be used as target sequence for the detection and identification of a specific microorganism in clinical samples. Additionally, clinically relevant characteristics of that particular strain, such as its genes encoding for resistance determinants and its array of virulence factors can also be detected. DNA is a stable molecule in the absence of nucleases or restriction enzymes, which makes it very suitable as a diagnostic target, especially since it can be isolated relatively easily from a variety of biological matrices. Over the past decades, a wide variety of nucleic acid amplification techniques has been developed and implemented in microbiological diagnostic laboratories. Clear benefits over traditional culture-based assays are, in particular, reduced time to identification and improved sensitivity and specificity. Polymerase chain reaction (PCR) is the most commonly used platform. PCR was developed by Kary Mullis and colleagues in 1987 (54). Since then, applications of PCR have expanded to clinical -, food -, environmental - and essentially all biological research areas.

The most significant optimisation in PCR diagnostics has been the development of a closed-tube, real-time detection system, thereby significantly reducing the risk of PCR product contamination (55-57). Real-time PCR continuously monitors the amplification process, in contrast to the end point detection of the amplification product in conventional PCR. Real-time PCR provides sensitive and quantitative detection of PCR products in a rapid turnaround time and strongly contributes to the strict requirements of clinical diagnostics. Real-time PCR also allows for the quantification of the bacterial target DNA by using an internal calibration curve. Nowadays, molecular quantification by real-time PCR is very common and important in virology to establish and monitor the viral load during antiviral therapy to improve patient management (HIV) (58). The determination of bacterial load potentially allows for the monitoring of antimicrobial therapy response or discrimination between infection and colonization (59). Results can be obtained on the same day of specimen collection, allowing adequate focusing of antibacterial therapy and reduction of unnecessary use of antibiotics.

Real-time PCR depends on different detection strategies. Fluorescent technologies employed are either non-specific or specific. Non-specific fluorescence uses dyes such as SYBR Green or SYBR Gold, which efficiently intercalate into the PCR product during the annealing phase of amplification. Specific fluorescence technologies use probes to detect target sequence amplification. A number of different fluorescent probe chemistries have been developed for real-time PCR assays, based on 2 main principles, hydrolysis probes (e.g. TaqMan probes, molecular beacons, Scorpion probes or minor groove binding probes) and hybridisation probes (e.g. Fluorescence Resonance Energy Transfer probes (FRET)). Different methods of fluorescence detection are shown in **Figure 2**.

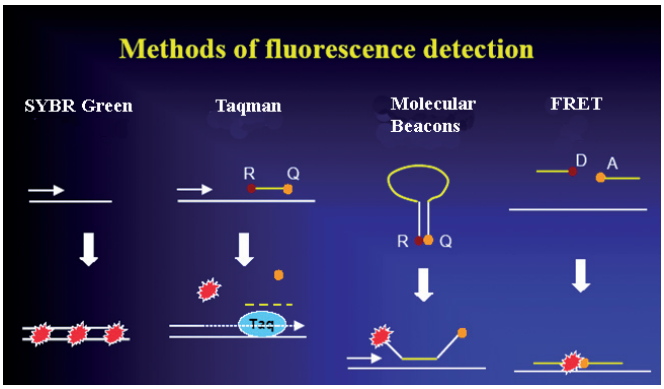

Figure 2. Methods of fluorescence detection.
R=reporter, Q=quencher, D=donor, A=acceptor.

A wide variety of alternative, commercial, amplification-based technologies has been described for the detection of an extended number of bacteria. These tests are too complex to be developed for an in-house format and involve nucleic acid sequence based amplification (NASBA), strand displacement amplification (SDA), multiplex ligation-dependent probe amplification (MLPA), loop-mediated isothermal amplification (LAMP) and many others (**Table 1**). SDA allows for 10^{10}-fold isothermal amplification of a DNA target in less than 15 minutes (60). SDA was the first nucleic acid amplification technology to be coupled with real-time fluorescence-based detection for routine diagnostic application in the clinical laboratory. The isothermal nature of the reaction process offers distinct advantages with regard to the cost and simplicity of the instrumentation.

Table 1. Commonly used nucleic acid amplification techniques in clinical microbiology.

Amplification technique	*Strengths*	*Weaknesses*	*Example(s)*	*References*
Polymerase chain reaction (PCR)	- Efficient - Sensitivity	- Limited in multiplex	Methicillin resistant *Staphylococcus aureus*	(54-57, 61-62)
Nucleic acid sequence-based amplification (NASBA)	- Efficient - Isothermal	- Low specificity	Respiratory tract infections	(62-64)
Strand displacement amplification (SDA)	- Isothermal - Rapid	- Low specificity - Short target sequences	Sexually transmitted diseases	(60, 62, 65)
Ligase chain reaction (LCR)	- Specific	- High risk of contamination - Low reproducibility	HIV, sexually transmitted diseases	(66-67)
Loop-mediated isothermal amplification (LAMP)	- Rapid - Specific - Isothermal	- Complex	*Clostridium difficile*, *Mycobacterium tuberculosis*	(68-71)
Multiplex ligation-dependent probe amplification (MLPA)	- Multiplex	- Costs - Post-amplification analysis - Slow	Respiratory tract infections, sexually transmitted diseases	(72-74)

However, in contrast to the obvious advantages of nucleic acid amplification techniques, there is a risk of false-positivity or false-negativity. False-positive results are primarily due to contamination of reactions by spill-over of other samples or products of previous amplifications. Nucleic acid amplification is highly sensitive and will pick up even the tiniest amounts of a contaminant. This means that contamination prevention has a high priority in the diagnostic laboratory by using different laboratories for PCR mix setup, nucleic acid isolation, PCR setup, amplification and detection. Next to this, contamination can also be prevented by using Uracil-DNA Glycosylase (UNG) prior to the thermal cycling in combination with the use of dUTP in the PCR amplification. Another factor causing false-positivity may be the result of cross-reactivity, indicating a low specificity of the diagnostic test.

False-negative results may be the consequence of insensitivity of the test; only high quantities of the target will lead to a positive result. Variability of the target sequence (e.g. SCC*mec* variance in MRSA) may also result in false-negativity. In such cases, the assay should be redesigned. Another cause of false-negative results is the presence of inhibitors of nucleic acid amplification in the clinical sample. Blood, urine and feces are well-known examples of complex biological samples, with many different inhibitors (61-62). The inhibitory compounds of the clinical sample interact with one or more of the components of the PCR reaction mixture. For instance, anticoagulants, added to blood samples, chelate Mg^{2+} ions, hereby reducing DNA-polymerase effectiveness (63). Blood compounds such as haemoglobin, lactoferrin, heme and IgG may also act as inhibitors (64). As a consequence of inhibition, extensive nucleic acid purification is often required to generate a high quality and high yield of nucleic acids (65). Morata et al demonstrated that incorporation of additional washing steps into their DNA extraction protocol removed inhibitors to allow successful PCR, although it resulted in a lower DNA yield (66). Purification adds to the time and expense of sample preparation, as well as to the loss of nucleic acids. Therefore a balance needs to be found between time, costs and purity (67).

Nucleic acid amplification assays have a number of limitations that restrict their applicability. Contamination of samples and PCR reagents remains an issue. These assays require careful validation. Validation includes sampling, DNA extraction, amplification and interpretation of the results. Furthermore, it implies the inclusion of positive and negative controls, testing of inhibition controls, pre-treatment step(s) to ensure good extraction of nucleic acids from bacterial cells and regular testing of all reagents, such as primers, probe(s), polymerase etc.

Bacterial typing

After detection and identification of (antibiotic-resistant) bacteria, identification at the strain level (also known as typing) may be necessary in order to trace potential transmission routes and decide whether infection prevention measures are mandatory. In addition, typing is essential for elucidation of (inter)national dissemination of bacterial clones.

Since *S. aureus* is one of the most important human pathogens and its antibiotic resistance is continuously increasing worldwide, many of the most relevant typing techniques have been developed and validated for (methicillin-resistant) *S. aureus*.

Currently, a wide variety of genetic typing methods are in use in the diagnostic laboratory, but pulsed-field gel electrophoresis (PFGE) of macro restriction fragments of genomic DNA is preferred because of its high discriminatory power (68-70). However, PFGE is technically demanding, with limited portability due to low reproducibility (70).

The introduction of a commercially available automated rep-PCR system, the DiversiLab system (bioMérieux) offers advances in standardization and reproducibility of the procedure over manual fingerprint-generating systems (71). However, although two independent studies concluded that the DiversiLab system is a rapid and reproducible technique, it clearly lacks resolution for typing of Gram-positive bacteria. DiversiLab analysis does not differentiate genetically among epidemiologically unique MRSA strains, which is needed for adequate outbreak analysis (72-73).

Multilocus variable number of tandem repeat analysis (MLVA) is a high-throughput genotyping technique that can be used for hospital- and (inter)national genotyping, but the discriminatory power depends on the number and types of loci analyzed (74-75).

Sequence-based approaches, such as spa sequence typing and multilocus sequence typing (MLST), have resulted in large sequence databases (76-78). The determination of sequence polymorphism of the *spa* gene encoding the staphylococcal surface protein A (*spa* sequence typing) has become the most popular MRSA typing system, thanks to high-throughput capacity and an excellent reproducibility, which allows portability of data and comparison worldwide (79). However, *spa* sequence typing has a moderate discriminatory power and misclassification of particular sequence types is possible due to recombination. MLST has great interlaboratory reproducibility thanks to standardised nomenclature, but comes with limited discriminatory power and low throughput. Therefore, MLST is more suited for long-term epidemiological studies, such as population structure analysis, phylogeny and long-term surveillance.

Whole genome sequencing (WGS) provides a complete inventory of micro-evolutionary changes within bacterial genomes, but this approach is as yet impractical for routine diagnostic laboratories due to high costs and mandatory technical expertise. Mwangi and colleagues used WGS to identify steps in the evolution of multidrug resistance in *S. aureus* isolates recovered from the bloodstream of a patient. Sequencing the first vancomycin susceptible isolate

and the last vancomycin non-susceptible isolate identified genome wide only 35 point mutations in 31 loci (80). In a recent paper, Harris et al described a new high-throughput genomics approach based on full-genome sequencing, which provides a high-resolution view of the epidemiology of MRSA with the potential to trace person-to-person transmission within a hospital environment (81).

Raman spectroscopy (SpectraCell*RA*, River Diagnostics, Rotterdam, The Netherlands) has been described as a promising tool for phenotypical identification and typing of microorganisms (82-83). This vibrational spectroscopic technique does not require any labels and is fast, non-invasive and highly reproducible. Its high throughput and ease of use enables it suitable for use in routine diagnostic laboratories.

Currently available molecular typing methods are described in **Table 2**.

Table 2. Comparison of the main currently available molecular typing methods.

Method	***Principle/target***	***Strengths***	***Weaknesses***	***Example(s)***	***References***
Pulsed-field gel electrophoresis (PFGE)	Restriction polymorphism of the whole genome	- High discriminatory power	- Technically demanding - Slow - Limited portability - Multiple nomenclatures	Methicillin resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*	(82-83, 98-99)
Multilocus sequence typing (MLST)	Sequence determination of allelic variants of housekeeping genes	- Phylogenetic structure of core genome - Portability - Standard nomenclature	- Limited discriminatory power - Low throughput - Expensive	*Candida albicans*, MRSA	(90-91, 100)
spa-sequence typing (for*S. aureus* typing only)	Polymorphism of number and sequence of repeat elements of the hypervariable gene	- Rapid - High throughput - Portability - Standard nomenclature	- Moderate discriminatory power - Misclassification of STs* due to recombination	(methicillin resistant) *S. aureus*	(92, 101)
rep-PCR typing (Diversilab)	Polymorphism in chromosomal inter-repeat element spacers	- Rapid - High throughput - Portability	- Limited discriminatory power for Gram-positive cocci - No validated interpretation criteria - No standard nomenclature	MRSA, multi-drug resistant bacteria, *P. aeruginosa*	(85-86, 102-103)
Multilocus VNTR analysis (MLVA)	Polymorphism in number of chromosomal VNTR** elements	- Rapid - High throughput - Standard nomenclature	- Limited discriminatory power	*Mycobacterium tuberculosis*, *Bacillus anthracis*, MRSA	(104-108)
Raman spectroscopy	Overall molecular composition as spectroscopic fingerprints	- High discriminatory power - Rapid - High throughput - Portability	- No standard nomenclature	MRSA, *P. aeruginosa*	(96, 109)

* ST: Sequence Type

** VNTR: Variable Number of Tandem Repeat.

Quality control

A challenge for high-quality molecular-based diagnostics is to monitor the performance of the participating laboratories and to standardize assays between laboratories. There are a limited number of commercially standardized molecular assays available, compared with the high variety of ill-controlled "in-house" real-time PCR assays. Different diagnostic microbiological laboratories may use different assays with different levels of sensitivity and specificity. One way of tackling this problem is by implementing external quality assessment (EQA) programs. This can be done by engaging both manufacturers and diagnostic laboratories in well-defined and robust EQA programs organized by independent institutes.

Depending on the technological and financial possibilities, a clinical microbiological laboratory should optimize its diagnostic strategy by applying a combination of conventional with real-time amplification tests for the detection of microbiological agents. When implementing such a strategy, a balance between performance criteria (sensitivity, specificity) and convenience criteria (clinical utility, turn-around time and costs) will have to be defined. In the end, this should result in the optimization of clinical patient management.

During the past ten years, a shift from classical technologies to refined molecular techniques could be observed in clinical microbiology. Rapid identification has been established, however, AST is still slow. Furthermore, there is a need for EQA and further automisation and communication of data into the clinic followed by action in the clinic. This thesis will cover various aspects of the abovementioned challenges.

Outline of the thesis

The scope of this thesis was to explore new applications of modern diagnostic tools within the setting of a clinical microbiology laboratory.

Introduction

Currently available microbiological tests for the detection of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Legionella* spp., the clinical performance of these tests and their future in the clinical microbiology laboratory are outlined in **Chapter 2**.

Biomarkers

The first part of **Chapter 3** describes procalcitonin and neopterin as suitable biomarkers for exclusion of severe *Plasmodium falciparum* disease at the initial clinical assessment of travellers with imported malaria. The diagnostic accuracy of procalcitonin -, neopterin – and sTREM-1-levels was evaluated in a cohort of 104 travellers with imported malaria (26 patients with non-*P. falciparum* malaria, 64 patients with uncomplicated *P. falciparum* malaria and 14 patients with severe *P. falciparum* malaria).

The second part of **Chapter 3** focuses on the diagnostic relevance of procalcitonin- and neopterin levels as biomarkers for a bacterial or a viral infection in a cohort of 69 ill-returned febrile travellers after a stay in the (sub)tropics (Dengue virus [n=33], *Salmonella enterica* serovar [para]Typhi [n=17] and *Rickettsia africae* [n=19]).

Culture

Chapter 4 describes the *in vitro* evaluation of the performance of Granada broth for detection of Group B Streptococcal (GBS) colonization, both direct and after transport. Simulating conditions in everyday practice, we have compared the sensitivity of Granada tube broth (GT) (bioMérieux) with that of classical Amies transport medium (AT) (bioMérieux) *in vitro*. A total of 1,485 GT and 1,485 AT were tested with 33 well-characterized GBS strains in three different concentrations, five different incubation times and three different temperatures.

Nucleic acid amplification

An update on molecular diagnostics and typing of MRSA is provided in **Chapter 5**.

Bacterial typing

Chapter 6 discusses typing of MRSA. We have analyzed a representative selection of the HARMONY MRSA strain collection originating from 11 European countries with the DiversiLab system, pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST).

Chapter 7 evaluates the application of Spectracell*RA* (Raman spectroscopy) for typing of MRSA isolates. We have retrospectively tested a well-documented MRSA collection, consisting of 113 MRSA isolates, originating from 54 households.

Quality control

Chapter 8 focuses on External Quality Assessments (EQA) of molecular diagnostics and genotyping of MRSA. Two multicentre EQA studies for molecular detection and genotyping of MRSA were produced and arranged in cooperation with Quality Control for Molecular Diagnostics (QCMD) in Glasgow. Firstly, eleven samples containing various amounts of inactivated MRSA strains, methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant coagulase-negative staphylococci (MRCoNS) or *Escherichia coli*, were distributed to 82 laboratories for MRSA detection. Secondly, a panel for MRSA typing, consisting of 10 samples (2 identical, 3 genetically related and 5 unique MRSA strains) was distributed to 19 laboratories.

Discussion and future perspectives

Chapter 9 provides an overview of diagnostic tools (Matrix assisted laser desorption ionisation-time of flight mass spectrometry [MALDI-TOF MS], electronic nose and Raman spectroscopy) that are currently being developed and implemented for fast and reliable identification or typing of bacteria in clinical laboratories. Furthermore, the most important candidates for multiplex analysis are discussed.

Finally, in **Chapter 10**, the preceding chapters are discussed, accompanied by a perspective on future developments within the field of diagnostic microbiology.

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Chapter 2

Specific diagnostic tests for atypical respiratory tract pathogens

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Abstract

This chapter reviews the microbiological diagnostic tests that are currently available for the detection of *M. pneumoniae*, *C. pneumoniae* and *Legionella* spp., their clinical performance and their future in the clinical microbiology laboratory. When implementing a strategy, a balance between performance criteria (sensitivity, specificity) and convenience criteria (clinical utility, turn-around time and costs) will have to be defined. In the end, this should result in the optimization of clinical patient management.

Introduction

The term "atypical pneumonia" most commonly refers to pneumonia caused by *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae* or *Legionella* species. Although *Bordetella pertussis/parapertussis*, *Franscissella tularensis*, and several respiratory parasites, fungi and viruses are also part of the spectrum of causative agents, these organisms will not be discussed further in this chapter. *C. pneumoniae*, *M. pneumoniae* and *Legionella* spp. are increasingly recognized as frequent and important pathogens in (acute) respiratory tract infections (RTI), such as community-acquired pneumonia and exacerbations of chronic bronchitis (1-2).

For the detection of these pathogens, serology is still considered as the gold standard (2). However, serological results are often unreliable and usually available too late to have an impact on patient management. Optimal serological testing for individual patient care depends on the age of the patient, timing of serum collection, whether paired (acute and convalescent) sera are obtained, availability of appropriate equipment and experience of the laboratory personnel. The latest developments in diagnostic strategies include the application of Nucleic Acid Amplification Techniques (NAATs), such as Nucleic Acid Sequence Based Amplification (NASBA), (real-time) Polymerase Chain Reaction (PCR), Strand Displacement Amplification (SDA), Multi Ligation-dependent Probe Amplification (MLPA) and others in the detection of an extended number of agents responsible for RTI. Advantages of real-time PCR over traditional PCR include a more rapid turn-around time and the complete lack of post-amplification analysis. Results can be obtained within the same day of specimen receipt, allowing appropriate focusing of therapy and reduction of unnecessary antibiotic therapy in RTI (3-4).

Viruses cause a large proportion of RTI and are responsible for extensive morbidity and mortality. The availability and use of these new molecular diagnostic tools in virology has contributed tremendously to a better understanding of the viral etiology of RTI. This, however, depends on the populations studied, the geographical location and seasonality. Next to the increasing importance of viral agents, the role of the bacterial pathogens *C. pneumoniae*, *M. pneumoniae* and *Legionella* spp. in RTI is becoming more clear (5-7). Novel diagnostic tests are more frequently presented and these also cover bacterial agents of RTI (8).

The overall diagnostic approaches for these bacterial pathogens are depicted in **Table 1** and will be described in separate sections in this chapter. The quality of different types of specimens commonly collected to detect pathogens causing RTI and different processing routes, have been compared and are important features in the diagnostic setting. These will also be discussed in forthcoming sections.

Depending on the technological and financial possibilities, a microbiological laboratory should optimize its diagnostic strategy by applying a combination of classical and/or real-time amplification tests for the detection of viruses and the atypical bacterial agents. When implementing such a strategy, a balance between performance criteria (sensitivity, speci-

Table 1. Diagnostic approaches for the detection of bacterial atypical RTI pathogens.

Pathogen	Clinical sample	"New" test	Gold standard
Legionella spp.	Urine	Antigen test	
	Respiratory samples	NAAT	Culture
	Serum		IgG/IgM
C. pneumoniae	Respiratory samples	NAAT	Culture
	Serum		IgG/IgM
M. pneumoniae	Respiratory samples	NAAT	Culture
	Serum		IgG/IgM

ficity) and convenience criteria (clinical utility, turn-around time and costs) will have to be defined. In the end, this should result in the optimization of clinical patient management. This chapter reviews the microbiological tests that are currently available for the detection of *M. pneumoniae*, *C. pneumoniae* and *Legionella* spp., their clinical performance and their future in the clinical microbiology laboratory.

Mycoplasma pneumoniae

Historically, serology has been the most common laboratory method for the diagnosis of *M. pneumoniae* infections. An infection is defined as a fourfold increase in antibody titre in acute and convalescent sera and is still considered the "gold standard" for reliably defining infection (9). Although culture and PCR are also used, non-clinical persistence of the viable organism or dead cells for variable periods of time following acute infection remains a challenge in the assessment of the significance of a positive culture or PCR assay result.

Culture

The time required for microbiological culturing, ranging from a few days to 3 weeks, renders cultivation impractical for patient management. Hence, culture expertise is not widely available, except in specialized research and reference laboratories.

Serological testing

M. pneumoniae has both lipid and protein antigens which can induce antibody responses that can be detected after about one week of infection, peaking at 3-6 weeks, followed by a gradual decline. This response facilitates several different types of serological assays, based on these different antigens and technologies. Serology is a useful epidemiologic tool in circumstances where the likelihood of RTI caused by *M. pneumoniae* is high, but it is not optimally suited for direct management of individual patients. Its main disadvantage is the

need for both acute and convalescent paired sera collected 2-4 weeks apart from each other that should be tested simultaneously for IgM and IgG to confirm sero-conversion indicative for infection. In a recent study, it was demonstrated that the percentage of persons with acute infection who demonstrate a positive IgG response in the acute phase was <50% (10). This low sensitivity could be explained by the presence of specific IgG from previous infections or slow IgG increase in the individuals tested. However, when convalescent sera were tested, the number of IgG positive specimens increased to 82%. A single measurement of IgM might indicate an acute infection if the test is performed after at least 7 days after onset, but the test could be false-negative if the test is performed before. This same study revealed that only 14 out of 27 (52%) of acute-phase sera tested positive by various IgM assays. However, this number increased to 39 (88%) when convalescent sera were tested. IgM antibodies can persist for several weeks to months. Another study showed that the IgM ImmunoCard (Meridian Bioscience, Cincinnati, Ohio) had a sensitivity of only 32% for the detection of acute *M. pneumoniae* infection in sero-positive children suffering from pneumonia (11). Again, the sensitivity increased to 89% when paired sera were analyzed. These findings suggest that diagnosis of RTI should not be based on a single IgM measurement in patient's serum, as was suggested in several other studies (12-13). It is important to realize and to emphasize that the antibody response may also be delayed in some infections or can even be absent if the patient is immuno-suppressed or immuno-deficient.

M. pneumoniae is a mucosal pathogen and, for that reason, IgA is produced in an early state of the infection. Measurement of serum IgA either alone or in combination with IgM may therefore be an alternative for the diagnosis of an acute infection. Unfortunately, very few commercial assays include reagents for IgA detection. The limited number of studies that involved detection of IgA, generally documented improved detection of acute infection, especially in adults (14-16). However, one study measured IgG, IgM and IgA antibodies in healthy blood donors and in patients with various infections caused by microorganisms other than *M. pneumoniae*, using various commercial enzyme immuno assays (EIA) (15). It was documented that 23% of the blood donors and 54% of the patients with various non-*Mycoplasma* infections were positive for IgA, raising doubts about its value to accurately diagnose a current *M. pneumoniae* infection. Talkington et al showed that single-use EIAs were better able to identify seropositive samples than several plate-type EIAs (10). However, plate-type EIAs may be more efficient and cost effective in laboratories that need high-throughput.

Molecular testing

Owing to the relative insensitivity and prolonged time needed for the detection of *M. pneumoniae* by culture, the need for paired acute and convalescent sera collected 2-4 weeks apart for optimal serological diagnosis and other problems inherent to serological assays as described before, PCR gained considerable interest very soon after its introduction in the late

80s of the previous century. The first reports of PCR suited for detection of *M. pneumoniae* appeared in 1989 (17-18). Since then, more than 200 papers describing the use of classical PCR for detection of *M. pneumoniae* in human infections were published. Gene targets used in various PCR-protocols for *M. pneumoniae* detection include the ATPase operon, the P1 adhesin, 16S rRNA gene, the elongation factor Tu coding gene (*tuf gene)* and the repetitive element repMP1 (17-21) (see **Table 2**). The sensitivity of PCR is very high, theoretically corresponding to a single organism. However, its major advantage is the exclusive gain of time.

Several real-time PCR assays have also been described (21-27). Comparison of (real-time) PCR with culture and/or serology has yielded various results that are not always concordant. As would be expected, molecular-based assays often demonstrate superior sensitivity for detection of acute infection over serology and culture (13, 23, 28), although this is not always the case (25, 29). Positive PCR results in culture-negative persons without evidence of respiratory tract disease suggest inadequate assay specificity, persistence of the organism after infection or asymptomatic carriage. Quantitative studies may be required before we can draw final conclusions on the usefulness and clinical applicability. Positive PCR results in sero-negative patients could be the result of an inadequate immune response, to earlier successful antibiotic treatment or sampling prior to specific antibody response. Negative PCR results in culture-proven or sero-positive patients might be caused by the presence of inhibitors or technical problems with the assay and its targetgene. If antibiotics have been administered, PCR results may be negative even though serology is positive.

Because *M. pneumoniae* is only one of a variety of fastidious and/or slow-growing pathogenic microorganisms responsible for RTI with clinically similar manifestations, there has been considerable interest and effort to develop multiplex PCR assays for single step detec-

Table 2. Original references on NAAT for atypical RTI pathogens.

Gene target	Authors	Reference
M. pneumoniae		
ATPase operon	Bernet et al	(18)
P1 adhesin	Jensen et al	(17)
16S rDNA	van Kuppeveld et al	(19)
tuf	Luneberg et al	(20)
repMP1	Dumke et al	(21)
Legionella spp.		
16S rDNA	Jonas et al	(46)
23S-5S	Herpers et al	(47)
5S rDNA	Kessler et al	(48)
mip	Lindsay et al	(49)
C. pneumoniae		
ompA	Kaltenboeck et al	(50)
pstI	Campbell et al	(51)
pmp4	Mygind et al	(52)
16S rDNA	Gaydos et al	(53)

tion of multiple causative agents. Most assays now include gene targets for *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila* and, occasionally, other organisms (26, 30-40). Still, monoplex assays seem to have higher sensitivity and specificity than multiplex assays (41).

NASBA, which is a technique based on isothermal RNA amplification, has also been applied for the detection of *M. pneumoniae* in clinical samples (42). Initial studies have shown that the performance of NASBA is comparable to PCR in terms of sensitivity. A multiplex NASBA assay targeting *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* has also been described (43). Other techniques such as multiplex reverse transcription PCR have been described recently (44) and will be developed further over the years to come (45).

In 2002 and 2004 the diagnostic performance of laboratories for the molecular detection of *M. pneumoniae* was investigated (41). For these two quality control exercises with a 2-year interval, specimens were spiked with *M. pneumoniae*. In 2002, only 2 out of 12 participants obtained 100% correct results, 2 out of 12 produced false-positive results and 10 out of 12 had between 0 out of 9 and 8 out of 9 correct positive results. In 2004, correct results were obtained in 15 out of 18 participating laboratories and no false-positive results were reported. Multiplex PCR and NASBA formats scored less samples positive than the monoplex reactions. This shows that the quality of the molecular tests is improving and that one should carefully consider whether single tests or multiplex tests will be used.

Diagnostic efficacy in different clinical samples

Clinical samples suitable for *M. pneumoniae* PCR include nasopharyngeal and oropharyngeal secretions, sputa, bronchoalveolar lavages (BALs) and throat swabs. Many patients with *M. pneumoniae* infection do not produce significant amounts of sputum; especially children fail to do so. Michelow et al evaluated nasopharyngeal and oropharyngeal samples obtained from children with serologically proven *M. pneumoniae* pneumonia and reported that either specimen type was equally effective for bacterial detection by PCR (29). Still, combining results from both clinical sites provided the most significant diagnostic yield. One group of investigators found sputa to be superior sources over nasopharyngeal aspirates and throat swabs in young adults with serologically proven *M. pneumoniae* infection (46). However, others found no difference in the detection efficiency of *M. pneumoniae* using PCR in the various anatomic sites (47). Gnarpe et al compared nasopharyngeal swabs and oropharyngeal swabs for the detection of *M. pneumoniae* by PCR (48). A total of 7 patients were sero-positive for *M. pneumoniae* and, of these, 6 were positive from oropharyngeal swabs and only 2 were positive from nasopharyngeal swabs. Another study compared nasopharyngeal swabs to oropharyngeal swabs in children and found no significant difference in the detection of *M. pneumoniae* by PCR (47). The authors did note that nasopharyngeal swabs were more likely to be problematic than oropharyngeal swabs because of the presence of PCR inhibitors or lack of respiratory epithelial material. Honda et al applied capillary PCR to sputum, BALs

and oropharyngeal swabs (49). The highest rate of detection was shown for oropharyngeal swabs.

Loens et al combined the results of two studies on LRTIs. From 25 patients both an oropharyngeal swab and a sputum were available for NAAT analysis and culture (43, 50). In both studies, sputa were the preferred specimens. Räty and co-workers collected sputum, a nasopharyngeal aspirate and an oropharyngeal swab from 32 young military employees suffering from pneumonia during an *M. pneumoniae* outbreak and applied PCR (46). This study also concluded that sputum is the best sample to detect *M. pneumoniae*. Dorigo-Zetsma et al confirmed this finding (51). Care should be taken when applying NAAT to sputum samples since inhibitors occur frequently in sputum and these may be difficult to eliminate (52).

In conclusion, if a sputum sample is available, this might be the most optimal specimen for *M. pneumoniae* detection by culture and NAAT. A nasopharyngeal swab, nasopharyngeal aspirate or oropharyngeal swab might be the second best option for analysis by NAAT.

***Legionella* spp**

Legionella pneumophila was identified as an important causative agent of severe RTI. The first cases of Legionnaires' disease or Legionellosis were discovered among attendants of an American Legion convention in Philadelphia in 1976 (53). Pontiac fever is caused by the same bacterium. This produces milder respiratory illness without pneumonia.

Legionella spp. are water borne bacterial species that can be found in tap water to which the first outbreak was attributed, but also in water from cooling towers, air-conditioning, ventilators and humidifiers (54).

Culture

Culture-based isolation of *Legionella* spp. from body fluids is still considered the gold standard for the diagnosis of Legionellosis. Culture requires special enriched media, adequate processing of specimens and technical expertise. Several days are required to obtain a positive result, with most *Legionella* spp. colonies being detected within 4-7 days. *Legionella* species other than *L. pneumophila* may grow at a slower rate and may, therefore, be detectable only after 10 days of incubation (55-56). Some *Legionella* spp. have unusual colony morphology and may easily be overlooked. The standard medium to culture *Legionella* is buffered charcoal yeast extract (BCYE) agar supplemented with α-ketoglutarate, with or without selective antimicrobial agents. The antibiotics most commonly added are polymyxin to control commensal flora, anisomycin against yeasts and cefamandole or vancomycine against Gram-positive bacteria (57).

Serological testing

Indirect immunofluoresence assay (IFA) was used to detect antibodies in patients from the Philadelphia outbreak which turned out to be instrumental in determining the cause of the illness. Since then, a number of serological tests has been developed and evaluated (58-68). Of the various antibody detection methods that are available, IFA and enzyme-linked immunosorbent assays (ELISA) are the most commonly used. Nowadays, many laboratories prefer ELISA assays over IFA testing, because they are less subjective, more accurate than IFA testing and have the potential for automated high-throughput performance. The reported sensitivities vary from 41% to 94% (56). A recent study showed sensitivities of 64%, 61% and 44% for ELISA, IFA and rapid microagglutination assays, respectively (69). In the ELISA assay, half of the patients showed a seroconversion in IgM and the other half showed a seroconversion in IgG. Other studies have shown that the early immune respons primarily involves IgM and that IgM tests must be included for optimal sensitivity (70-73). Seroconversion may take several weeks, which is a major limitation of serological testing. In most cases, a fourfold increase in antibody titre is detected within 3-4 weeks, but in some cases this may take more than 10 weeks (74). Obviously, this severely compromises the timeline of serological testing. Acute-phase IFA antibody titres of ≥256 during pneumonia were once considered sufficient for a presumptive diagnosis, but this has been shown to be unreliable, given the high prevalence of *Legionella* antibody positivity in persons without clinical evidence for Legionellosis (75). Another disadvantage of serological testing is its incapability to accurately detect all *Legionella* species and serogroups. In addition, a diagnosis by a fourfold IgG or IgM titre increase can only be made retrospectively and cannot support patient management. Therefore, there is a urgent need for additional tests for *Legionella* diagnosis in the early stage of disease.

Antigen detection

The detection of *Legionella* antigen in urine was developed shortly after the first outbreak in Philadelphia (76). *Legionella* antigen can be detected in urine as early as 24 hours after onset of symptoms and antigen shedding persists for days to weeks. The detected antigen is a component of the lipopolysaccharide portion of the *Legionella* cell wall (77-78). The urinary antigen tests combine reasonable sensitivity (80-95%) and high specificity (95-100%) with very rapid results (79-80). Nowadays, it is the most commonly used laboratory test for *Legionella* diagnosis (81-82). In Europe, the proportion of cases diagnosed by the urinary antigen detection has increased rapidly from 15% in 1995, 33% in 1998 and 74% in 2004 to more than 90% in 2006 (83).

Commercial kits that use both radioimmunoassay (RAI) and EIA methodologies have been available for several years and have similar performance characteristics (55). Agglutination assays have also been introduced, but these have not yet provided an acceptable level of

sensitivity and specificity (84). In addition, immunochromatographic assays (IA) have been developed that have similar sensitivity and specificity as compared to EIA (85). The majority of IA is most sensitive for the detection of the Pontiac monoclonal antibody type of *L. pneumophila* serogroup 1 (up to 90%), less sensitive for other monoclonal antibody types of *L. pneumophila* serogroup 1 (to 60%) and poorly sensitive (to 5%) for other *L. pneumophila* serogroups and other *Legionella* species (86-87). An important feature of these assays is its high specificity (>99%).

The sensitivity of urinary antigen detection appears to be associated with the clinical severity of the disease (88). Yzerman et al tested two enzyme immunoassays, Binax *Legionella* Urinary Antigen EIA (Binax, Portland, Maine) and Biotest *Legionella* Urin Antigen EIA (Biotest, Dreieich, Germany) and one immunochromatographic assay, Binax NOW Urinary Antigen Test (Binax, Portland, Maine), using urine samples from outbreak related Legionellosis patients. For patients with mild Legionellosis, the test sensitivities ranged from 40-53%, whereas for patients with severe Legionellosis, the sensitivities reached 88-100%. These findings have implications for the diagnostic process in patients with mild pneumonia and suggest that patients with mild pneumonia may be underdiagnosed if only the urine antigen test is used. The use of concentrated urine samples increased sensitivity without decreasing the specificity. Since this concentration step is timely and laborious, some laboratories only use this approach in case of equivocal results or strong clinical indication.

Another association between test sensitivity and certain defined subpopulations has been described by Helbig et al (85). The clinical utility of *Legionella* urinary antigen assays for the diagnosis of Legionnair's disease has been assessed by using samples from 317 culture-proven cases. The sensitivities of the Binax EIA and Biotest EIA urinary tests were found to be 94% and 94% for travel-associated infection and 87% and 76% for community-acquired infection, but only 44% and 46% for hospital-acquired infection.

Several new immunochromatographic urinary antigen tests for the detection of *L. pneumophila* serogroup 1 have been developed (79, 89). The Binax NOW urinary antigen test, in concordance with the findings of previous studies, has excellent sensitivity and specificity. The performance of some new tests is below the acceptable level for diagnostic assays (**Table 3**) (89).

Molecular testing

The first assay designed to detect DNA of *L. pneumophila*, was based on RIA in which a radiolabeled ribosomal probe specific for all strains of *Legionella* spp. was applied. Researchers reported a varying sensitivity and specificity for this assay (90-91). The use of this probe for the detection of Legionellosis at one hospital resulted in 13 false-positive cases and the assay was removed from the market soon after it falsely recorded this pseudo outbreak (92).

Table 3. Performance of urinary antigen detection tests.

Urinary antigen test	Sensitivity		Specificity	Reference
	NCU	CU		
SAS Legionella Test (SA Scientific, San Antonio, Texas)	82,9%	NT	99,0%	(87)
Binax NOW Urinary Antigen Test (Binax, Portland, Maine)	91,4%	NT	100,0%	(87)
Binax Legionella Urinary Antigen EIA (Binax, Portland, Maine)	97,1%	NT	NT	(88)
Biotest Legionella Urin Antigen EIA (Biotest, Dreieich, Germany)	91,4%	NT	NT	(88)
Binax NOW Urinary Antigen Test (Binax, Portland, Maine)	94,3%	NT	NT	(88)
Binax Legionella Urinary Antigen EIA (Binax, Portland, Maine)	86,5%	NT	NT	(93)
Biotest Legionella Urin Antigen EIA (Biotest, Dreieich, Germany)	76,0%	NT	NT	(93)
Biotest EIA (Biotest, Dreieich, Germany)	94,6%	NT	100,0%	(94)
Bartels EIA (Bartels Inc. Trinity Biotech Company, Wicklow, Ireland)	74,1%	91,5%	100,0%	(95)
Biotest Legionella Urin Antigen EIA (Biotest, Dreieich, Germany)	51,7%	91,5%	100,0%	(95)
Biotest Legionella Urin Antigen EIA (Biotest, Dreieich, Germany)	71,0%	74,0%	NT	(96)
Binax Legionella Urinary Antigen EIA (Binax, Portland, Maine)	69,0%	79,0%	NT	(96)
Binax NOW Urinary Antigen Test (Binax, Portland, Maine)	72,0%	81,0%	NT	(96)
Rapid U Legionella Antigen Test (Diamondial, Sees, France)	71,2%	NT	96,6%	(97)
SD Bioline Legionella Urinary Antigen Test (Standard Diagnostics Inc., Kyonggi-do, Korea)	31,5%	NT	98,9%	(97)
Binax NOW Urinary Antigen Test (Binax, Portland, Maine)	91,8%	NT	100,0%	(97)

NCU: non-concentrated urine

CU: concentrated urine

NT: not tested

Legionella PCR is available in an increasing number of laboratories that use a variety of in-house or commercial assays. Diagnostic PCR assays target specific *Legionella pneumophila* DNA regions within 16S rRNA genes (93-100), the 23S-5S spacer (101), 5S rDNA (102-103) or the macrophage inhibitor potentiator (*mip*) gene (104-107). The application of PCR to non-respiratory samples seems particularly attractive for patients who do not produce sputum. *Legionella* DNA can be detected in urine, serum and leukocyte samples obtained from patients with Legionellosis, with sensitivities ranging from 30% to 86% (102, 108-110). The sensitivity of the detection of *Legionella* DNA in serum is relatively low (50-60%) in Legionellosis patients, but was shown to be higher (70-90%) in patients with more severe disease (108-109). When testing samples from the lower respiratory tract (bronchoalveolar lavage), PCR has repeatedly shown to have a sensitivity equal to or higher than culture (99-100, 111). Indeed, PCR has been considered by some authors to be the test-of-choice for patients who produce sputum (111). However, a number of false positive results has been reported, both with commercially available tests and with in-house tests (55, 99). A problem with the interpretation of these false positive results is the question whether these are truly false positive or whether it was the reference method that failed. It is difficult to solve this issue and, at present, there is only one study available were the authors have determined the exact sensitivity and specificity of *Legionella* PCR in patients with pneumonia of unknown aetiology (112). Diederen et al demonstrated that variation of DNA targets influences the test

performance. PCR designed on the detection of 16S RNA had a sensitivity and specificity of 86% and 95%, as for PCR designed to detect the *mip* gene, this was 92% and 98%, respectively. In conclusion, laboratory workers and clinicians must be cautious when interpreting results and should not hesitate to question the results, especially when these results are unexpected based on clinical presentation and local epidemiology. Only one study describes a multicenter comparison of molecular methods for detection of *Legionella* spp. (113). The authors compared the methods of 9 laboratories for 12 sputum samples with *L. pneumophila* or *Legionella longbeachae* and conclude that PCR targeting the *mip* gene is *L. pneumophila* specific and 16S rRNA gene amplification is genus-specific.

Relevance of sample type

Legionella spp. can be isolated from a variety of sample types, although sputum and bronchoalveolar lavage are the samples of choice. Sputum samples are generally considered to be optimal for isolation of *L. pneumophila* in patients with RTI. Culture results depend on the severity of illness, with the lowest result (15-25%) for mild pneumonia and the highest result (>90%) for severe pneumonia (111). A major limitation of culturing the pathogen from sputum is the fact that less than 50% of *Legionella* patients produce sufficient amounts of sputum (111, 114). Most patients with Legionellosis produce non-purulent sputum. Obviously, laboratories that reject sputum samples containing limited mucosal polymorphonuclear leukocytes may reject potential positive samples. Ingram and Plouffe demonstrated that up to 84% of *L. pneumophila* positive samples would have been discarded by using sputum purulence screens and they recommended acceptance of all aspects of sputum suspected for *Legionella* culture (115). Estimated sensitivities of sputum culture range from <10% to 80% and vary between individual laboratories (55, 111).

In conclusion, the urinary antigen detection is currently the most helpful rapid test for the diagnosis of *Legionella* infection. The use of the rapid urinary antigen tests reduces mortality and avoids unnecessary or inappropriate use of antibiotics in patients with CAP. However, combining test results from more than one sampling site appears to improve the diagnostic accuracy. Especially in this diagnostic segment, molecular testing may play an important (future) role.

Chlamydophila pneumoniae

Culture

For *C. pneumoniae*, culture on cell lines has traditionally been considered as a reference and standard diagnostic method. However, due to the important limitations in the cultivation of *C. pneumoniae* (technical complexity, limited viability of the bacteria, slow growth and variable diagnostic success) performance of culture remains restricted to specialized laboratories. Hence, the use of culture as a diagnostic tool is suboptimal and not often recommended. Still, the most common method for diagnosis of *C. pneumoniae* infection is serology. Assays available for detection of *C. pneumoniae* specific antibodies include Micro Immuno Fluorescence (MIF) tests, ELISAs and EIAs, each of which exists in a variety of in-house and commercial variations.

Recommendations by the Centre for Disease Control (CDC) and the practical guidelines of the Infectious Disease Society of America (IDSA) defined the main criteria for the diagnosis of acute *C. pneumoniae* infection as a single IgM titre of ≥1:64 or a fourfold increase in the IgG titre in acute serum and convalescent serum, measured 4 weeks apart from each other. The use of single IgG or IgA titres is discouraged because of the relatively high overall seroprevalence in healthy populations (116). However, several studies deviated widely from these guidelines and there are several inherent limitations to the serodiagnosis of *C. pneumoniae* infection (117-121).

Serological testing

Serological testing at best offers a retrospective diagnosis. In primary infections, IgM antibodies appear 2-3 weeks and IgG antibodies appear 6-8 weeks after infection, whereas in reinfections, IgM may be absent or of low titre and IgG appears earlier, within 2-3 weeks after infection.

The MIF assay has been repeatedly demonstrated to be insensitive and it showed a poor correlation with the detection of *C. pneumoniae* by culture or PCR, particularly in children. Only 1%-3% of culture positive children in a study by Hammerschlag met the serological criteria for acute infection (122). Wellinghausen et al. reported that 17 patients with CAP, had seronegative results with MIF (119).

Generally, the specificity of serological testing may be suboptimal as well. Serological evidence of acute infection was found in 19% of healthy adults who had negative results by culture and PCR (122). This lack of specificity may result from serological cross-reactivity with other *Chlamydia* species, as well as *Mycoplasma* spp. or *Bartonella* spp. In addition, other limitations of the MIF test relate to a lack of standardized reagents, technical complexity and subjective endpoint determination, all of which result in significant intra- and interlaboratory variation of test performance. One study evaluated the interlaboratory reliability of the MIF

test for measurement of *C. pneumoniae* specific IgA and IgG titres for 392 serum samples, using reagents and antigens obtained from a common source. The investigators observed agreement between IgA and IgG titres to be as low as 55% and 38%, respectively (123).

EIAs may overcome some of the limitations of the MIF test by being more objective in the interpretation of the results and less technically demanding. Hermann et al. compared 7 commercial EIAs or ELISAs with 4 MIF assays for detection of specific IgG antibodies (124). The authors used serum samples from 80 healthy subjects and reported sensitivities and specificities ranging from 42% to 100% and from 88% to 100%, respectively. The SeroCP ELISA (Savyon Diagnostic, Ashdod, Israel) and Quant EIA (Savyon, Ashdod, Israel) showed the best sensitivities, 96% and 92%, respectively, followed by the Vircell ELISA (Viva Diagnostica, Köln-Hürth, Germany) and the Labsystems EIA (Labsystems, Helsinki, Finland), which each had a sensitivity of about 75%.

Molecular testing

For reasons of the above-mentioned issues with culture and serology, PCR can be an interesting alternative for diagnosis of *C. pneumoniae* infections. The first reports of PCR application for the detection of *C. pneumoniae* appeared in 1990 and 1992 (125-126). Since then, more than 250 studies describing the use of PCR for detection of *C. pneumoniae* in human infections have been published. Gene targets used in various types of PCR for *C. pneumoniae* include the *ompA* gene (127), *pstI* (126), *pmp4* (128) and 16S rDNA (129). Real-time PCR assays have also been described (128, 130-134). Multicenter studies that use a large and diverse repertoire of clinical specimens and compare data independently are likely to provide important insights into the performance of new assays. Two such studies describing multicenter comparisons of the performance of various NAAT test for detection of *C. pneumoniae* in respiratory specimens have been published. Both studies revealed significant variations of test performance from laboratory to laboratory. Cherneskey et al compared a *C. pneumoniae* PCR kit from Abbot (Abbott Laboratories, North Chicago, Illinois) with 5 conventional PCR assays, using specimens spiked with pre-extracted DNA (135). Loens et al used spiked respiratory specimens to compare the performance of several in-house PCR assays (41). Correct results were produced in 12 out of 16 and 13 out of 18 tests in 2002 and 2004, respectively. Both of these studies revealed significant intercenter discordance of detection rates, using different or even the same tests. Both multiplex PCR and NASBA formats scored a smaller number of positive samples than the monoplex tests.

Relevance of sample type

The choice of a specimen from the respiratory tract has an impact on the sensitivity of *C. pneumoniae* isolation and detection by culture and PCR. In a study in which the authors

enrolled 260 previously healthy children (3-12 years), it was shown that the nasopharynx might be superior to the throat as a source of materials to be used for isolation of *C. pneumoniae*. Of 34 children from whom *C. pneumoniae* was isolated, nasopharyngeal swabs were positive for all children, but oropharyngeal swabs were positive for only 50% of the same group (136). During a *C. pneumoniae* outbreak, Boman et al collected sputum, oropharyngeal swabs and nasopharyngeal swabs from 116 patients presenting with RTI (137). When the authors compared the performances of PCR, culture and antigen detection for samples from three different niches in 61 patients for whom all samples were available, 20 patients were positive for *C. pneumoniae*, for whom 7 nasopharyngeal swabs, 10 oropharyngeal swabs and 20 sputum samples were considered to be true positives. Sensitivities of PCR, culture and antigen detection by EIA for sputum samples were 95%, 100% and 80%, respectively. Sensitivities for the other types of sampling sites were much lower (25%-50%). The clinical relevance of sputum for the detection of *C. pneumoniae* was confirmed by Kuoppa et al (131). In this study, a sputum sample, a nasopharynx aspirate and an oropharyngeal swab from 35 patients suspected of having a *C. pneumoniae* infection were examined by PCR. The majority of all samples had *C. pneumoniae* DNA copies below $1x10^4$ genome copies per ml, but the majority of the sputum samples contained higher inocula of *C. pneumoniae* DNA, with an average of $8.6x10^5$ copies/ml. However, these results are in contrast with those obtained by Verkooyen et al, who examined sputum, nasopharyngeal swabs, oropharyngeal swabs and throat wash specimens from 156 hospitalized CAP patients by PCR and culture (138). The highest sensitivity in this study was obtained by applying PCR on nasopharyngeal swabs (51.3%). Surprisingly, none of the sputum samples tested positive.

Gnarpe et al compared PCR results for *C. pneumoniae* from nasopharyngeal swabs and oropharyngeal swabs in 66 patients presenting with RTI (48). Of a total of 18 patients positive for *C. pneumoniae*, in 15 patients the oropharyngeal swab was the only positive specimen, whereas for 3 patients both the oropharyngeal swab and the nasopharyngeal swab yielded a positive PCR result.

In conclusion, sputum may be the preferred specimen for detection of *C. pneumoniae* by NAAT.

Concluding remarks

Historically, atypical agents of RTI were merely detected on the basis of microbiological culture. Without exception, sensitivity and specificity of such cultures did not meet criteria of excellence. Hence, a variety of antigen- or antibody mediated tests was developed. Unfortunately, none of these individual tests was in itself sufficient to reliably identify the causative infectious agents. Also the more recent availability of a variety of NAATs did not (yet) solve this problem. In today's clinical microbiology laboratories no widely accepted gold standard

for the detection of *M. pneumoniae*, *C. pneumoniae* and *Legionella* spp. is available. Technicians and physicians have to rely on a combination of test results that, together with clinical presentation of the patient, may lead to a presumptive identification of a causative agent at best. Although great diagnostic improvements have been made over the last twenty years, no final tool is as yet available. Future biomarker discovery is still required before the ultimate test for the diagnosis of atypical RTI agents will become available.

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Biomarkers

Chapter 3.1

Neopterin and procalcitonin are suitable biomarkers for exclusion of severe Plasmodium falciparum disease at the initial clinical assessment of travellers with imported malaria

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Abstract

Most clinicians in developed, non-malaria endemic countries have limited or no experience in making clinical assessments of malaria disease severity and subsequent decisions regarding the need for parenteral therapy or high-level monitoring in febrile patients with imported malaria. In the present study, the diagnostic accuracy of plasma soluble Triggering Receptor Expressed on Myeloid cells 1 (sTREM-1), neopterin and procalcitonin levels as biomarkers for severe *Plasmodium falciparum* disease was evaluated in 104 travellers with imported malaria (26 patients with non-*P. falciparum* malaria, 64 patients with uncomplicated *P. falciparum* malaria and 14 patients with severe *P. falciparum* malaria).

sTREM-1, neopterin and procalcitonin were determined in serum using commercially available ELISA or EIA tests. The diagnostic performance of these biomarkers for severe disease was compared with plasma lactate, a well-validated parameter for disease severity in patients with malaria, as reference. Severe malaria was defined according to the modified WHO criteria.

No significant differences in sTREM-1 levels were detected between the different patient groups. Patients with severe *P. falciparum* malaria had significantly higher neopterin and procalcitonin levels on admission when compared to patients with uncomplicated *P. falciparum* malaria or non-*P. falciparum* malaria. Receiver Operating Characteristic (ROC) curve analysis showed that neopterin had the highest Area-Under-the-ROC curve (AUROC 0.85) compared with plasma lactate (AUROC 0.80) and procalcitonin (AUROC 0.78). At a cut-off point of 10.0 ng/ml, neopterin had a positive and negative predictive value of 0.38 and 0.98 whereas procalcitonin, at a cut-off point of 0.9 ng/ml, had a positive and negative predictive value of 0.30 and 1.00.

Although the diagnostic value of neopterin and procalcitonin is limited, the high negative predictive value of both neopterin and procalcitonin may be helpful for a rapid exclusion of severe malaria disease on admission. This may be a valuable tool for physicians only occasionally dealing with ill-returned travellers from malaria-endemic regions and who need to decide on subsequent oral anti-malarial treatment or timely referral to a specialized centre for high-level monitoring and intensified parenteral treatment.

Introduction

Travellers from industrialized countries and inhabitants of malaria-endemic regions clearly represent two distinct worlds of malaria (1). The global burden of malaria is largely carried by the world's malaria-endemic regions with as many as 500 million cases annually and a death toll of 1 to 3 million children each year. Severe malaria in areas of endemicity is associated with a mortality of 15 to 40% (2-3). In many malaria-endemic regions, strict triage for admission to ICU facilities must be applied because the ICU capacity is usually limited. Recently, a 5-point Coma Acidosis Malaria (CAM) score based on only acidosis (base deficit) and cerebral malaria (measured with Glasgow Coma Scale) was introduced, which could identify adult patients with severe malaria who were at high risk of death (4).

In striking contrast, in non-endemic industrialized countries malaria is only seen as an occasionally imported disease (5) and is usually associated with a low case-fatality rate (6-7). Even in the pre-artesunate era, the mortality of severe malaria in non-endemic regions was significantly lower when compared with regions of malaria endemicity (6-8), probably reflecting the availability of adequate supportive care facilities in industrialized countries.

Industrialized countries, however, have to face other –more trivial- problems. For instance, the expertise on diagnosis and treatment of malaria is usually focussed in some specialized hospitals and institutes but many ill-returning travellers may present to non-specialized hospitals or even general practitioners. Making a proper diagnosis of malaria may be troublesome under these circumstances, for instance, by lack of experience in the examination of malaria thick and thin blood smears and in the assessment of parasite load. These non-specialized centres therefore often rely on rapid diagnostic tests for the diagnosis of malaria (9). Although sensitive in diagnosing *P. falciparum* malaria, these rapid tests do not provide any information about the severity of the infection. Moreover, although artesunate, which is now considered the parenteral drug of choice for treatment of severe falciparum malaria, is available as an orphan drug in The Netherlands, it is currently only in stock in some specialized centres but certainly not available in every Dutch hospital. Some of these general hospitals do not even have any drug in stock for the treatment of malaria (10). To prevent unnecessary delay in diagnosis of severe malaria and institution of proper parenteral treatment, a simple, well-validated, laboratory-based biomarker that predicts or excludes severe disease accurately would be of great help for those clinicians occasionally dealing with febrile travellers returning from malaria endemic regions. These clinicians have to decide on subsequent oral anti-malarial treatment or a timely referral to a specialized centre for high-level monitoring and intensified parenteral treatment. In the present study, the diagnostic accuracy of plasma soluble Triggering Receptor Expressed on Myeloid cells 1 (sTREM-1), neopterin and procalcitonin was evaluated as potential markers for malaria disease severity in travellers with imported malaria. These bio-substances are all involved in the systemic pro-inflammatory response of the host to invading pathogens. Some of these biomarkers

are already in use for the diagnosis and follow-up of sepsis or used in treatment algorithms, resulting in a successful reduction of antibiotic use and duration (11-12).

Materials and Methods

Study population

The Harbour Hospital is a 161-bed general hospital located in Rotterdam. It also harbours the Institute for Tropical Diseases, which serves as a national reference centre. In the period 1999-2008 almost 500 cases of imported malaria were diagnosed (13). For the majority of these cases, demographic, clinical and laboratory data and serum samples were available. For the present study, a representative sample of this cohort was taken and analysed.

Definitions

Patients were classified as having severe *P. falciparum* malaria if they met one or more of the WHO criteria for severe malaria, as modified by Hien *et al* (14):

- A score on the Glasgow Coma Scale (GCS) of less than 11 (indicating cerebral malaria).
- Anaemia (haematocrit <20%) with parasite counts exceeding 100,000/µl (roughly corresponding to 2% parasitaemia) on a peripheral blood smear.
- Jaundice (serum bilirubin >50 µmol/l) with parasite counts exceeding 100.000/µl on a peripheral blood smear.
- Renal impairment (urine output <400 ml/24 h and serum creatinine >250 µmol/l).
- Hypoglycaemia (blood glucose <2.2 mmol/l).
- Hyperparasitaemia (>10% parasitaemia).
- Systolic blood pressure <80 mm Hg with cold extremities (indicating shock).

Study design

In previous studies (6, 13, 15) these severity criteria were also used to define severe malaria in non-immune travellers. In the present study the occurrence of severe malaria was considered a primary end-point. This contrasts with the design of many studies in patients with severe malaria in regions of malaria endemicity where the severity criteria are used as an entry criterion. In the present study, plasma lactate was used as a surrogate parameter for acid-base disbalance and reference biomarker. It was evaluated in a previous study in non-immune travellers with imported malaria (15). The diagnostic performance of sTREM-1, procalcitonin and neopterin for malaria disease severity was compared with that of plasma lactate, which is routinely measured at the Institute for Tropical Diseases in ill-returning travellers.

Procedures

On admission, blood samples were taken for analysis of the red blood cell count, haematocrit, white blood cell count, platelet count, serum electrolytes, total bilirubin, serum creatinine, liver enzymes, and blood glucose. In addition, a serum sample was taken on admission which was stored at -20°C until analysis. For the determination of plasma lactate, a separate blood sample was drawn on admission without congestion and placed on melting ice after which it was immediately analysed after isolation of plasma. Malaria was diagnosed by QBC (Quantitative Buffy Coat) analysis, by a rapid diagnostic antigen test for malaria (Binax NOW® Malaria Test, Binax Inc., Maine, USA) and by conventional microscopy of stained thick and thin blood smears. In case of *P. falciparum* infections, parasite density was determined. When the parasitaemia was less than 0.5% infected erythrocytes, parasites were counted per 100 leucocytes in thick smears. When the parasitaemia was equal or higher than 0.5% infected erythrocytes, infected erythrocytes were counted in thin blood smear and expressed as a percentage of the total erythrocytes. The number of parasites per microliter was subsequently calculated from these data.

sTREM-1 and neopterin levels were determined in serum samples using commercially available ELISA tests (R&D Systems, Abingdon, UK; DRG, Marburg, Germany, respectively). Procalcitonin levels in serum samples were determined using a commercially available EIA test (VIDAS BRAHMS Procalcitonin, bioMérieux, Lyon, France). All tests were performed according to manufacturer's instructions. Detection limits were 3.88 pg/ml for sTREM-1, 0.2 ng/ml for neopterin and 0.05 ng/ml for procalcitonin, respectively. According to the manufacturers, normal serum values are <100 pg/ml for TREM-1, <3 ng/ml for neopterin and <0.1 ng/ml for procalcitonin.

Statistical methods

For comparison between groups, the Mann-Whitney U-test was used and p-values of <0.05 were considered statistically significant. The diagnostic performance of each biomarker was reported as sensitivity, specificity, positive and negative predictive value for severe *P. falciparum* malaria and their corresponding 95% confidence intervals. Of each test a Receiver Operating Characteristic (ROC) curve, a graphical plot of sensitivity (true positive rate) versus 1-specificity (false positive rate), was constructed as a summary statistic and the area under the ROC curve (AUROC) and its corresponding 95% confidence intervals were calculated. Youden's index J (J=sensitivity+specificity-1) was used to choose the most appropriate cut-off point for each biomarker. All statistical analyses were performed using SPSS 15.0.

Results

Patient characteristics

In total 104 travellers with imported malaria were included in this study, of which 26 patients were diagnosed with a non-*P. falciparum* infection (*Plasmodium malariae* n=2; *Plasmodium ovale* n=5; *Plasmodium vivax* n=19) and 78 patients were diagnosed with *P. falciparum* infection. The general characteristics of all patients are shown in **Table 1**.

Table 1. General characteristics and laboratory results on admission of patients with various species of malaria. Data are given as median (range).

	Non-P. falciparum	**P. falciparum**	
		Uncomplicated	Severe
	(n=26)	(n=64)	(n=14)
Demographics			
Male/female	20/6	51/13	6/8
Age, years	40 (17-62)	40 (11-67)	40 (26-57)
Continent of acquisition			
Africa	12 (46%)	60 (94%)	12 (86%)
Asia	9 (35%)	3 (5%)	1 (7%)
South America	5 (19%)	1 (2%)	1 (7%)
Vital signs on admission			
Body temperature, °C	38.8 (36.1-41.5)	38.7 (36.1-40.6)	38.8 (36.8-40.6)
Pulse rate, beats per minute	90 (60–130)	95 (68-120)	108 (78-140)
Systolic blood pressure, mm Hg	123 (100-196)	120 (95-185)	118 (80-160)
Laboratory data on admission			
Parasite load, throphozoites/µl	ND	5,502 (1.0-385,000) *	205,600 (80,500-860,000)
Plasma lactate, mmol/l	1.4 (0.7-3.0) *	1.5 (0.5-4.4) *	2.6 (0.9-5.8)
Haemoglobin, mmol/l	8.2 (6.1-10.1)	8.7 (5.3-11.1) *	7.6 (3.8-10.2)
Leucocytes, x 10^9/l	5.2 (1.9-9.3)	5.5 (1.8-11.3)	6.6 (3.2-18.5)
Platelets, x 10^9/l	93.0 (10.0-205.0) *	78.5 (16.0-247.0) *	27.0 (3.0-152.0)
C-reactive protein, mg/l	86.5 (18.0-208.0) *	109.0 (5.0-278.0) *	190.0 (91.0-265.0)
Serum creatinine, µmol/l	94.0 (66.0-149.0)	103.5 (63.0-208.0)	102.5 (70.0-199.0)
Total bilirubin, µmol/l	24.0 (6.0-84.0) *	25.0 (7.0-164.0) *	54.0 (20.0-269.0)

p-values as compared with severe *P. falciparum* malaria.

* indicates $p<0.01$

ND: not done

Characteristics of patients with severe malaria

Thirteen patients fulfilled the criteria for severe malaria at initial presentation. Another patient did not fulfil these criteria on admission, but the clinical course deteriorated shortly hereafter with impaired consciousness and hyperparasitaemia. Procalcitonin and neopterin levels were already increased on admission in this particular patient. Eventually, at admission to the ICU, all 14 patients fulfilled one or more of the severity criteria (GCS<11, n=1; anaemia

with a parasite count exceeding 100,000 trophozoites per µl, n=2; icterus with a parasite count exceeding 100,000 trophozoites per µl, n=8; acute oliguric renal insufficiency, n=0; hypoglycaemia, n=0; hyperparasitaemia, n=5 and shock, n=1, respectively). Five patients had an impaired conscious level but a GCS above 11; eight patients had a parasitaemia >5%, respectively. The first arterial blood gas analysis on ICU showed a median bicarbonate level of 22 mmol/l (range 17 to 26 mmol/l) and a median base deficit of 2 (range -3 to 8). Median GCS was 15 (range 9 to 15). One patient needed mechanical ventilation. Eleven patients received exchange transfusion as an adjunct therapy. No case fatalities were observed. The laboratory results on admission of travellers with imported severe *P. falciparum* malaria were further characterized by significantly lower platelet counts and haemoglobin levels and by significantly higher plasma lactate, bilirubin and C-reactive protein levels and erythrocyte sedimentation rates, respectively (**Table 1**).

Analysis of biomarkers for severe malaria

sTREM-1

No statistically significant differences were observed in sTREM-1 levels in serum, between patients with severe *P. falciparum* malaria, uncomplicated *P. falciparum* malaria and non-*P. falciparum* malaria (**Figure 1A**).

Neopterin

Neopterin levels on admission were significantly higher in travellers with severe *P. falciparum* malaria when compared to travellers with uncomplicated *P. falciparum* malaria (p<0.0001) and travellers with non-*P. falciparum* malaria (p<0.0001) (Figure 1B). ROC curve analysis showed an AUROC of 0.85 (95% Confidence Interval 0.76-0.94), suggesting a good accuracy (**Figure 2**). As shown in **Table 2**, at a cut-off point of 10.0 ng/ml, neopterin had an excellent sensitivity and negative predictive value but a poor specificity and positive predictive value for severe disease.

Table 2. Descriptive statistics of diagnostic accurary of neopterin, procalcitonin as compared with lactat for the diagnosis of severe falciparum malaria on admission. The Youden's index was used to choose an appropriate cut-off value.

	Neopterin		Procalcitonin		Lactate	
		95% Confidence Interval		95% Confidence Interval		95% Confidence Interval
Optimal cut-offvalue	10 ng/ml		0.9 ng/ml		1.8 mmol/l	
Youden's index	0.60		0.53		0.58	
Sensitivity	0.93	0.64-1.00	1.00	0.70-1.00	0.92	0.60-1.00
Specificity	0.67	0.54-0.78	0.53	0.40-0.66	0.66	0.51-0.78
Positive predictive value	0.38	0.23-0.56	0.30	0.17-0.47	0.39	0.22-0.59
Negative predictive value	0.98	0.86-1.00	1.00	0.87-1.00	0.97	0.83-1.00

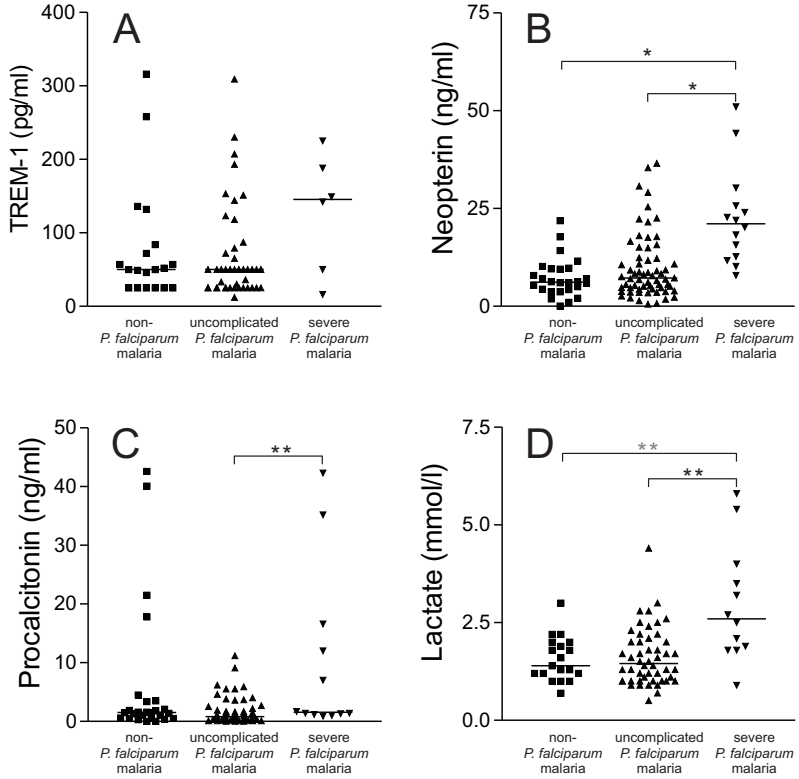

Figure 1. Concentrations of potential biomarkers for disease severity in malaria patients on admission.
Individual data are shown with the median value of each biomarker; TREM-1 (panel A), neopterin (panel B), procalcitonin (panel C) and plasma lactate (panel D). Significant differences in biomarker concentrations between patient groups (black square = non-*P. falciparum* malaria; black triangle up = uncomplicated *P. falciparum* malaria; black triangle down = severe *P. falciparum* malaria) with P values < 0.0001 and < 0.005 are indicated by * and **, respectively.

Procalcitonin

Procalcitonin levels were significantly higher in travellers with severe *P. falciparum* malaria when compared to travellers with uncomplicated *P. falciparum* malaria (p=0.0022). However, no significant differences were noted in comparison to travellers with non-*P. falciparum* infections (p=0.17) (**Figure 1C**). ROC curve analysis showed an AUROC of 0.78 (95% CI 0.66-0.91), compatible with a fair accuracy (**Figure 2**). At a cut-off point of 0.9 ng/ml, procalcitonin had an excellent sensitivity and negative predictive value, whereas specificity and positive predictive value for severe *P. falciparum* malaria was poor (**Table 2**).

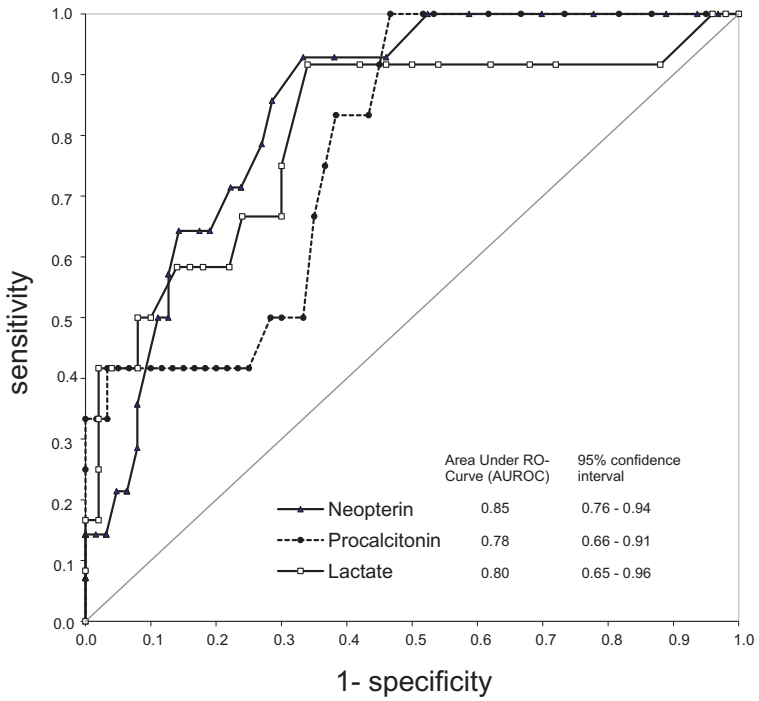

Figure 2. Receiver Operating Curves (ROC) characteristics of the diagnostic performance of neopterin, procalcitonin and lactate for severe *P. falciparum* malaria.

The ROC curve is a graph of sensitivity (true positive fraction) plotted against 1-specificity (false positive fraction). The performance of a diagnostic variable can be quantified by calculating the area under the ROC curve (AUROC). The ideal test would have an AUROC of 1, whereas a random guess would have an AUROC of 0.5.

Plasma lactate

Plasma lactate levels were significantly higher in travellers with severe *P. falciparum* malaria when compared to travellers with uncomplicated *P. falciparum* malaria (p=0.0012) and travellers with non-*P. falciparum* malaria (p=0.0040). ROC curve analysis of plasma lactate levels showed an AUROC of 0.80 (95% CI 0.65-0.96) compatible with a good accuracy (**Figure 2**). At a cut-off point of 1.8 mmol/l, lactate had an excellent sensitivity and negative predictive value, but a poor specificity and positive predictive value for severe *P. falciparum* malaria (**Table 2**), respectively.

Combinations of various biomarkers for severe falciparum disease

Analysis of various combinations of newly tested biomarkers and the use of different cut-off levels did not result in better discrimination of patients with severe *P. falciparum* malaria.

Discussion

Severe malaria is disreputable for its high case-fatality rate, but the outcome of severe *P. falciparum* infections has significantly improved since the introduction of artesunate as first line treatment of severe malaria, in particular in developing countries (2). In industrialized countries such as The Netherlands, the case-fatality rate of imported malaria is low and fatal cases are only occasionally reported. In the present study, the biomarkers sTREM-1, neopterin and procalcitonin were evaluated for their potential to be used as a marker for severe malaria disease upon admission. This contrasts with the design of many studies in regions of malaria endemicity where severe malaria is usually the entry criterion. For reasons of comparability, the same set of criteria for severe malaria was strictly applied for the diagnosis of severe malaria in this study, even though the study population comprised of presumably non-immune travellers and some authors even suggest a threshold of 5% in stead of 10% parasitized erythrocytes to define hyperparasitaemia in non-immune individuals.

The quantification of sTREM-1 levels on admission did not result in proper discrimination of severe *P. falciparum* malaria from uncomplicated *P. falciparum* malaria and non-*P. falciparum* malaria. In contrast, travellers with severe *P. falciparum* malaria had significantly higher levels of neopterin and procalcitonin on admission as compared with travellers with uncomplicated *P. falciparum* malaria or non-*P. falciparum* malaria, respectively. These findings correspond with the results of several other studies performed in semi-immune malaria patients living in malaria-endemic regions (16-18). When the ROC curve characteristics of neopterin and procalcitonin were compared to that of plasma lactate, the AUROC of neopterin appeared superior whereas the AUROC of procalcitonin appeared inferior to that of lactate, suggesting that neopterin provided the most accurate diagnostic performance for severe *P. falciparum* malaria in this cohort of travellers.

Unfortunately, the applicability of these tests in the initial clinical assessment of patients with severe *P. falciparum* malaria will probably be limited by the poor positive predictive value of neopterin and procalcitonin indicating that neither test can serve as a valuable tool for the diagnosis of severe *P. falciparum* malaria. For illustration, applying a procalcitonin level > 0.9 ng/ml or a neopterin level > 10.0 ng/ml as a guide to intensified monitoring and treatment would result in more than 20 of 64 patients with uncomplicated *P. falciparum* malaria receiving more intensive monitoring and treatment than strictly necessary. On the other hand, the high negative predictive value of both neopterin and procalcitonin suggests that these tests can still be of value by providing a tool for exclusion of severe disease. With either a procalcitonin level of less than 0.9 ng/ml or a neopterin level of less than 7.9 ng/ml in serum on admission as a cut-off point for severe *P. falciparum* malaria, no patient with severe disease would have been denied access to high-level monitoring and intensive treatment. In a previous study, in which a semi-quantitative 'point-of-care' procalcitonin test as a diagnostic tool for severe *P. falciparum* malaria was evaluated prospectively, all 6 patients

with severe *P. falciparum* malaria had procalcitonin values classified as either "moderate" or "high" (corresponding to a procalcitonin level ≥ 2 ng/ml), but never as "normal" or "low (18). This is compatible with the findings of the current retrospective serum sample-based study in which procalcitonin was measured quantitatively.

Although severe or fatal malaria rarely results from infections with the non-sequestering *Plasmodium* species *vivax*, *ovale* and *malariae*, increased neopterin and procalcitonin serum levels were also observed in the majority of these patients, although levels were lower than compared with severe *P. falciparum* malaria patients. Although speculatively, these observations suggest that the mechanism whereby neopterin and procalcitonin levels increase in malaria, is not specific for severe *P. falciparum* malaria alone. Therefore, it may not accurately reflect the pivotal pathophysiological events in complicated *P. falciparum* malaria, such as the sequestration of infected red blood cells in the microcirculation of vital organs and disturbance of microcirculatory flow. Whereas an increased plasma lactate level conceivably reflects a significant reduction in microcirculatory flow in vital organs, the elevated neopterin and procalcitonin levels are probably the result of activation of a common inflammatory host response evoked by infection with the respective *Plasmodium* parasites. In fact, some reports even suggest that *P. falciparum* malaria per se is not associated with a stronger host response than *P. vivax* or *P. ovale* malaria, but that the parasite burden of the causative *Plasmodium* species may also modulate the extent of the host inflammatory response (19).

In conclusion, although neither neopterin nor procalcitonin can probably serve as a useful single diagnostic tool for severe *P. falciparum* malaria, the high negative predictive value of both neopterin and procalcitonin may be helpful for a rapid exclusion of severe *P. falciparum* malaria on admission. This may be a valuable tool – particularly if available as a rapid diagnostic test - for physicians only occasionally dealing with ill-returned travellers and who need to decide on subsequent oral anti-malarial treatment or a timely referral to a specialized centre for high-level monitoring and intensified parenteral treatment.

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Chapter 3.2

Procalcitonin- and neopterin levels do not accurately distinguish bacterial from viral infections in ill-returned febrile travellers

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Abstract

The diagnostic performance of procalcitonin and neopterin as markers for bacterial and viral cause of fever was evaluated in a cohort of 69 febrile travellers with known etiological agents. Our aim was to establish a decision rule to minimize empirical antibiotic treatment. Compared with C-reactive protein (CRP) and leukocyte (differential) counts, procalcitonin and neopterin had a disappointing diagnostic accuracy. Refraining antibiotics in case of combined presence of lymphocytosis and/or CRP ≤ 10 mg/l would result in an 85% reduction in unwanted antibiotic treatment in patients with viral disease but in adequate antibiotic coverage of all patients with bacterial disease.

Introduction

The initial clinical evaluation of ill-returned travellers with fever should focus on infections that are treatable, transmissible and that may cause serious sequelae or even death. As a dogmatic rule, malaria remains the most important infection to exclude in anyone who returns with fever after visiting a tropical destination, necessitating investigation without delay (1). After exclusion of malaria, the clinician must subsequently decide whether the infection is likely to be caused by bacteria or viruses and whether the patient should be admitted for intensified treatment including empirical treatment with antibiotics. The most commonly differential diagnoses in this subpopulation of ill-returning travellers involve Dengue fever, enteric fever and rickettsial disease. Discrimination between these diseases may be difficult because of overlapping geographical endemicity patterns and clinical features. Results of routine laboratory findings may provide additional clues for a diagnosis. Traditionally, leukocytosis is considered to be suggestive of bacterial infection, whereas leukopenia may suggest viral infection. Unfortunately, in a number of bacterial infections, including uncomplicated typhoid fever, brucellosis and rickettsial infections, leukocytosis is not a characteristic feature of the disease. As a consequence, the clinician can not rely on these traditional parameters for decision making in the acute care setting. In order to reduce the need for empirical antibiotic treatment, we evaluated the diagnostic power of the biomarkers procalcitonin and neopterin to accurately predict a bacterial or a viral cause of fever in a cohort of ill-returned febrile travellers with a confirmed diagnosis of viral or bacterial disease. The performance of these biomarkers was compared to that of the parameters leukocyte differential count and C-reactive protein (CRP), respectively.

Materials and Methods

In this pilot study we evaluated procalcitonin and neopterin levels in stored serum samples taken on admission of 69 patients with a confirmed diagnosis (Dengue virus infection n=17, enteric fever [*Salmonella enterica* serovar Typhi n=9; Paratyphi A n=7 and B n=1] and *Rickettsia africae* infection n=19) in the period January 2005 to December 2009, respectively. Procalcitonin levels were determined using a commercially available EIA test (VIDAS BRAHMS Procalcitonin, bioMérieux, Lyon, France). Neopterin levels were determined using a commercially available ELISA test (DRG, Marburg, Germany). Normal serum values are <3 ng/ml for neopterin and <0.1 ng/ml for procalcitonin. All other laboratory parameters were previously established on admission using routine procedures.

Results

As shown in **Table 1**, there was a considerable overlap in leukocyte differential counts between patients with viral or bacterial disease. The bacterial indicators leukocytosis, segmentosis and an increased proportion of neutrophil band forms did not discern bacterial from viral causes. However, the viral indicators leukopenia, lymphocytosis and atypical lymphocytes were more frequently observed in patients with viral than in patients with bacterial disease. The PPV of these indicators varied between 61 and 80 percent. The corresponding likelihood ratios ranged from 2.1 to 5.3 indicating that presence of these predictors resulted in a 2 to 5-fold increase in odds favouring viral cause of fever. Determination of CRP levels also performed well. At a level above 11 mg/L, CRP was associated with a likelihood ratio of 3.7 for bacterial disease. The good performance of CRP for bacterial disease became even clearer in Receiver

Table 1. The diagnostic performance of the viral and bacterial predictors for a viral and bacterial cause of fever in ill-returning febrile patients with a known etiological agent.

Parameter on admission	**Viraldisease**	**Bacterialdisease**		**Predictor**	**Statistics**					
	Dengue (n=33)	Typhoidfever (n=17)	Rickettsioses (n=19)		Sensitivity	Specificity	PPV	NPV	LR	P-value
Leukocytes, n (%)										
<4.0 (x10^9/l)	*14 (42)*	*2 (12)*	*2 (11)*	*Viral*	*44*	*89*	*78*	*64*	*3.9*	P=0.005
4.0 - 10.0 (x10^9/l)	17 (52)	15 (88)	16 (84)							
>10.0 (x10^9/l)	*1 (3)*	*0 (0)*	*1 (5)*	*Bacterial*	*3*	*97*	*50*	*47*	*n.a.*	n.s.
Segments, n (%)										
<45 (%)	13 (52)	3 (19)	2 (12)							
45 – 75 (%)	11 (44)	11 (69)	14 (82)							
>75 (%)	*8 (32)*	*2 (13)*	*1 (6)*	*Bacterial*	*9*	*96*	*75*	*44*	*n.a.*	n.s.
Lymphocytes, n (%)										
<20 (%)	3 (12)	4 (25)	3 (18)							
20 – 50 (%)	14 (56)	10 (63)	14 (82)							
>50 (%)	*8 (32)*	*2 (13)*	*0 (0)*	*Viral*	*32*	*94*	*80*	*65*	*5.3*	P=0.014
Band forms, n (%)										
0 – 5 (%)	20 (80)	11 (69)	16 (94)							
>5 (%)	*5 (20)*	*5 (31)*	*1 (6)*	*Bacterial*	*18*	*80*	*55*	*43*	*n.a.*	n.s.
Atypical lymphocytes,										
n (%)	11 (44)	12 (75)	12 (71)							
Absent	*14 (56)*	*4 (25)*	*5 (29)*	*Viral*	*56*	*73*	*61*	*69*	*2.1*	P=0.033
Present										
C-reactiveprotein, n (%)										
< 11 (mg/l)	22 (79)	0 (0)	7 (41)							
≥ 11 (mg/l)	*6 (21)*	*17 (100)*	*10 (59)*	*Bacterial*	*79*	*79*	*82*	*76*	*3.7*	P<0.0001
Procalcitonin, n (%)										
<0.14 (ng/ml)	27 (87)	3 (21)	16 (89)							
≥0.14 (ng/ml)	*4 (13)*	*11 (79)*	*2 (11)*	*Bacterial*	*41*	*87*	*76*	*59*	*3.1*	P=0.002
Neopterin, n (%)										
<3.0 (ng/ml)	14 (44)	1 (6)	8 (44)							
≥3.0 (ng/ml)	*18 (56)*	*16 (94)*	*10 (56)*	*Viral*	*56*	*26*	*41*	*39*	*n.a.*	n.s.

Operating Characteristics (ROC) curve analysis where the AUROC was 0.83 (95% Confidence Interval 0.71-0.92) (**Figure 1**).

In contrast, the performance of the biomarkers procalcitonin and neopterin for a respective bacterial or viral cause of fever was inferior to that of CRP. Even though procalcitonin levels were significantly higher in bacterial than in viral infections (0.66 ± 2.21 ng/ml *vs.* 0.07 ± 0.13 ng/ml, p=0.0355) and procalcitonin levels above 0.14 ng/ml were associated with a likelihood ratio of 3.1, ROC curve analysis indicated a poor accuracy of procalcitonin for bacterial cause of fever (AUROC 0.61 [95% CI 0.47-0.73]). The performance of neopterin for viral cause of fever was even worse. Neopterin levels were significantly higher in patients with bacterial infections than in viral infections (10.2 ± 6.4 ng/ml *vs.* 4.9 ± 4.3 ng/ml, p=0.0084). Furthermore, neopterin had an AUROC of 0.33 (95% CI of 0.20-0.48), suggesting a very poor accuracy.

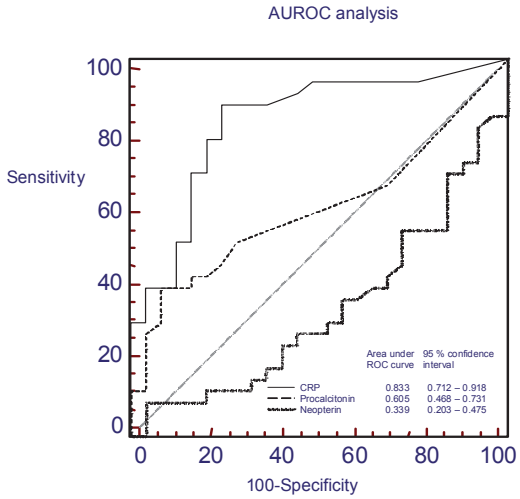

Figure 1. Receiver Operating Curves (ROC) characteristics of the diagnostic performance of CRP and procalcitonin for bacterial infection and neopterin for viral infection. The ROC curve is a graph of sensitivity (true positive fraction) plotted against 1-specificity (false positive fraction). The performance of a diagnostic variable can be quantified by calculating the area under the ROC curve (AUROC). The ideal test would have an AUROC of 1, whereas a random guess would have an AUROC of 0.5.

Discussion

The value of procalcitonin and neopterin in clinical decision making was shown in several studies (1-7), however not in all (8). In the present study we found a disappointing diagnostic accuracy of both procalcitonin and neopterin for a bacterial and viral cause of fever in ill-returning travellers, especially when the respective AUROCs were considered a measure of diagnostic performance. Moreover, in this study neopterin even had a more accurate performance for bacterial than for viral disease. These findings may be explained by the ob-

servation that *S.* Typhi and Paratyphi and *Rickettsia* species are intracellular bacteria and that other intracellular bacteria like tuberculosis and melioidosis also lead to increased neopterin levels (6).

Of the more traditional leukocyte differential count-based parameters, only presence of leukopenia, lymphocytosis and presence of atypical lymphocytes were suggestive of viral disease. Its use in clinical practice is hampered by its considerable overlap with the findings in the bacterial infections. When the indication for empirical antibiotic treatment was based on a single parameter, at least 11 of 33 patients (maximally 18 of 33 patients) with viral disease would receive unnecessary antibiotic treatment whereas a minimum of 7 of 36 patients (maximally 35 of 36 patients) with documented bacterial disease were withheld antibiotic treatment. Interestingly, when empirical antibiotic treatment was withheld on admission on the basis of combined presence of lymphocytosis and/or CRP ≤ 10 mg/l, only 5 of 33 patients with Dengue would receive unnecessary antibiotic treatment but all patients with enteric fever and rickettsial diseases would receive antibiotic treatment; an almost 85% reduction of unnecessary empirical antibiotic treatment.

Since the present findings were based on a relatively small number of selected patients with possibly inherent study limitations like selection bias and lack of power, the suggested clinical decision rule for empirical antibiotic treatment in returning febrile travellers without leukocytosis on admission needs validation in properly designed prospective studies.

Acknowledgments

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Culture

Chapter 4

In vitro evaluation of the performance of Granada selective enrichment broth for the detection of group B streptococcal colonization

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Abstract

A broth for the screening of group B streptococcocal (GBS) carriage during pregnancy is about to be introduced. Simulating conditions in everyday practice, we have compared the sensitivity of this Granada tube broth (GT) with that of classical Amies transport medium (AT) in vitro.

A total of 1,485 GT and 1,485 AT were tested with 33 well-characterized GBS strains in three different concentrations, five different incubation times and three different temperatures. After initial incubation at room temperature (RT) or 4°C, GT were placed at 37°C. GT were scored for the presence of orange pigment. GT and AT were subcultured on blood agar (BA).

Pigment was observed in 98% of GT incubated at 37°C. GBS could be cultured in 91%, 73%, and 55% of GT incubated at 37°C, RT, or 4°C, respectively. For AT, these percentages were only 20% at 37°C, 52% at RT, and 59% at 4°C. When GT initially incubated at RT or 4°C were subsequently incubated at 37°C, the sensitivity improved significantly.

We conclude that GT is a more sensitive GBS transport and culture medium than the conventional method, especially for low inocula and prolonged transport/incubation times.

GT does not exclude the presence of GBS, and should always be incubated at 37°C and subcultured on solid agar for optimal sensitivity.

Introduction

Group B streptococci (GBS) has been known as a human pathogen since 1938. In the course of pregnancy and the postpartum period, GBS may cause a variety of serious infections in both the mother and neonate (1-2). Neonatal early-onset GBS disease (GBS-EOD) presents in the first week of life and is usually acquired during delivery by neonates born from mothers colonized with GBS in the rectovaginal tract. Up to 35% of pregnant women are colonized with GBS in the rectovaginal tract, most often without symptoms (3-5). Approximately 1% of neonates born to colonized mothers develop GBS-EOD and up to 40% of the surviving neonates suffer serious sequelae, such as mental retardation or seizures (2). Mortality is high among preterm infants, with average case-fatality rates of approximately 20% (2). These rates vary from as high as 30% among those children born before 33 weeks of gestation to 2-3% among full-term infants (2).

Schrag et al. demonstrated that a prevention strategy based on routine screening for GBS carriage prevents more cases of GBS-EOD than an approach based on risk-factor assessment (6). Therefore, screening for GBS carriage during pregnancy is the key in many guidelines to prevent GBS-EOD (2, 7). Consequently, sensitive and specific GBS cultivation is essential and laboratory procedures need to be streamlined. Little attention has been paid to the consequences of transport and transport delay of swabs to the laboratory. Revised Centers for Disease Control and Prevention (CDC) guidelines state specifically that GBS isolates remain viable in Amies transport medium for several days at room temperature. However, the recovery of isolates declines over a period of 1-4 days, particularly at elevated temperatures. Even when appropriate transport media are used, the sensitivity of culture is highest when the specimen is stored at 4°C before culture and processed within 24 h of collection (2, 8-11).

More rapid and, especially, more sensitive methods than the currently available and recommended transport and culture methods would improve the effectiveness of the screening-based approach. A new transport and enrichment broth, called Granada tube broth (GT) (bioMérieux, Marcy l'Etoile, France) is about to be introduced. In this broth an orange pigment is produced in the presence of GBS (12-13).

The aim of this study was to investigate whether the use of GT would improve the sensitivity of GBS cultures in comparison with the current gold standard under various culture conditions. We also investigated the reliability of GT after prolonged transport times.

Materials and methods

Bacterial isolates

A subset of 33 colonizing and invasive GBS isolates representing all seven of the important subtypes was obtained from a reference collection (**Table 1**) (14). All isolates were previously identified as GBS using both biochemical and molecular methods (14). Strains were stored at -80°C. Prior to testing, strains were subcultured twice on Columbia III agar with 5% sheep blood (bioMérieux) for 18-24 h at 37°C.

Table 1. Group B streptococci (GBS) isolates used (ref. 14).

Serotype	Invasive	Colonizing
Ia	2	3
Ib	0	5
II	0	4
III	3	2
IV	0	4
V	3	2
VI	0	5

Variables and culture

Phase I

In the first phase of the study, we studied the viability of the GBS strains using GT and Amies transport medium (AT) under different circumstances (five different incubation times [1, 2, 3, 4 and 7 days], three different temperatures [37°C, room temperature (RT) (±20°C), and 4°C] and three different inocula). Of each strain, a suspension of 0.5 McFarland (~ $1.5x10^8$ colony-forming units [CFU]/ml) was prepared in sterile saline. This suspension was diluted until three concentrations were obtained: $1.5x10^6$, $1.5x10^4$ and $1.5x10^2$ CFU/ml, respectively. All suspensions were subcultured on blood agar (BA) for purity checking and growth control. For every strain, 45 GT and 45 AT were inoculated with 100 µl of suspension. After incubation, GT were scored for the presence of orange pigment by two individuals and subcultured on BA for the detection of growth. The presence of orange pigment was checked after 1, 2, 3, 4 and 7 days. All changes in colour were compared to a negative control tube, which was processed similarly to the inoculated tubes. After incubation, both GT (using 10 µl of broth) and AT were subcultured onto BA using the four-quadrant technique, to allow semi-quantification of the number of GBS colonies. Growth was recorded after 1 and 2 days of incubation at 37°C and was graded as negative (no growth), weakly positive (growth in the first quadrant), or positive (growth in the second, third or fourth quadrants).

Phase II

Phase II of the study was performed in parallel to phase I. GT initially incubated at 4°C or RT in phase I were subsequently incubated at 37°C for 1 and 2 days and were checked for the presence of orange pigment as described previously. After this second incubation period, BA were inoculated and both GBS growth detection and quantification were performed as described above.

Results

Phase I

Overall, orange pigment was detected in 98% of all GT after incubation at 37°C for 1-7 days, with the lowest detection rate at day 3 for 10^2 CFU/ml (90%). After incubation at RT, orange pigment was observed after 3 days of incubation and in 10-40% of the GT (**Figure 1**). At 4°C, in none of the GT was orange pigment detected.

Comparisons of GT with AT for growth detection for the different incubation times, different incubation temperatures, and different inocula are shown in **Figure 2** and **Table 2**. Data for semi-quantification via the four-quadrant technique showed no differences (data not shown).

Table 2. Overall detection of GBS after subculture of Granada tube broth (GT) and Amies transport medium (AT) on blood agar (BA) after incubation for 1, 2, 3, 4, or 7 days at 37°C, room temperature (RT) or 4°C using three different inocula (study phase I). A p-value <0.05 is considered to be statistically significant.

10^6 CFU/ml	GT culture-positive (%)	AT culture-positive (%)	p-value
37°C	94	43	<0.0001
RT	98	98	NS
4°C	100	100	NS
10^4 CFU/ml			
37°C	93	13	<0.0001
RT	80	55	NS
4°C	63	75	NS
10^2 CFU/ml			
37°C	84	5	<0.0001
RT	41	4	0.0002
4°C	3	3	NS

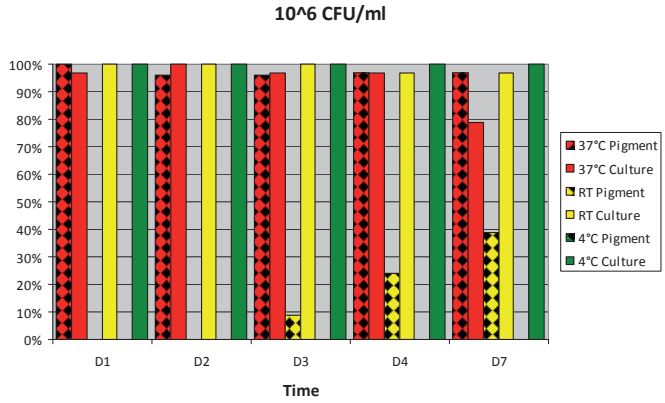

Figure 1A. 10^6 CFU/ml

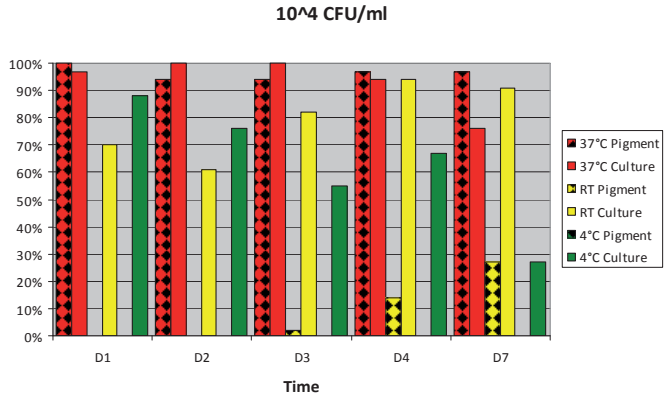

Figure 1B. 10^4 CFU/ml

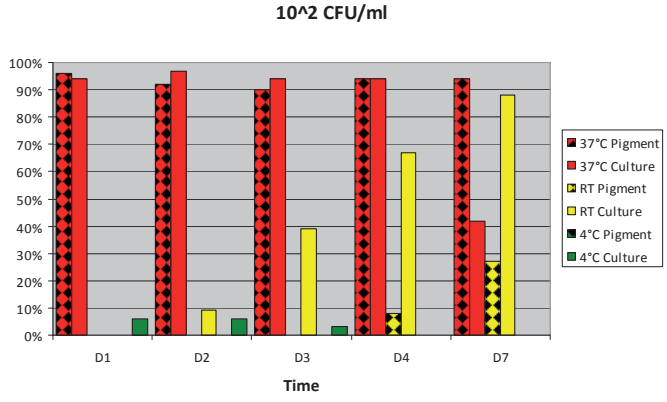

Figure 1C. 10^2 CFU/ml

Figure 1. Detection of orange pigment and group B streptococci (GBS)-positive cultures in Granada tube broth (GT) after incubation for 1, 2, 3, 4, or 7 days at 37°C, room temperature (RT), or 4°C using three different inocula (study phase I). In none of the GT at 4°C, was orange pigment detected.

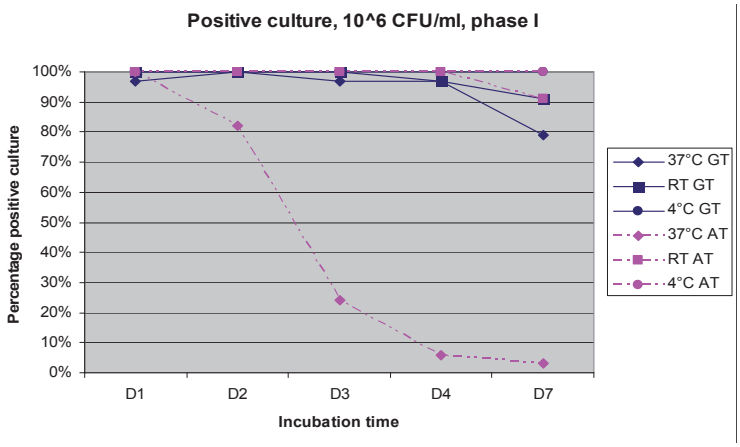

Figure 2A. 10^6 CFU/ml

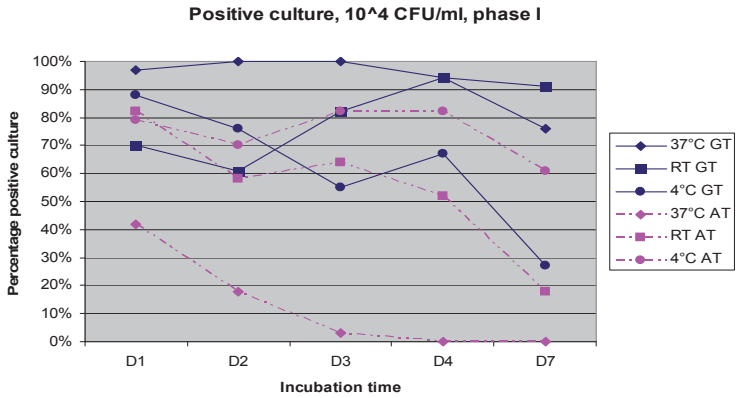

Figure 2B. 10^4 CFU/ml

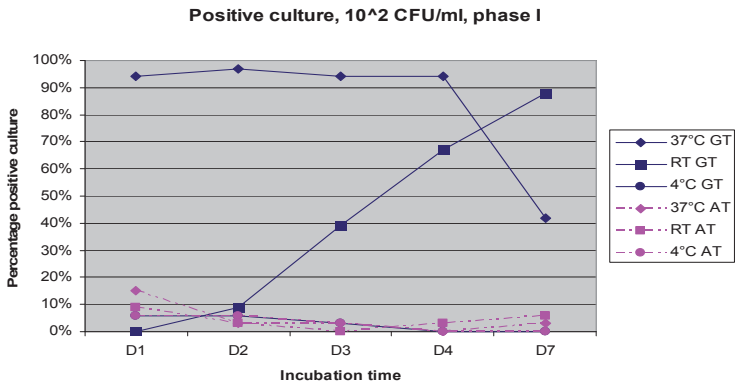

Figure 2C. 10^2 CFU/ml

Figure 2. Detection of GBS after subculture of GT and Amies transport medium (AT) on blood agar (BA) after initial incubation for 1, 2, 3, 4, or 7 days at 37°C, RT, or 4°C using three different inocula (study phase I).

Phase II

Data on the observation of orange pigment after subsequent incubation at 37°C and the detection of GBS after subculture on BA are shown in **Figure 3**.

No differences were observed in the presence of orange pigment and positive cultures between 1 and 2 days subsequent incubation (data not shown). Furthermore, no differences were observed between the different virulence types or between the different serotypes (data not shown).

Discussion

Granada agar has long been used in Europe to detect GBS in pregnant women. Most strains of beta-hemolytic GBS produce an orange carotenoid pigment on this agar, usually within 24h of incubation. Variable results on the sensitivity of this culture medium have been reported, with some authors considering it to be unacceptably low (15-18). However, our results cannot be compared with the results of these studies as we tested Granada broth instead of agar.

On average, we observed the production of orange pigment in 98% of all GT that were incubated at 37°C for 1-7 days, with the lowest detection rate at day 3 for 10^2 CFU/ml (90%). At room temperature, orange pigment was observed only after 3 days of incubation and in only 10-40% of the GT. At 4°C, in none of the GT was orange pigment detected. As the overall sensitivity for GT for GBS culture was 95%, independent of the incubation-temperature and the production of orange pigment, our results show that the absence of orange pigment does not exclude the presence of GBS. Furthermore, because the GBS pigment is linked to hemolysin activity, less or non-hemolytic strains may not be detected with GT (19). Therefore, it may be reasonable to subculture all GT (both positive and negative) on BA. This allows the recognition of non-hemolytic GBS strains and other GBS that may not produce the orange pigment. This is important for identification, susceptibility testing and potential typing of the cultured strailn our simulation of transport conditions, GBS could be cultured only in 20%, 52%, and 59% of AT incubated at 37°C, RT, or 4°C, respectively. These percentages correspond with those found by Rosa-Fraile et al., Stoner et al., and with revised CDC guidelines (2, 10-11). The sensitivity of GT was significantly higher than that of AT, especially for low inocula and extended transport/incubation time. This is important, since specimens may be exposed to high temperatures during transport, especially when swabs are obtained outside the hospital.

Direct incubation of GT at 37°C resulted in the highest yield of GBS. No difference in the growth of GBS was observed between transport at RT or at 4°C. Without incubation at 37°C after transport, the growth of GBS was seen in 73% and 55% of GT transported at RT or 4°C,

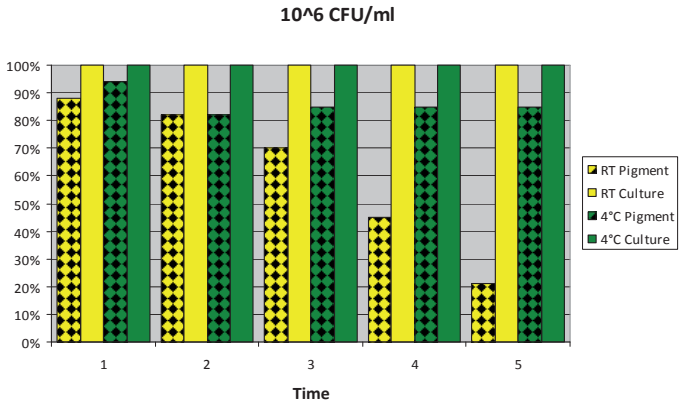

Figure 3A. 10^6 CFU/ml

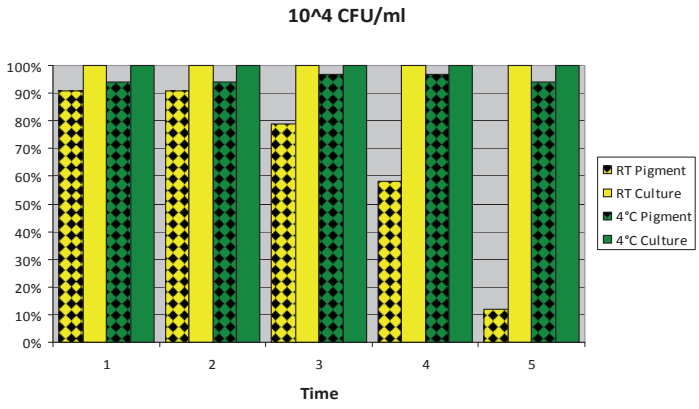

Figure 3B. 10^4 CFU/ml

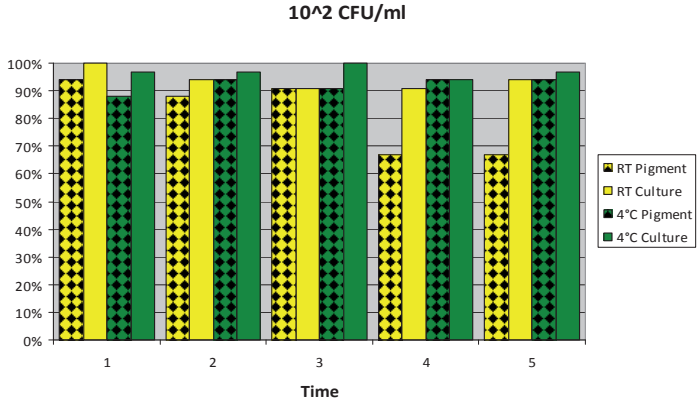

Figure 3C. 10^2 CFU/ml

Figure 3. Detection of orange pigment and GBS-positive cultures in GT after subsequent incubation at 37°C for 24 h after an initial incubation for 1, 2, 3, 4, or 7 days at RT or 4°C using three different inocula (study phase II).

respectively. When GT were subsequently incubated at 37°C, after transport at RT or 4°C, the sensitivity increased to almost 100%.

The study is limited in that it is performed in vitro and that the application of purified isolates of GBS in GT and onto AT may not reflect GBS survival on swabs containing vaginal and/or rectal flora. Nonetheless, we can conclude that GT is a highly sensitive transport and culture medium to detect GBS in pregnant women. The survival of GBS was significantly better in GT when compared to AT.

GT may not be suitable for the direct detection of GBS, as the absence of orange pigment does not conclude in the absence of GBS. Therefore, GT should always be subcultured for optimal use. Furthermore, GT should always be incubated at 37°C to improve its sensitivity.

GT may especially be suited for the transport of swab specimens from general practitioners and midwifery practices to the laboratory, which may take 2-3 days. Specifically, if a woman has low-density GBS colonization, extended transport times of swab specimens at RT or higher could reduce the culture sensitivity for AT but possibly not for GT.

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The Granada tubes, Amies transport media, and Columbia III agar with 5% sheep blood used in this study were provided by bioMérieux free of charge.

Conflicts of interest

Alex van Belkum is an employee of bioMérieux. There are no conflicts of interest to disclose.

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Nucleic acid amplification

Chapter 5

Molecular diagnostics and genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA): an update

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Expert Reviews of Molecular Diagnostics, 2010; 10: 375-80

The organism

Staphylococcus aureus is a significant human pathogen that can cause a wide variety of diseases due to its ability to acquire and express various virulence factors and antimicrobial resistance determinants. Colonization is an important step in the pathogenesis of *S. aureus* infection. Approximately 20-30% of the general human population is persistently colonized with *S. aureus*, most frequently in the anterior nares, although other body sites, such as the perineum, skin and throat, may also be colonized. Another 30% of the general population is intermittently colonized and the remaining 40-50% appear to be not susceptible to *S. aureus* carriage (1-2).

Persistent nasal carriers of *S. aureus* have a three to six times higher risk of health-care associated infections with *S. aureus* than non-carriers (3-5). More than 80% of health-care associated *S. aureus* infections are endogenous (6-8). Recently it has been shown that the number of surgical-site *S. aureus* infections acquired in the hospital can be reduced by rapid screening and decolonization of nasal carriers of *S. aureus* on admission (9).

Following the introduction of methicillin in 1959, methicillin-resistant *S. aureus* (MRSA) has quickly and widely emerged as a major nosocomial burden worldwide (10). Today, MRSA continues to be a major issue in hospitals but has also emerged as a problem in the community (11). Methicillin resistance in *S. aureus* is encoded by the *mecA* gene, which is embedded within a mobile staphylococcal cassette chromosome (SCC) element known as SCC*mec*. MRSA can emerge from methicillin-susceptible *S. aureus* (MSSA) upon site-specific integration of SCC*mec* into the *orfX* locus in the chromosome. To date, nine major types of SCC*mec* have been recognized in *S. aureus* (12).

Phenotypic detection and identification of MRSA

Rapid, high-throughput diagnostic tools are needed to detect infections caused by MRSA strains and to contain their spread. The traditional screening cultures require at least 48 hours until a negative test result for MRSA can be reported. These methods have been superseded by the introduction of selective agar cultivation methods and latex agglutination tests for the specific detection of the product of the *mecA* gene, Penicillin Binding Protein 2a (PBP 2a). Most of these tests have been evaluated in different studies (13-26). Sensitivity and specificity for agar cultivation ranged from 40-98% and 80-100%, respectively. For latex agglutination tests, both sensitivity and specificity were almost 100%.

The first commercial example of a rapid screening test for detection and identification of MRSA, the BacLite Rapid MRSA assay (Acolyte Biomedia, United Kingdom) is documented as a sensitive (90.4%) and specific (95.7%) test for the detection of MRSA nasal colonization

within 5 hours (27). The test measures adenylate kinase activity in a selective broth during a 4h growth episode.

Recently, Stenholm et al described the results of a new culture-based method for MRSA screening (28). This new method employs the two-photon excited fluorescence (TPX) technology to provide a quantitative *S. aureus*-specific fluorescence signal in a single separation-free process. Different fluorescence signal progressions are recorded for MRSA and MSSA when bacterial growth under conditions of antibiotic pressure is monitored online by continuous or repeated measurements. When monoclonal antibodies were used, the assay was 100% sensitive and 100% specific for screening for MRSA from pure cultured samples and results were available within 8 to 12 hours.

However, most phenotypic techniques still require 4-24 hours of cultivation before detection and identification of *S. aureus* and testing for methicillin resistance can be initiated and may therefore not be suitable for fast screening of patients.

Genotypic detection and identification

Nucleic Acid Amplification Techniques (NAATs) offer clear benefits over traditional culture-based assays, in particular a reduced time to identification and an improved specificity and sensitivity. Over the past decade, a range of commercial and in-house developed NAATs has been introduced (For an overview, see **Table 1**) with 2 main strategies: identification of MRSA via detection of *S. aureus*-specific genes, such as *spa*, *nuc* and *fem* in combination with the detection of the *mecA* gene or via detection of the SCC*mec* region. However, sensitivity and specificity of these assays may be compromised due to the presence of methicillin-resistant coagulase negative staphylococci (MRCoNS), variability within the used *S. aureus* specific genes or variability within the SCC*mec* cassette. This may lead to false-negative results (e.g. gene mutation or SCC*mec* variants) or false-positive results (e.g. deletion of the *mecA* gene from the SCC*mec* region) (29-32).

The detection of the *mecA* gene by (real-time) PCR is widely recognized as the gold standard for identification of MRSA. A variety of in-house assays performed on several different instruments has been described in the literature with variable results (**Table 1**). Most of these are performed on an overnight selective enrichment broth. The major limitations of this PCR strategy for direct testing in clinical samples were the necessity of enrichment and the inability to directly link identification of *S. aureus* with *mecA* gene detection, because of the confounding effect caused by MRCoNS. In 2004, Huletsky et al described a novel real-time PCR assay (33). This assay is able to distinguish between MRSA and mixtures of MSSA and MRCoNS and therefore suitable for direct identification of MRSA in clinical specimen. The assay became commercially available as the IDI-MRSA assay and is currently marketed as the

Table 1. Commercial molecular tests for MRSA detection and identification.

Commercial					
Testkit (supplier)	Test principle	Sensitivity (%)	Specificity (%)	TAT (1)	Reference
Culture					
Genotype MRSA (Hain)	PCR and reverse line blot detection of *mecA* and *S. aureus* specific sequence	91	99	4h	(51)
Direct from clinical specimen (swabs)					
HyplexStaphyloResist (Alpha Omega)	PCR-ELISA for detection of *mecA*, *coa* and conserved*S. aureus* housekeeping gene	92	90	4h	(52)
GenoType MRSA direct (Hain)	PCR and reverse line blot detection of SCC*mec*/orfX	95	99	4h	(53)
GeneOhm IDI-MRSA (BD)	Multiplex Real-time PCR for detection of SCC*mec*/*orfX*	95	94	2h	(33, 54-55)
GeneXpert MRSA assay	Multiplex Real-time PCR for detection of SCC*mec*/*orfX*	90	97	80'	(35)
In-house					
Enrichment					
NA (2)	Multiplex PCR (agarose gel) detecting *femB* and *mecA*	100	100	3h	(56-57)
NA (2)	Real-time PCR detecting *mecA*	100	100	2h	(58)
NA (2)	High-throughput real-time PCR detecting *mecA* and *nuc*	100	100	2,5h	(59)
NA (2)	Real-time PCR detecting *mecA*and *SA442*	100	100	4h	(60)
NA (2)	Isothermal signal amplification (CytAMP) detecting *coa* and *mecA*	83	93	3,5h	(61)
NA (2)	Real-time PCR detecting *nuc* and *mecA*	93	90	2,5h	(62)
Direct from clinical specimen (swabs)					
NA (2)	Real-time PCR detecting *mecA* and *femA*	100	91	6h	(63)
NA (2)	*S. aureus*Immunocapturing (protein A) and quantitative PCR detecting*mecA, femA and femB*	100	64	6h	(64)
NA (2)	Real-time PCR detecting SCC*mec*/*orfX*	93 (98)*	100 (100)*	3,5h	(65)

*) Percentage sensitivity and specificity between brackets are after overnight selective enrichment.

(1) TAT: turn-around-time

(2) NA: not applicable

BD GeneOhm MRSA assay (BD GeneOhm, San Diego, California). When testing nasal swabs, the detection limit was suggested to be 325 CFU per swab. However, in an External Quality Assessment (EQA) performed by Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland) in 2009, the limit of detection was found to be 10^3-10^4 CFU/ml (34). Also, when evaluating the performance of this test, Bartels et al described how a common variant of SCC*mec* type IVa was not detected by the BD GeneOhm assay (30).

Rossney et al evaluated a commercial platform based on the BD GeneOhm principal for direct detection of MRSA in clinical samples, the GeneXpert MRSA assay (Cepheid Diagnostics,

Sunnyvale, California) (35). In this fully automatic, but low-throughput assay, cells are lysed using ultrasonics and DNA from samples is extracted, amplified and detected in separate chambers of single-use disposable cartridges which contain freeze-dried beads with all reagents required for the real-time process. Sample preparation time is minimal and the PCR assay time is 75 minutes maximum. The authors reported an average limit of detection of 610 CFU/ml with a sensitivity, specificity, positive predictive value and negative predictive value of 90%, 97%, 86% and 98%, respectively.

In the QCMD EQA study of 2009, eleven samples containing various amounts of inactivated MRSA cells, methicillin-susceptible *S. aureus* (MSSA), MRCoNS or *Escherichia coli*, were distributed to 82 laboratories (34). When compared with previous EQA studies on molecular diagnostics of MRSA, a statistically non-significant decrease was observed in the overall test sensitivity. However, a minor improvement was observed for the 'specificity' and the 'true-negative' samples. In this EQA, one normal MSSA strain and two MSSA samples harbouring a SCC*mec* cassette lacking the *mecA* gene were included. There was a marked difference in the percentage of correct results for the MSSA strain containing the *mecA* gene compared to the two strains lacking it.

In conclusion, the quality of direct molecular diagnostic tests still needs improvement. Every assay should be evaluated and continuously monitored to determine the assay's usefulness. Furthermore, positive results should always be confirmed by a culture method or a second molecular test.

Genotyping

After MRSA detection, genetic typing may be necessary in order to assess whether transmission of MRSA occurred and whether infection-preventive measures are mandatory. In addition, genetic typing is essential for elucidation of (inter)national dissemination of MRSA clones. Currently, many different genotyping methods are in use in the diagnostic laboratory, but pulsed-field gel electrophoresis (PFGE) of *Sma*I macro restriction analysis of genomic DNA is preferential (36). However, PFGE is a technically demanding method, with limited portability due to lack of reproducibility.

The development of a commercially available automated rep-PCR assay, the DiversiLab system (bioMérieux, Marcy l'Etoile, France) offers advances in standardization and reproducibility over manual fingerprint generating systems (37). However, although two independent studies concluded that the DiversiLab system is a rapid and reproducible technique, it also lacks resolution. DiversiLab analysis does not differentiate genetically and epidemiologically unique MRSA strains, which is needed for adequate outbreak analysis (38-39).

Multi-locus variable number of tandem repeat analysis (MLVA) is a high-throughput genotyping technique that can be used for hospital, national and international genotyping

of MRSA, but the discriminatory power depends on the number and types of loci analyzed (40-41).

Sequence-based approaches, such as spa sequence typing and multi-locus sequence typing (MLST), have resulted in large sequence databases for MRSA. The determination of sequence polymorphism of the *spa* gene encoding the staphylococcal surface protein A (spa sequence typing) has become the most popular MRSA typing system, thanks to high-throughput capacity and an excellent reproducibility, which allows portability of data and comparison worldwide (42). MLST defines variation within a very small sample of the genome and often cannot distinguish between closely related isolates.

Full-genome sequencing provides a complete inventory of micro-evolutionary changes, but this approach is impractical for routine diagnostic laboratories. In a recent paper, Harris et al described a new high-throughput genomics approach based on full-genome sequencing, which provides a high-resolution view of the epidemiology of MRSA with the potential to trace person-to-person transmission within a hospital environment (43).

New techniques

New tools for identification and typing of MRSA include different applications of spectroscopy, such as PCR/Electrospray Ionization-Mass Spectrometry (44-45) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (46). MALDI-TOF MS is cost-effective, analyzes samples in minutes and requires little hands-on-time.

Also Raman spectroscopy has been described as a promising tool (47-48). Raman spectroscopy is a fast and reproducible typing technique, which provides strain-specific optical fingerprints in a few minutes instead of several hours to days, as is the case with genotyping methods. Its high throughput and ease of use make it suitable for use in routine diagnostic laboratories. Efforts to develop these technologies for the analysis of single cells are currently in full progress and may in the end compete effectively with the currently preferred nucleic acid based technologies.

Instead of detecting *S. aureus* itself, new strategies may be to look at the host immune response or to look at genetic variation in the host.

The rapid detection of antibody levels against *S. aureus* with Luminex technology showed that antibody levels were associated with the presence of toxin genes in infectious *S. aureus* isolates (49). And Emonts et al (50) showed that persistent carriage of *S. aureus* is influenced by and associated with genetic variation in host inflammatory response genes.

Both approaches can be useful in fast screening for (susceptibility to) (methicillin-resistant) *S. aureus* carriage or infection.

Concluding remarks

MRSA is responsible for a large and still growing number of both health-care and community-associated infections, resulting in increased morbidity and excessive healthcare costs. Screening of individuals combined with an aggressive infection control program has become the standard for management of these infections. Rapid screening methods that allow reliable detection of MRSA within hours are now available. The short time-to-result is a clear advantage that has provided a tool for successful infection control strategies. However, every assay should be evaluated against the local MRSA diversity before being introduced in the diagnostic microbiological laboratory. Continuous evolution of SCC*mec*, constrains continuously monitoring of the assay performance and positive results of direct MRSA testing should always be confirmed by a culture method or a second molecular test. For laboratories with high false positivity rates or in regions with low prevalence of MRSA, confirmation is essential.

In conclusion, the quality of molecular diagnostic tests and (geno)typing techniques is still under discussion. Adequate internal and external quality control and international standardization for MRSA diagnostics should be developed over the years to come. To improve the performance and quality of molecular detection, identification and typing of MRSA, both laboratories and manufacturers should be encouraged to participate in EQAs.

Genetic and functional studies in large populations are warranted to clarify the contribution of new strategies such as different applications of spectroscopy and spectrometry, determination of genetic variability in humans or rapid antibody – and/or antigen-detection.

Potential conflicts of interest

Alex van Belkum is member of the scientific advisory board of Cepheid (Cepheid Diagnostics, Sunnyvale, California).

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Bacterial typing

Chapter 6

Comparison of the DiversiLab system, pulsed-field gel electrophoresis and multi-locus sequence typing for the characterization of epidemic reference MRSA strains

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Abstract

We have analyzed a representative selection of the HARMONY methicillin-resistant *Staphylococcus aureus* strain collection originating from 11 European countries (Cookson, B.D. et al. 2007, J. Clin. Microbiol. 45: 1830-1837) with the DiversiLab System, pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Simpson's diversity indices were 0.905, 0.877 and 0.860 for PFGE, MLST and DiversiLab, respectively. All methods displayed concordant classification of the MRSA strains, although with divergent resolution and reproducibility.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates emerged soon after the introduction of methicillin and has become an increasing problem in both healthcare settings and in the community worldwide (1). The impact on patient morbidity, mortality and healthcare-associated costs is significant (2-6). Therefore, controlling the spread of this pathogen by screening patients, personnel and the environment remains a high priority in infection control programs. Tracing the source and transmission routes of MRSA relies on typing methods as tools for the genetic characterization of isolates (7). Pulsed-field gel electrophoresis (PFGE) has been accepted as the reference method for molecular strain typing of MRSA. PFGE is known to be highly discriminatory and therefore frequently used for outbreak analysis (8). However, this strategy is labor-intensive, time-consuming and technical instability has an adverse effect on reproducibility (9). Therefore, results are not interchangeable. Other molecular fingerprint-generating strain typing methods such as manual rep-PCR (9-10) and randomly amplified polymorphic DNA (RAPD) analysis generate similar problem (11). Multilocus sequence typing (MLST) defines unambiguous strain types and results can easily be exchanged between different laboratories (12). MLST has a moderate resolution to delineate genetically unique strains and has been proposed for population structure analysis or phylogeny studies (13). MLST is a relatively expensive method and therefore not an option for many clinical laboratories (14).

Repetitive sequence-based PCR (rep-PCR) uses primers that target non-coding repetitive sequences interspersed in bacterial and fungal genomes (15-17). When separated by electrophoresis, the amplified DNA fragments constitute a genomic fingerprint that can be employed for subspecies discrimination and strain delineation of bacteria and fungi (18). The development of a commercially available, automated rep-PCR assay system, the DiversiLab System (bioMérieux, Marcy l'Etoile, France), offers advances in standardization and reproducibility over manual fingerprint generating systems (19).

The aim of the current study was to compare the performance (discriminatory power and reproducibility) and the feasibility (interchange of data, rapidity and cost) of the DiversiLab system and two worldwide used *S. aureus* typing methods, PFGE and MLST.

Materials and methods

A representative selection of ninety-three MRSA-isolates of the HARMONY collection was cultured on trypticase soy (TSA) II agar with 5% sheep blood for 18 hours at 37°C. All strains were tested in triplicate. From each isolate DNA was extracted using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA) according to manufacturer's in-

structions. The presence and concentration of genomic DNA was estimated with NanoDrop® ND-1000 Spectrophotometer (Isogen, IJsselstein, The Netherlands) and DNA concentration was adjusted to 25-50 ng/µl for each sample.

All DNA samples were amplified using the DiversiLab *Staphylococcus aureus* kit for DNA fingerprinting (bioMérieux) following the manufacturer's instructions. Briefly, 2 µl of genomic DNA (final concentration 25-50 ng/µl), 0.5 µl (or 2.5 U) of AmpliTaq® polymerase (Applied Biosystems, Foster City, CA, USA), 2 µl kit-supplied primer mix and 2.5 µl of 10X PCR buffer (Applied Biosystems) were added to 18 µl of the kit-supplied rep-PCR master mix (MM1) for a total of 25 µl per PCR mixture. Thermal cycling parameters were as follows: initial denaturation at 94°C for 2 min; followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, extension at 70°C for 90 sec; and a final extension at 70°C for 3 min. Separation and detection of rep-PCR products was done by micro-fluidic chips of the DiversiLab System (bioMérieux) and analysis was performed with DiversiLab software version v.r.3.3.40. The resulting DNA fingerprint patterns were viewed on a personal DiversiLab website as electropherograms. The reports included a dendrogram with similarity matrix, scatterplot and a virtual gel image of the fingerprint for each sample.

The DiversiLab software used the Pearson correlation coefficient to analyze and calculate genetic similarity coefficients among all samples. The unweighted pair-group methods of averages (UPGMA) was employed to automatically compare the rep-PCR profiles and create corresponding dendrograms (19). Reports included computer-generated virtual gel images, scatterplots and selected demographic fields to aid interpretation of the data. Guidelines have been suggested by the manufacturer for determining the strain-level discrimination (typing). Cluster analysis, based on peak height and presence or absence of the peaks, was done by DiversiLab software. Percentage similarity for *S. aureus* was set at 98% similarity.

Results

The 93 MRSA isolates were differentiated in 7 clusters and 8 unique rep-PCR types by the DiversiLab system, comprising 15 different fingerprints in total (see **Figure 1** and **Table 1**). The technical reproducibility of DiversiLab as determined with the average similarity of the triplicate samples was >99% (95-100%). Data for PFGE and MLST results were retrieved from a manuscript by Cookson et al. (20). PFGE (*Sma*I digestion) resolved 28 pulsotypes and MLST differentiated 16 sequence types in 6 clonal complexes (CCs). Rep-PCR cluster VII included half of the total collection (n=47), consisting of 7 different STs and 12 different pulsotypes. Next to this, 2 other large rep-PCR clusters were found. Cluster II with 10 isolates, consisting of 1 ST and 3 pulsotypes and cluster III, with 14 isolates, composed of 3 STs and 6 pulsotypes.

The discriminatory power calculated as Simpson's Index of Diversity (DI) was 0.860, 0.877 and 0.905 for DiversiLab, MLST and PFGE, respectively.

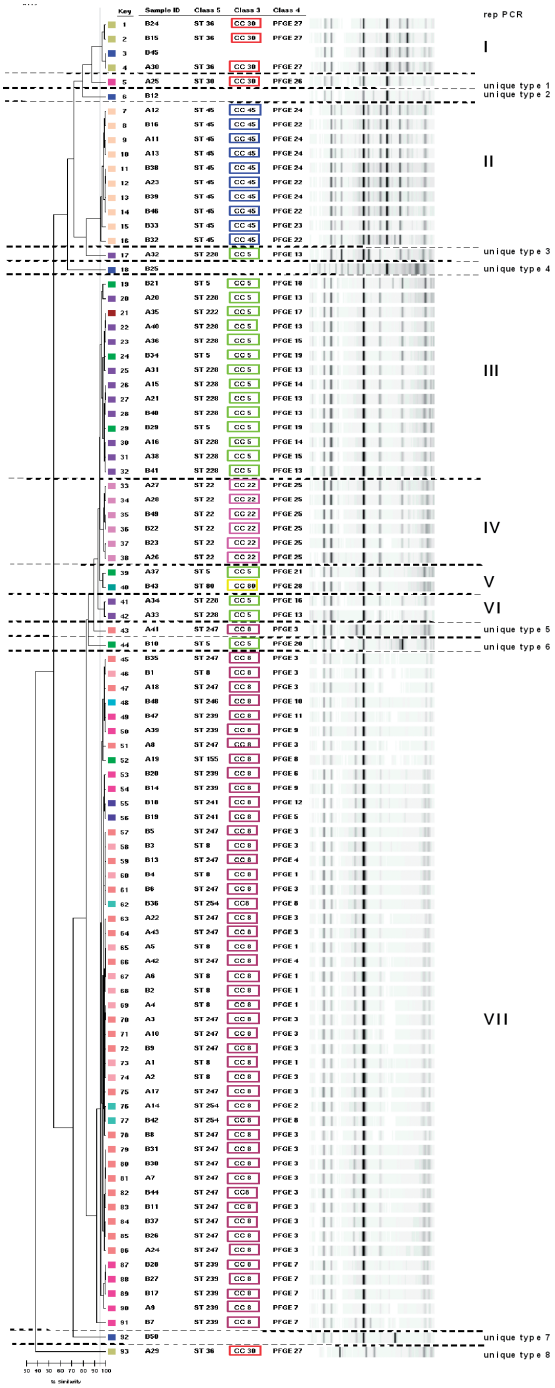

Figure 1. Pearson cluster analysis of rep-PCR generatedfingerprintsobtainedfor the 93 MRSA isolates.
PFGE and MLST data are included in the dendrogram. Clonalcomplexes are depicted in colouredboxes. A Similarity Index cutoff value of 95% was used to define genetic classification.

Table 1. MRSA strain typing results obtained with rep-PCR, PFGE and MLST.

rep PCR Cluster (n)	MLST ST (n)	CC	PFGE type (n)
I (4)	36 (3)	30	27 (3)
II (10)	45 (10)	45	22 (4), 23 (1), 24 (5)
III (14)	5 (3), 222 (1), 228 (10)	5	13 (6), 14 (2), 15 (2), 17 (1), 18 (1), 19 (2)
IV (6)	22 (6)	22	25 (6)
V (2)	5 (1), 80 (1)	5, 80	21 (1), 28 (1)
VI (2)	228 (2)	5	13 (1), 16 (1)
VII (47)	8 (9), 155 (1), 239 (9), 241 (2), 246 (1), 247 (22), 254 (3)	8	1 (6), 2 (1), 3 (24), 4 (2), 5 (1), 6 (1), 7 (5), 8 (3), 9 (1), 10 (1), 11 (1), 12 (1)
unique 1	30	30	26*
unique 2	Nd	Nd	Nd
unique 3	5	5	13
unique 4	Nd	Nd	Nd
unique 5	247	8	3
unique 6	5	5	20*
unique 7	Nd	Nd	Nd
unique 8	36	30	27

MLST: multi-locus sequence typing; PFGE: pulsed-field gel electrophoresis (after *sma*I restriction); ST: sequence type; CC: clonal complex; nd: not determined

*: unique PFGE types

Discussion

The primary aim of this study was to assess the performance and feasibility of the DiversiLab system for discrimination of MRSA isolates in a microbiological diagnostic routine setting and to compare evaluation criteria with these obtained with PFGE and MLST. The HARMONY isolates were collected to represent circulating nationwide epidemic nosocomial isolates from 1981 to 1998 in 11 European countries. These isolates represent the major clones causing hospital-acquired MRSA outbreaks in Europe (13).

PFGE (S*ma*I digestion) resolved 28 pulsotypes and MLST differentiated the collection into 16 STs representing 6 CCs. The HARMONY collection is divided into 3 genetic homogenous clonal clusters and 2 clonal clusters showing genetic heterogeneity. CC 30, CC 45 and CC 22 are very homogenous clusters, also defined with DiversiLab and PFGE. CC 5 and CC 8 are heterogeneous clusters, subdivided into several types with both MLST and PFGE, but not with rep-PCR. These observations confirmed the result of a previous *S. aureus* population structure analysis (21). DiversiLab results are fully concordant with the CC classification as defined with MLST, but displayed less concordance with STs and pulsotypes. The discriminatory power of the DiversiLab system was comparable to MLST, while PFGE was the most discriminatory technique. The acceptable level of discrimination will depend on a number of factors, such as

the epidemiological question, but a DI >0.900 might be desirable if the typing results are to be interpreted with confidence (22).

The reproducibility of the DiversiLab system was excellent. The feasibility of the DiversiLab system with respect to labour intensity and rapidity was good. The most labour-intensive step is the extraction, which requires almost half a working day hands-on time, depending on the number of isolates being tested. Some technical skill is necessary, especially for the preparation of the micro-fluidic chips used for separation and detection of PCR products. Occasionally, air bubbles form as DNA is loaded onto the chip. Costs of PFGE, MLST and DiversiLab are comparable and are approximately €46, €60 and €48 per sample, respectively. The rapidity of DiversiLab is better than MLST and PFGE. Total turn-around-time is one working day with a maximum of 36 samples, which is less than PFGE (3 to 5 days) and MLST (approximately 3 days). Web-based software is user-friendly, provides standardized comparisons among the isolates and generates customized reports.

In summary, DiversiLab is a rapid and non labour-intensive technique, but lacks resolution to differentiate genetically and epidemiologically unique MRSA strains, needed for outbreak analysis. It may be useful as a library typing system for long-term epidemiological studies.

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Chapter 7

Good performance of the SpectraCell*RA* system for typing of methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

After methicillin-resistant *Staphylococcus aureus* (MRSA) detection, typing remains necessary in order to assess whether transmission of MRSA occurred and to what extend infection prevention measures need to be taken. In this study, the SpectraCell*RA* system (SCRA; River Diagnostics, Rotterdam, The Netherlands) was evaluated for typing of MRSA strains. SCRA determines clonal relationships between bacterial isolates by Raman spectroscopy.

We have analyzed a well-documented MRSA collection of 113 MRSA strains, collected from 54 patients and their household members. The epidemiological relationship between the MRSA strains within one household was used as the "gold standard" as the *a priori* chance of being MRSA carrier is very low in The Netherlands. Discrepant findings between the result of SCRA and the epidemiological data were analysed using pulsed-field gel electrophoresis (PFGE) of *Sma*I macro restriction fragments of genomic DNA.

Results of SCRA analysis corresponded with epidemiological data for 108 of 113 strains, a concordance of SCRA and the gold standard of 95.6%. However, when results were analyzed at the household level, results of SCRA were correct for 49 out of 54 households; a concordance of 90.7%. This indicates that the discriminatory power of SCRA may be too high for adequate outbreak analysis. Reproducibility was found to be 100%.

We conclude that the SpectraCell*RA* system is a fast, easy to use and highly reproducible typing platform for outbreak analysis that can compete with the currently used typing techniques.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be a major problem in hospitals. Following laboratory detection of MRSA, typing remains necessary in order to assess whether transmission of MRSA occurred and what kind of infection prevention measures are needed. An ideal typing system for epidemiological surveillance of MRSA should be user-friendly, fast and reliable, combined with high-throughput capacity (1). However, next to these the two most important requirements for such a typing system are high discriminatory power and reproducibility (1). Currently, different typing methods are in use in the diagnostic laboratory, with pulsed-field gel electrophoresis (PFGE) of *SmaI* macro restriction analysis of genomic DNA still being considered as the gold standard for typing of bacteria because of its high discriminatory power (2-3). However, PFGE is technically demanding with limited portability due to low inter-center reproducibility, with a long time-to-result (4). Therefore, alternative typing techniques such as staphylococcus aureus protein A typing (*spa* typing), multi-locus sequence typing (MLST) or multi-locus variable number of tandem repeats analysis (MLVA) are used for outbreak analysis.

Raman spectroscopy (SpectraCell*RA* [SCRA], River Diagnostics, Rotterdam, The Netherlands) has been described as an adequate tool for typing of bacteria (5). This vibrational spectroscopy-based technique does not require any labels or dyes and is fast. Its high throughput and ease of use enables SCRA as a valuable tool for outbreak analysis in routine diagnostic laboratories.

Following detection of MRSA in many, if not all, Dutch hospitals the patient (primary case) is isolated to prevent transmission. No eradication therapy is offered during the in-hospital period due to lower success rates during this period. After discharge, eradication therapy is started as soon as the patient has no wounds, no antibiotics or drains or when there is no urgent need for treatment. In the Erasmus MC (Rotterdam, The Netherlands), all household members of the patient are tested for MRSA colonization before therapy. All positive household members (secondary cases) are then treated simultaneously with the index patient. Since the prevalence of MRSA is still low in The Netherlands (6-7), there is a very low *a priori* chance for these household members of acquiring MRSA from another source. Furthermore, Mollema et al showed in 2010 that all isolates from MRSA positive household members had the same PFGE type as the isolate from their index person (8). Therefore, the presence of MRSA carriers within one household is considered to be a consequence of household transmission

The primary aim of this study was to assess whether the SpectraCell*RA* system indeed can determine clonal relationships between MRSA strains isolated from household members infected or colonized with MRSA and determined the performance of this typing system. PFGE was used as a second typing method for verification purposes in case of discrepancies between SCRA typing and the gold standard of epidemiological data and initial PFGE results.

Materials and methods

Bacterial strains

A total of 113 MRSA strains from a well-documented set of 54 households were included in the analysis (8). Strains were collected in 2005, 2006 and 2007 from primary cases (index patient, n=54) and corresponding secondary cases (household members of index, n=59). Households consisted either of 4 members (n=3), 3 members (n=14), 2 members (n=22) or 1 member (n=15).

Cultures for MRSA were performed at the diagnostic laboratory of the Department of Medical Microbiology and Infectious Diseases (Erasmus MC). Results were confirmed by using Accuprobe (Gen-Probe Incorporated, San Diego, USA) and the MRSA-screen latex agglutination test (Denka Seiken Co., Tokyo, Japan). Furthermore, all isolated MRSA strains were sent to the National Institute for Public Health and the Environment (RIVM; Bilthoven, The Netherlands) for confirmation and PFGE-typing.

For all MRSA isolates, epidemiological data including household information, date of admission and sampling date and PFGE results were available. We defined all secondary cases as having acquired their strain from the index case of their household. Epidemiological household relations and PFGE results were defined as the gold standard. Verification of discrepant results between SCRA and the gold standard (epidemiological data + initial PFGE) was performed by new PFGE of *Sma*I macro restriction analysis of genomic DNA, as previously described (2). Furthermore, we analysed discrepant household members for possibilities of "exo-household" (from a source other than the household) acquisition of MRSA by looking into the epidemiological data.

SpectraCellRA analysis

Clonal relationships among the MRSA isolates were tested by SCRA. Cultures, sample preparation and SCRA measurements were performed according to the Operators Manual (version 1.7) (5) .

Briefly, isolates were inoculated on blood agar (BD Diagnostics). After incubation for 18-20 h, isolates were sub-cultured for 20 h on Trypticase Soy Agar (TSA agar) plates. Biomass was taken from this culture to fill a 1 µl loop. This biomass was suspended in 5 µl of distilled water and 3 µl of this suspension was transferred onto a MicroSlide sample carrier.

Similarities between spectra

Pearson correlation coefficients (R^2 values) were calculated between replicate measurements of the same isolate and between spectra of different isolates. The R^2 values between repli-

cates account for any signal variance due to differences in culturing, sample preparation, or actual Raman measurements. To be able to discriminate 2 isolates, the R^2 value between the isolates had to be lower than the R^2 values between replicate measurements of these isolates.

Hierarchical cluster analysis (HCA)

SCRA type analysis of sets of spectra was performed using the pair wise similarities as a distance matrix in combination with Ward's cluster algorithm. This results in a dendrogram in which each node represents the lowest correlation coefficient (or similarity) between all isolates combined in the cluster defined by this node.

Reproducibility

To establish reproducibility, 3 reference isolates were processed on 5 different, independent days during the study by 2 different technicians. The reference isolates were obtained from a reference MRSA strain collection that was used to study interlaboratory reproducibility of PFGE (4). Reference isolates 811 and 814 are genetically related isolates, reference isolate 806 was chosen as a unique isolate. Furthermore, 26 MRSA isolates from the study were analyzed in duplicate on 2 different days by 2 different technicians. Reference isolates and duplicates were performed as full biological replicates; isolates were processed from the freezer on different days of the study.

Workflow and costs

Estimation per test was made on the basis of total turn-around time, hands-on-time and costs (in Euro's) incurred by SCRA analysis. These parameters were then compared with those of PFGE.

Results

Similarities between spectra

The distribution of the R^2 values between replicates (red bars) and between isolates (blue bars) is given In **Figure 1**. When the R^2 value for two isolates is below 0.9995 (region 1), these isolates are non-related in the SCRA analysis. When two isolates have a R^2 value above 0.9996 (region 3), these isolates are indistinguishable in the SCRA analysis. R^2 values in between these values (region 2) indicate a possible relatedness between isolates. A similarity cut-off is chosen in region 2, based on the presumption that 95% of all replicates must have a R^2 value above this cut-off. This similarity cut-off in this study was set to 0.9996.

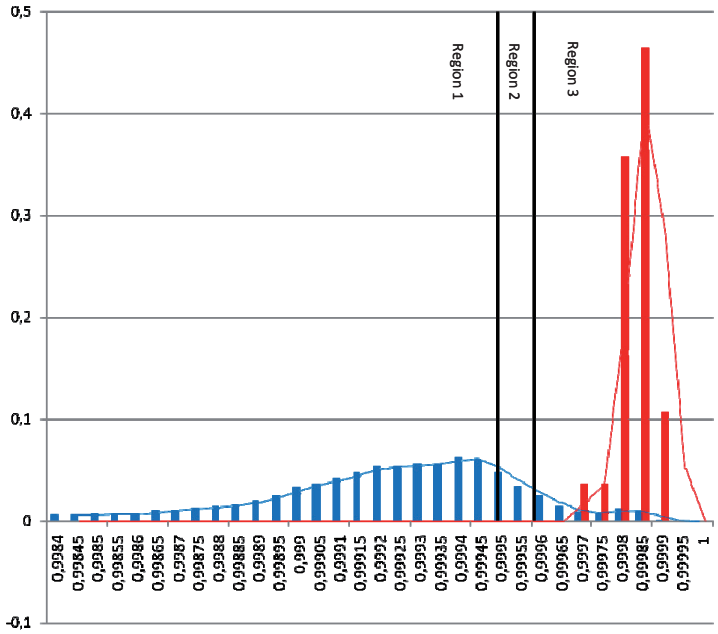

Figure 1. Histogram of R2 values found for our study.

R^2 values between replicates are indicated by red bars, R^2 values between isolates are indicated by blue bars. Region 1 indicates non-related isolates in the SCRA analysis. When isolates are in region 3, these isolates are indistinguishable in the SCRA analysis. Isolates in between these regions (in region 2), are possibly related. The cut-off is chosen in region 2, based on the presumption that 95% of all replicates must have a R^2 value above this cut-off. This similarity cut-off is set to 0.9996.

Hierarchical cluster analysis (HCA)

Among the 113 MRSA isolates, 59 SCRA types could be distinguished (**Table 1**). Based on the epidemiological data, results of SCRA analysis were correct for 108 of 113 strains. This results in a concordance of 95.6%.

When SCRA types were analyzed at the level of household clusters, results of SCRA analysis were concordant for 49 out of 54 households (90.7%).

All MRSA isolates within household cluster 14 (n=3) were non-typeable using PFGE. However, SCRA resulted in 1 SCRA type.

Discrepancy analysis

Five discrepant results were observed between SCRA analysis and epidemiological data. PFGE-typing results as reported by the RIVM of these discrepancies were fully concordant with epidemiological data. PFGE was performed on all the isolates from the discrepancy-households 7, 19, 20, 47 and 52. Household clusters 7, 19, 20 and 47 consisted of 1 primary case and 1 secondary case each. PFGE showed 2 identical isolates within each of these clus-

Table 1. Results of SCRA analysis.

Household cluster	Household members (n)	SCRA type	PFGE cluster as reported by the RIVM
1	3	2	155
2	2	57	55
3	4	11	18
4	3	10	18
5	3	21	27
6	2	69	37
7	2	48, 67	68
8	2	41	55
9	3	28	109a
10	2	43	587
11	3	9	23, 28
12	1	37	28
13	2	62	293
14	3	68	NT
15	1	34	263
16	1	3	15
17	3	16	23
18	4	39	22, 68, 665
19	2	47, 58	55
20	2	56, 59	55
21	2	52	50a, 675
22	1	19	22
23	1	60	23
24	2	22	24
25	1	51	25
26	3	50	26
27	2	65	27
29	2	35	50a
30	1	73	30
31	3	25	31
32	3	6	32
33	1	54	381
34	1	76	34
35	2	30	35
36	2	29	199
37	2	5	37
38	2	71	38
39	1	31	39
40	3	75	40
41	1	32	41
42	2	77	42
43	3	82	43
44	1	27	44
45	4	14	27, 28
46	2	12	46

Table 1. Contineud

Household cluster	Household members (n)	SCRA type	PFGE cluster as reported by the RIVM
47	2	4, 18	47
49	2	36	49
50	1	17	50
52	3	40, 45	52
54	2	61	54
55	2	55	55
57	3	49	15
58	1	79	58
59	1	80	59
Reference 806	5	29	
Reference 811	5	51	
Reference 814	5	50	

NT: Non-typeable with PFGE.

ters, whereas SCRA analysis resulted in 8 unique isolates. Household cluster 52 consisted of 3 members. For this household, PFGE showed 3 identical isolates, whereas SCRA resulted in 2 identical isolates and 1 unique isolate. Overall concordance of SCRA analysis was 95.6% on isolate level and 90.7% on household cluster level. Analysis of the discrepant household members for exo-household acquisition of MRSA did not result in any possible transmission of another known source or contact with a person with known risk factors as described in the Dutch national guidelines (WIP).

Reproducibility

Mean correlation coefficients and standard deviation (SD) were calculated for the 5 independent measurements of each reference isolate. For sample 806, 811 and 814 mean correlation coefficients (SD) were 0.9998 (±0.0), 0.9998 (±0.0) and 0.9999 (±0.0). Furthermore, all duplicate measurements of the 26 isolates resulted in identical results. Therefore, reproducibility was 100%.

Workflow

Total hands-on-time for 24 samples was around 3 h for SCRA and 7 h for PFGE. Total turnaround-time was 36-48 h for SCRA and 96 h for PFGE, with a maximum of 72 samples for SCRA and 50 samples for PFGE.

SCRA analysis requires a subculture on TSA agar on day 0 (15 min. for inoculation, 15 min. for documentation, 18-20 h incubation). On day 1, secondary subcultures on TSA were performed (15 min. for inoculation, 20 h incubation). Then, isolates were processed and

measured on day 2 followed by analysis of the results (30 min. for preparation of the slides, 60 min. for measurements and 30 min. for analysis).

Costs of SCRA and PFGE are comparable and are approximately €50 per sample, including personnel expenses and consumables.

Discussion

Results of SCRA analysis at the isolate level were concordant with the gold standard of epidemiology for 95.6% (108/113). However, when our results were analyzed at the household cluster level, results were concordant for 90.7% (49/54). In other words, when using SCRA in practice, for 9% of the small clusters SCRA would have given discrepant results. Analysis of discrepant results using PFGE showed equal results as epidemiological data. When the discrepant households were analyzed for possibilities of exo-household acquisition of MRSA, no possible transmission of another known source or contact with a person with known risk factors were found. This indicates that the discriminatory power of the SCRA system might be too high for adequate outbreak analysis of small clusters.

During transmission and acquisition of MRSA, micro-evolution may take place (9-10). PFGE may not be able to detect this micro-heterogeneity, where Raman spectroscopy does. However, since a household can be considered as a close community, we can assume that transmission and detection have occurred in a short time period. Therefore, it is very likely that the discrepancy between SCRA and the gold standard is not due to micro-evolution of the organism.

Multiple independent measurements of 3 reference isolates and the duplicate measurements of 26 MRSA strains resulted in a reproducibility of 100%. This indicates that SCRA is stable over a longer period of time, which has been published before (5). Typeability (as indicated by household cluster 14) was 100% for SCRA and <100% for PFGE.Our findings are similar to those found in another study, where the observed concordance between SCRA and the gold standard of epidemiological data and PFGE was 97% (11). Reproducibility in our study was better (100% vs. 95%). In both studies isolates were tested as full biological replicates at different points in the study.

PFGE is still being considered as the gold standard for typing of bacteria because of its high discriminatory power (2-3). However, PFGE is technically demanding with limited portability due to low inter-center reproducibility (4). Therefore, alternative typing techniques are currently used for outbreak analysis. However, although *spa* typing, MLST and MLVA have a good portability due to standard nomenclature, the discriminatory power of these methods is too limited for adequate outbreak analysis (12-13). Furthermore, these methods cannot be used in routine diagnostic laboratories easily because of the required technical expertise. A commercially available automated rep-PCR system, the DiversiLab system (bioMérieux,

Marcy l'Etiole, France), offers advances in ease-of-use and reproducibility of the procedure over manual typing systems (14). However, although three independent studies concluded that the Diversilab system is a rapid and reproducible technique, it clearly lacks resolution for typing of gram-positive bacteria such as MRSA (15-17).

The feasibility of the SCRA system with respect to hands-on-time (~ 3 h) and time-to-result (36-48 h) was good and better than PFGE. Time-to-result may be improved by applying 1 subculture instead of 2 subcultures. In this way, results will be available the next day, what is enormous valuable for prevention control.

Mistyping of strains by using the SCRA system for outbreak analysis is a risk. Isolates epidemiologically assigned to one cluster could now be considered as unique isolates and as a single event each, which has serious consequences for infection prevention measures. Prevention measures to a single event, or 2 separate single events, are different than the response on an outbreak (2 or more MRSA cases belonging to one cluster). For 90-95% of cases a considerable amount of time for implementing prevention measures is gained. For the remaining 5-10%, further analysis will be necessary.

Many studies on typing of MRSA focus on a small set of MRSA isolates and the relevant epidemiological data of the isolates are often (partially) unknown. We had the unique opportunity to use the epidemiological relationships within households as the gold standard, together with PFGE.

Although the high discriminatory power of SCRA may lead to discrepancies, and accompanying consequences, in 5-10% of epidemiologically related MRSA clusters, we conclude that the SpectraCell*RA* system is a highly reproducible, easy-to-use and fast typing platform that can compete with the currently used typing techniques.

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Quality control

Chapter 8

External Quality Assessment of molecular diagnostics and genotyping of methicillin-resistant *Staphylococcus aureus*

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Abstract

Two multicentre External Quality Assessments (EQA) for molecular detection and genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) were arranged.

Firstly, eleven samples containing various amounts of inactivated MRSA strains, methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant coagulase-negative staphylococci (MR-CoNS) or *Escherichia coli*, were distributed to 82 laboratories. Samples containing 10^2 or 10^3 MRSA cells were correctly scored in only 16% and 46% of the datasets returned, respectively. Two of the used MSSA strains contained a SCC*mec* cassette lacking the *mecA* gene. There was a marked difference in the percentage of correct results for these two MSSA strains (37% and 39%), compared to the MSSA strain lacking the SCC*mec* cassette (88%). The MRCoNS sample was correctly scored negative by 40 out of 45 (89%) commercial tests used and by 23 out of 33 (70%) in-house assays. The *E. coli* sample was incorrectly reported as positive in 9 (11%) of the datasets.

Secondly, a panel for MRSA genotyping, consisting of 10 samples (2 identical, 3 genetically related and 5 unique strains) was distributed to 19 laboratories. Seventy-three percent of the datasets recorded all samples correctly. Most pulsed-field gel electrophoresis (PFGE) protocols proved to be suboptimal, resulting in inferior resolution in the higher or lower fragment regions.

Performance of molecular diagnostics for MRSA showed no significant changes since our first EQA in 2006. The first molecular typing results are encouraging. Both assessments indicate that programme expansion is required and that major diagnostic and typing performance discrepancies still continue to exist between diagnostic microbiology laboratories.

Introduction

Adequate infection control of methicillin-resistant *Staphylococcus aureus* (MRSA) strongly depends on the speed and quality of (molecular) identification and characterisation strategies used by the clinical microbiological laboratory (1-2).

Over the past 4-5 decades, cultivation assays have been primarily used for detection and subsequent identification of MRSA. However, cultivation requires prolonged incubation periods, and, in general, clinically relevant methicillin resistance still needs to be confirmed by the detection of the *mecA* gene or its product. Nucleic acid amplification techniques (NAATs) offer benefits over traditional culture-based assays, in particular a reduced time to identification and an improved specificity and sensitivity. Over the past decade, a range of commercial and in-house developed NAATs has been introduced. Sensitivity and specificity of these assays may be compromised, as a result of inhibition or false-positivity due to the presence of methicillin-resistant Coagulase Negative *Staphylococci* (MRCoNS) or variability within the *mec*-resistance cassette. This may lead to false-negative results (new Staphylococcal Cassette Chromosome mec [SCC*mec*] variants) or false-positive results (deletion of the *mecA* gene) (3-6). After MRSA detection, genetic typing may be necessary to assess whether local cross infection occurs and whether preventive measurements are mandatory.

Currently, many different genotyping methods are in use in the diagnostic laboratory, but pulsed-field gel electrophoresis (PFGE) of *Sma*I digested genomic DNA still remains the most frequently used method (7). Only when outbreaks are properly defined, adequate infection control measurements can be implemented.

The current multicentre External Quality Assessment (EQA) study determined the performance of molecular assays to detect MRSA and genotyping techniques to differentiate MRSA strains. The studies were coordinated by Quality Control for Molecular Diagnostics (QCMD) in Glasgow, Scotland.

Materials and methods

EQA for molecular MRSA detection and identification

In August 2009, the EQA MRSA panel for MRSA detection and identification was distributed to 80 participating laboratories in 15 countries, along with detailed sample processing instructions. Participants were given 6 weeks to examine the samples and to report their results to QCMD by using an online data collection system. The QCMD MRSA panel consisted of six samples containing 10^6, 10^5 (n=2), 10^4, 10^3 and 10^2 CFU/ml MRSA bacterial cells, one methicillin-susceptible *S. aureus* (MSSA) sample, one sample containing MRCoNS, two samples containing MSSA harbouring a SCC*mec* cassette lacking the *mecA* gene and one sample

containing *Escherichia coli* (**Table 1**). The production laboratory quantified the contents of the samples on the basis of colony counting, optical density measurements and real-time molecular amplification results. All bacterial samples were heat-inactivated for 10 minutes at 100°C.

Table 1. Composition of the QCMD 2009 panel for MRSA detection and identification.

Sample	Sample content	Target value CFU/ml	Sample status
MRSA09-01	MRCoNS c	1x10E7	Negative
MRSA09-02	MSSA d	5x10E6	Negative
MRSA09-03	MSSA b	5x10E6	Negative
MRSA09-04	MRSA a	5x10E5	Positive
MRSA09-05	MRSA a	5x10E6	Positive
MRSA09-06	MRSA a	2.5x10E2	Positive
MRSA09-07	MRSA a	2.5x10E4	Positive
MRSA09-08	MRSA a	5x10E5	Positive
MRSA09-09	MSSA e	1x10E7	Negative
MRSA09-10	MRSA a	2.5x10E3	Positive
MRSA09-11	*E. coli* f	5x10E6	Negative

MRSA a: Methicillin-resistant *Staphylococcus aureus* N315.
MSSA b: Methicillin-sensitive *Staphyloccocus aureus* ATCC 29213.
MRCoNS c: Methicillin-resistant Coagulase-negative Staphylocci 634.
MSSA d: Methicillin-sensitive *Staphyloccoous aureus* (mecA negative 92-1866 [SCCmec III]).
MSSA e: Methicillin-sensitive *Staphyloccoous aureus* (mecA negative 93-2886 [SCCmec I]).
E. coli f: *Eschericia coli* ATCC 35218.

EQA for MRSA genotyping

The EQA panel for MRSA genotyping was distributed to 19 participants in 8 countries in August 2009. The panel consisted of 10 samples of viable MRSA strains in Müller Hinton broth. Genetic relatedness of the MRSA panel was originally determined with PFGE (8). The current panel consisted of two identical strains, three genetically related strains and 5 unique strains (**Table 2**). Genotype and subtype were reported by the production laboratory. A different letter signified the detection of a different genotype, whereas a different number signified the detection of a different subtype. All data were reported in relation to the reference strain in panel sample MRSATP09-01.

The QCMD Neutral Office analysed the data, which was anonymously released to all participants in a detailed EQA final report.

Table 2. Genotyping results per panel member and technology type of the QCMD 2009 panel for MRSA genotyping.

		Total datasets		PFGE a		AFLP b		SPA c	
	Expected	n=15		n=11		n=2		n=2	
Sample	genotype	n	%	n	%	n	%	n	%
MRSATP09-01	A	15	100	11	100	2	100	2	100
MRSATP09-02	B	11	73.3	8	72.7	1	50.0	2	100
MRSATP09-03	C	14	93.3	11	100	1	50.0	2	100
MRSATP09-04	D	14	93.3	11	100	1	50.0	2	100
MRSATP09-05	A	13	86.7	10	90.9	1	50.0	2	100
MRSATP09-06	E	13	86.7	11	100	2	100	0	0.0
MRSATP09-07	F	12	80.0	9	81.8	1	50.0	2	100
MRSATP09-08	G	14	93.3	11	100	1	50.0	2	100
MRSATP09-09	G1	11	73.3	11	100	0	0.0	0	0.0
MRSATP09-10	G2	11	73.3	11	100	0	0.0	0	0.0

PFGE a: bioRadGenePath Group 1 reagent kit (n=2), in-house PFGE (n=8) and Double Locus Sequence Typing (n=1).
AFLP b: In-house AFLP (n=2).
SPA c: In-house *spa* typing (n=2).

Results

EQA for molecular MRSA detection and identification

Out of the 80 participants, 68 (85%) responded. Twelve participants did not return results. Five of these withdrew officially, indicating 'assay not offered' (n=2), 'internal issues' (n=1) and 'other' (n=2) as the reason for withdrawal.

The following commercial amplification assays were used for MRSA detection: BAG Healthcare hyplex Staphyloresist (n=1) (BAG Healthcare, Lich, Germany), BD Diagnostic GeneOhm MRSA Assay (n=13) (BD Diagnostics – GeneOhm, San Diego, California), BD Diagnostics GeneOhm Staph SR Assay (n=5) (BD Diagnostics – GeneOhm), Cepheid IDI MRSA (n=1) (Cepheid, Sunnyvale, California), Cepheid Xpert MRSA Test (n=11) (Cepheid), Cepheid Xpert MRSA/SA Test (n=2) (Cepheid), Roche LightCycler MRSA Advanced Test (n=4) (Roche Diagnostics, Basel, Switzerland), TIB MOLBIOL LightMix Kit MRSA (n=2) (TIB MolBiol, Berlin, Germany) and Hain Lifescience GenoQuick MRSA (n=2) (Hain Lifescience, Nehren, Germany). This diversity overlaps with the spectrum of currently available commercial tests. All results are summarised in **Table 3**.

Results for the panel samples with 10^6 MRSA cells (MRSA09-05), 10^5 MRSA cells (MRSA09-04 and MRSA09-08) and 10^4 MRSA cells (MRSA09-07) were reported correctly in 100%, 100%, 99% and 98% of the datasets, respectively. The samples containing lower amounts, MRSA09-10 (10^3 CFU/ml) and MRSA09-06 (10^2 CFU/ml), were reported correctly in only 46% and 16% of the datasets, respectively. No statistically significant differences in sensitivity or specificity could be seen between the different tests or between commercial and in-house testing. MR-

CoNS sample MRSA09-01 was correctly reported as MRSA negative by 87% (40 out of 46) of the commercial PCR tests and in 70% (23 out of 33) of the in-house PCR assays. MSSA sample MRSA09-03 was correctly reported as MRSA negative by 89% (41 out of 46) of commercial PCR tests and in 85% (28 out of 33) of datasets generated by in-house PCR assays, respectively. The MSSA samples containing the SCC*mec* cassette but lacking the *mecA* gene (MRSA09-02 and MRSA09-09) were both incorrectly reported as positive by commercial PCR tests in 87% (40 out of 46). For in-house assays these samples were reported incorrectly in 24% (8 out of 33) and 30% (10 out of 33), respectively. These percentages of incorrect results underscore the need for improved specificity of these MRSA tests and therefore positive results should always be confirmed by a culture method or a second molecular test. For laboratories with high false positivity rates or in regions with low prevalence of MRSA, confirmation is essential (9). For the *E. coli* sample, commercial PCR results were reported correctly in 87% (39 out of 45), whereas in-house PCR tests recorded correct results in 97% (29 out of 30).

Table 3. Number of correct qualitative results per panel member and technology type on the QCMD 2009 panel for MRSA detection and typing.

Sample	Sample content	Total		PCR								NASBA e		Other f	
				Conventional				Real-time							
		datasets		Commercial a		In-house b		Commercial c		In-house d					
		n=82		n=1		n=3		n=45		n=30		n=1		n=2	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%
MRSA09-05	MRSA	**82**	**100**	**1**	**100**	**3**	**100**	**45**	**100**	**30**	**100**	**1**	**100**	**2**	**100**
MRSA09-04	MRSA	**82**	**100**	**1**	**100**	**3**	**100**	**45**	**100**	**30**	**100**	**1**	**100**	**2**	**100**
MRSA09-08	MRSA	**81**	**98.8**	**1**	**100**	**3**	**100**	**44**	**97.8**	**30**	**100**	**1**	**100**	**2**	**100**
MRSA09-07	MRSA	**80**	**97.6**	**1**	**100**	**3**	**100**	**45**	**100**	**28**	**93.3**	**1**	**100**	**2**	**100**
MRSA09-10	MRSA	**38**	**46.3**	**0**	**0.0**	**2**	**66.7**	**22**	**48.9**	**13**	**43.3**	**0**	**0.0**	**1**	**50.0**
MRSA09-06	MSSA	**13**	**15.9**	**0**	**0.0**	**0**	**0.0**	**6**	**13.3**	**6**	**20.0**	**0**	**0.0**	**1**	**50.0**
MRSA09-03	MSSA	**72**	**87.8**	**1**	**100**	**3**	**100**	**40**	**88.9**	**25**	**83.3**	**1**	**100**	**2**	**100**
MRSA09-09	MSSA	**30**	**36.6**	**1**	**100**	**3**	**100**	**5**	**11.1**	**20**	**66.7**	**1**	**100**	**0**	**0.0**
MRSA09-02	MSSA	**32**	**39.0**	**1**	**100**	**3**	**100**	**5**	**11.1**	**22**	**73.3**	**1**	**100**	**0**	**0.0**
MRSA09-01	MRCoNS	**66**	**80.5**	**0**	**0.0**	**2**	**66.7**	**40**	**88.9**	**21**	**70.0**	**1**	**100**	**2**	**100**
MRSA09-11	*E. coli*	**73**	**89.0**	**1**	**100**	**1**	**33.3**	**39**	**86.7**	**29**	**96.7**	**1**	**100**	**2**	**100**

Commercial a: BAG Healthcare hyplexStaphyloresist (n=1).

In-house b: Details not presented.

Commercial c: BD Diagnostics details not provided (n=7),BD Diagnostics GeneOhm MRSA Assay (n=13), BD Diagnostics GeneOhm Staph SR Assay (n=5), Cepheid IDI MRSA (n=1), Cepheid Xpert MRSA Test (n=11), Cepheid Xpert MRSA/SA Test (n=2), Roche LightCycler MRSA Advanced Test (n=4), TIB MolBiolLightMix Kit MRSA (n=2).

In-house d: Details not presented.

NASBA e: Details not presented.

Other f: HainLifescienceGenoquick MRSA (n=2).

EQA for MRSA genotyping

Out of the 19 potential participants, 14 (74%) responded. Four of the non-responders withdrew officially indicating 'panel used for research' (n=1) and 'assay not offered' (n=3). The

majority of datasets were generated by PFGE (n=11), with the remainder generated by AFLP (n=2) and spa typing (n=2). Only eight participants (73%) scored all samples correct: all with PFGE (**Table 2**).

Discussion

To maintain high-quality clinical care, quality control of molecular diagnostics is very important. The primary aim of our EQA programme was to assess the proficiency of laboratories in the molecular detection and characterisation of MRSA strains. Here, we conclude that the molecular detection of MRSA in samples with high CFU counts is reliable, which can and has been implemented in various laboratory settings with confidence. All tests performed equally well. However, for direct molecular diagnostics, we have to conclude that current commercial and in-house tests do not meet the requested quality criteria. The sensitivity of many tests is relatively low and the specificity needs to be improved. The FDA submission in 2004 of the IDI-MRSA test had a detection limit of 325 CFU/swab (10). This is much more sensitive than documented in this study. Pre-enrichment of clinical samples leads to concentrations of MRSA exceeding 10^9 CFU/ml, which is higher than the concentrations of MRSA likely to be found in a patient sample, and those in this EQA panel. However, pre-enrichment reduces one of the major advantages offered by NAATs, namely rapidity. In this year's panel, only inactivated cells were present. As a consequence, pre-enrichment was not possible. This may have influenced the results of some laboratories. For the years to come, viable cells will be distributed, which is more similar to the real clinical situation.

This is the fourth year that QCMD has offered the MRSA DNA EQA Programme and the number of participants has increased from 51 in 2006, 61 in 2007 and 74 to 2008 to 80 in 2009 (11). Over the years, we observe a statistically non-significant decrease in overall test sensitivity. The most pronounced discrepancies were observed in the low concentration panel samples (10^3 and 10^2 CFU/ml). Conversely, the percentage of correct results showed an overall improvement for the "specificity" samples (containing MSSA and MRCoNS or *E. coli*) and the "true negative" samples (**Table 4**). Again, this was not statistically significant. Still, these incorrect results underscore the need for improved specificity of molecular MRSA tests and therefore positive results should always be confirmed by a culture method or a second molecular test. For laboratories with high false positivity rates or in regions with low prevalence of MRSA, confirmation is necessary.

In 2009, two MSSA samples harbouring a SCC*mec* cassette, but lacking the *mecA* gene, were included. There was a marked difference in the percentage of correct qualitative results for the MSSA strain containing the *mecA* gene compared to the two strains lacking it. These data show that confirmation on results generated using assays that only target the SCC*mec* cassette is mandatory.

Table 4. Comparison of performance on like samples in the QCMD 2006, 2007, 2008 and 2009 MRSA EQA Programmes

Sample content	Sample concentration	Percentage correct results			
	CFU/ml	2006	2007	2008	2009
MRSA a	1x10E9	96.6	96.3	93.2	NIP
MRSA a	5x10E6	82.8	96.3	91.9	100
MRSA a	5x10E5	NIP	NIP	NIP	100
MRSA a	5x10E5	NIP	NIP	NIP	98.8
MRSA a	2.5x10E4	NIP	NIP	NIP	97.6
MRSA a	2.5x10E3	51.7	72.2	54.1	46.3
MRSA a	1x10E3	51.7	66.7	55.4	NIP
MRSA a	2.5x10E2	12.1	37.0	20.3	15.9
MSSA b	1x10E9	87.9	92.6	89.2	NIP
MSSA b	5x10E6	NIP	NIP	NIP	87.8
MSSA b	1x10E3	NIP	94.4	95.9	NIP
MRCoNS c	1x10E7	82.8	88.9	94.6	80.5
MSSA b + MRCoNS c	1x10E3 + 1x10E5	94.8	83.3	86.5	NIP
MSSA b + MRCoNS c	1x10E3 + 1x10E4	96.6	77.8	83.8	NIP
MSSA d	5x10E6	NIP	NIP	NIP	36.6
MSSA e	1x10E7	NIP	NIP	NIP	39.0
E. coli f	5x10E6	93.1	92.6	97.3	89.0
S. aureus Neg medium	-	NIP	92.6	98.6	NIP

NIP: not in panel.

MRSA a: Methicillin-resistant *Staphylococcus aureus* N315.

MSSA b: Methicillin-sensitive *Staphyloccoous aureus* ATCC 29213.

MRCoNS c: Methicillin-resistant Coagulase-negative Staphylocci 634.

MSSA d: Methicillin-sensitive *Staphyloccoous aureus* (mecA negative 92-1866 [SCCmec III]).

MSSA e: Methicillin-sensitive *Staphyloccoous aureus* (mecA negative 93-2886 [SCCmec I]).

E. coli f: *Eschericia coli* ATCC 35218.

In conclusion, the quality of molecular diagnostic tests still needs improvement and proper and regular quality control and international standardisation for MRSA diagnostics should be mandatory for the years to come.

We present the first QCMD EQA program for the genetic characterisation of MRSA strains. Clear differences in resolution were observed between the datasets. Some PFGE protocols, which were implemented by most of the participating laboratories, proved to be suboptimal as a low level of discrimination in the high and/or low molecular weight fragments was observed in the majority of the results reported. This suggests the need for optimisation of the PFGE program. This lack of resolution was most evident within the group of closely related MRSA strains in the panel (MRSATP09-08, MRSATP09-09 and MRSA09-10). These strains were incorrectly reported in 27% of datasets. Participants reported using a range of criteria for determining genotype and subtype. The guidance according to Tenover *et al* was the most prominent method (12).

To improve the performance and quality of molecular diagnostics, both laboratories and manufacturers should be encouraged to participate in EQAs. The availability of EQA

panels for detection and typing should also be developed for other important (nosocomial) infectious agents including vancomycin resistant *enterococci* (VRE) and extended spectrum β-lactamase (ESBL) producing *Enterobacteriaceae*.

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Discussion and future perspectives

Chapter 9

New clinical microbiological diagnostics

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Abstract

Current diagnostics of infectious diseases is based on conventional microscopy and culture-methods. Essentially, laboratories can perform simple tests on a large number of clinical samples on a daily basis, using inexpensive culture media and simple techniques. This strategy also provides bacterial isolates, which can be further characterized e.g. more detailed identification of the species, analysis of their antibiotic susceptibility profiles and, in special cases, epidemiological typing for outbreak analysis can be obtained. Many new diagnostic techniques have been developed during the past two decades, all with the objective to save time and to improve accuracy. Next to nucleic acid amplification techniques, there is a trend towards biophysical technologies such as matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), the "electronic nose" and Raman spectroscopy. These will be discussed in this chapter.

Multiplex screening offers significant advantages with respect to time, costs, sample requirements and the amount of data that can be generated. Many different strategies are (highly) suited for multiplex detection and candidates for implementation in clinical diagnostic laboratories are discussed in this chapter as well.

Molecular diagnostics

Introduction

Pathogens can be detected in different ways in the daily routine of a clinical microbiological laboratory. Most prominently used methods are culture, microscopy and (indirect) antigen- and antibody detection. Molecular diagnostics can also be of great value to clinical microbiological diagnostics. By using molecular techniques, pathogens can be detected by species-unique nucleic acid targets. Within the molecular diagnostics laboratory, Polymerase Chain Reaction (PCR) is the most widely used method (1). Real-time PCR can be considered as the most important recent innovation within microbiological diagnostics (2-4). This technique combines target amplification with detection of the product by using a fluorescent probe in a closed system. Amplification and detection take 1-2 hours, which is faster than conventional PCR and corresponding post-amplification process. The risk of contamination is marginal and performing real-time PCR demands less hands-on-time and expertise.

In contrast, there are drawbacks for PCR. There is a chance of false-positive (low specificity, contamination) or false-negative (low sensitivity, variability in the target, inhibition) results.

Bacterial typing

After detection and identification of (drug resistant) bacteria, typing is necessary to assess the occurrence of bacterial transmission and whether infection-prevention measures are mandatory. In addition, typing is essential for elucidation of local or (inter)national dissemination of clones. Currently, many different genotyping methods are in use in the diagnostic laboratory. Examples are pulsed-field gel electrophoresis (PFGE) of macro restriction fragments of genomic DNA, multi-locus variable number of tandem repeat analysis (MLVA) and direct nucleotide sequence-based approaches, such as *spa*-sequence typing and multi-locus sequence typing (MLST) (**Table 1)**. Due to the complex, expensive and technical protocols of these techniques, typing is mainly performed retrospectively and only when there is a sufficient number of cases (e.g. ≥5 patients).

Multiple new diagnostic techniques have been developed during the last decade and there is an increasing interest in biophysical techniques. These analytical platforms are more and more adapted for identification and typing of microorganisms. A significant example is matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). This technique identifies microorganisms by their proteomes. Another example with potency in microbiology is the "electronic nose"; biosensors that recognize specific bacterial odours by which bacteria can be identified. For bacterial typing, Raman spectroscopy will be discussed in this chapter. With this technique it is possible to determine clonal relationships (also called typing) between bacteria, but also between isolates of specific yeast or fungus species.

Finally, different candidates for implementation of rapid multiplex screening in clinical diagnostic laboratories will be discussed.

Table 1. Comparison of the main currently available molecular typing methods.

Method	***Principle/target***	***Strengths***	***Weaknesses***	***Example(s)***	***References***
Pulsed-field gel electrophoresis (PFGE)	Restriction polymorphism of the whole genome	- High discriminatory power	- Technically demanding - Slow - Limited portability - Multiple nomenclatures	MRSA, *Pseudomonas aeruginosa*	(5-8)
Multilocus sequence typing (MLST)	Sequence determination of allelic variants of housekeeping genes	- Phylogenetic structure of core genome - Portability - Standard nomenclature	- Limited discriminatory power - Low throughput - Expensive	*Candida albicans*, MRSA	(5-7)
spa-sequence typing (for *S. aureus* typing only)	Polymorphism of number and sequence of repeat elements of the hypervariable gene	- Rapid - High throughput - Portability - Standard nomenclature	- Moderate discriminatory power - Misclassification of STs* due to recombination	*S. aureus* (MRSA)	(8-9)
rep-PCR typing (Diversilab)	Polymorphism in chromosomal inter-repeat element spacers	- Rapid - High throughput - Portability	- Limited discriminatory power for gram positive cocci - No validated interpretation criteria - No standard nomenclature	MRSA, multi-drug resistant bacteria, *P. aeruginosa*	(10-13)
Multilocus VNTR analysis (MLVA)	Polymorphism in number of chromosomal VNTR** elements	- Rapid - High throughput - Standard nomenclature	- Limited discriminatory power	*Mycobacterium tuberculosis*, *Bacillus anthracis*, MRSA	(14-18)

* ST: Sequence Type

** VNTR: Variable Number of Tandem Repeat.

MALDI-TOF MS

Introduction

MALDI-TOF MS is a relatively new technology within microbiology (5-6). The most common bacterial pathogens, yeasts and fungi that are currently isolated from clinical samples in a clinical microbiology laboratory can be identified using MALDI-TOF MS (e.g. *Enterobacteriaceae*, staphylococci, streptococci and *Nocardia* spp) (7-8). The practical procedure of MALDI-TOF MS is simple and robust. The sample is transferred onto a sample slide and the cells are embedded into a matrix. The principle of the procedure is illustrated in **Figure 1**.

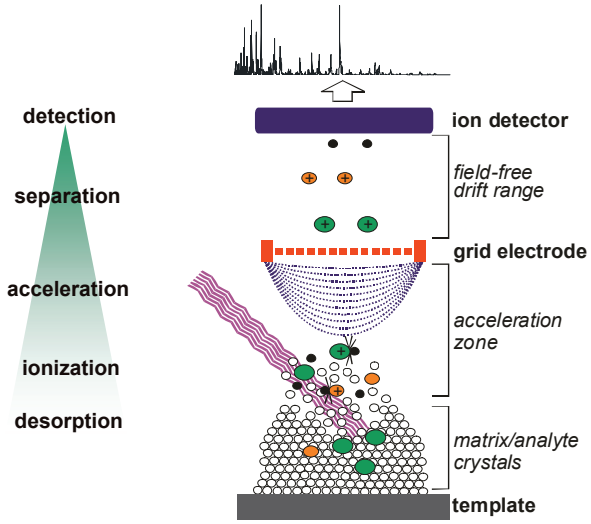

Figure 1. Principle of MALDI-TOF MS (Adapted with permission from bioMerieux, Marcy l'Etoile, France).

The matrix is essential for two reasons. Firstly, the matrix must absorb the energy of the laser and, secondly, the matrix stimulates ionisation of the microbial fragments after laser excitation. The ions that form are accelerated in an electromagnetic field between the sample and a detector. After passing two electrodes, the ions are transferred into a vacuum tube, which contains a detector at the end. The "time-of-flight" of the ions is inversely proportional to the mass-charge ration of the ions. A complete cycle consists of desorption, ionisation, acceleration, separation and detection. Depending on the microorganism, the sample is processed 40-100 times to accumulate a stable, average mass spectrum. The actual data acquisition with MALDI-TOF MS is performed in an automated way. The laser scans the sample and accumulates a number of mass spectra by launching a defined number of laser pulse cycles.

In **Figure** 2, the different spectra that can be observed between different species are illustrated.

Certain bacterial species and yeasts are more difficult to identify with MALDI-TOF MS than via direct transfer of the colony. Special protocols have been developed to improve the quality of the spectra. These protocols use chemical agents to break the cell walls to expose intracellular proteins, which greatly improves the ability of MALDI-TOF MS to identify yeasts (8-9).

The essential step for species identification is comparing the unknown mass spectrum of the sample to a database containing reference mass spectra. Obviously, the quality and completeness of such a database are crucial for an optimal performance. This requires reference mass fingerprints of all species of interest and mass fingerprints of multiple strains per species (10).

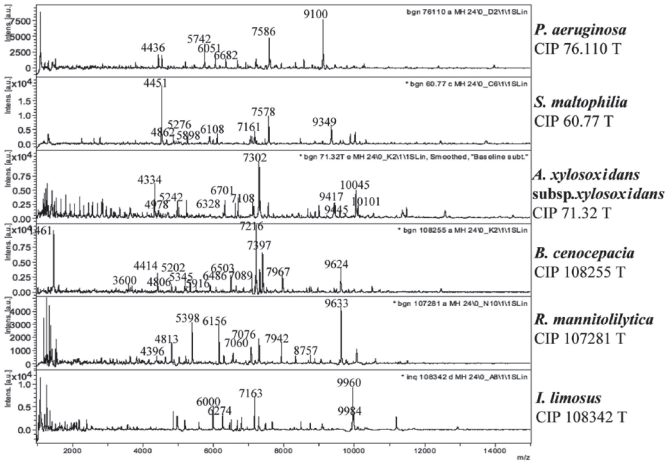

FIG. 1. MALDI-TOF-MS spectra of six reference strains.

Figure 2. Examples of MALDI-TOF MS generated spectra. Distinct differences between different, related species can be observed (Adapted with permission from Ferroni et al).

During the last five years, two commercial systems with extended and secure databases have been developed for routine diagnostics: MALDI Biotyper from Bruker (Bremen, Germany) and Vitek MS from bioMérieux (Marcy l'Etoile, France) (29-30). With both systems, batches of 100 samples can be analyzed simultaneously in less than one hour; the analysis of 1.000 samples per day with a single instrument is realistic. Throughput may be enhanced even further by efficient automation of the sample preparation process.

Possible drawbacks

Naturally, MALDI-TOF MS-based identification of microorganisms has limits. Some limits are of a technical nature and include the amount of biomass required for a reliable result (10^4-10^5

cells). Consequently, culturing of bacteria prior to identification is needed, which results in delay in diagnosis. Another technical drawback is variability between duplicate measurements (11). Other limits are of a biological nature and concern the taxonomic resolution that can be achieved. Examples are the failure to discriminate between *Escherichia coli* and *Shigella* spp. or difficulties with discriminating species of the *Streptococcus mitis* complex (7, 12). Furthermore, "coagulase negative staphylococci" and "Gram-negative non-fermenting bacteria" can be identified difficulty by using MALDI-TOF MS. On the one hand, the analyzed ribosomal proteins provide little discriminatory information; on the other hand this may be caused by an incomplete database.

However, in the near future, these shortcomings can and will be solved. Moreover, the applicability of MS for clinical microbiological diagnostics will be expanded further. A number of studies has evaluated the possibility to differentiate drug resistant strains from sensitive strains of the same species, such as methicillin resistant *S. aureus* (MRSA) and methicillin sensitive *S. aureus* (MSSA) (13-14). Also, an approach to detect enzymes that are responsible for antibiotic resistance has been published (15).

Multiple studies have evaluated applications of MALDI-TOF MS to analyze mixed bacterial species (16-17). Despite the fact that two or three bacterial species can be separated and identified simultaneously, the discriminatory power of MALDI-TOF MS is restricted when one single species predominates in the mixture. The background caused by commensal bacteria hinders the detection of potential pathogens in clinical samples. For urinary tract infections (UTI), where usually one single species predominates, reliable identification of UTI causing pathogens has been described (18). Identification of pathogens directly from patient blood is difficult due to the low titre, but recently pathogenic bacteria have been efficiently detected by MS directly from positive blood cultures (19).

Currently, possibilities for typing with MALDI-TOF MS are limited as a consequence of the limited resolution. Optimization of sample preparation and new approaches in data analysis or MS technology will possibly allow the discriminatory power of MALDI-TOF MS to increase.

Conclusion

MALDI-TOF MS is currently settling in clinical microbiological diagnostics. During the next 5 years, currently used biochemical identification techniques will be replaced by a combination of MALDI-TOF MS and automated antibiotic susceptibility testing, such as Vitek (bioMérieux) or Phoenix (BD Diagnostics, Breda, The Netherlands). The addition of automated sample preparation will make MALDI-TOF MS an indispensible identification platform in clinical microbiology.

Electronic nose

Introduction

One of the aspects of conventional bacteriological diagnostics is the specific odour of some bacterial species, such as *Clostridium difficile* (smells like "stable"), *Haemophilus influenzae* ("burned caramel") and *Pseudomonas aeruginosa* ("blossom"). Based on the simplicity and ease-of-use of odour-based diagnostics, this may be an attractive alternative for identification of bacteria.

The concept of an electronic nose was developed during the seventies of the 20th century. The availability of computers made it possible to recognize patterns, analogous to human smell perception, when measuring complex mixtures of compounds with biosensors. Hence the name electronic nose.

The metabolism of microorganisms proceeds via different routes, depending on the (genetic) possibilities of a microorganism and on the available nutrients in the environment. As a consequence, microorganisms produce a wide variety of volatile organic compounds (VOCs) during growth. By measuring these VOCs, analysis of the metabolic activity can lead to the development of a fast identification system.

E-nose

In all papers describing the classification of bacteria with electronic noses the analysis models used a single sensing device (20-21). A company called C-it (Zutphen, The Netherlands) has developed an electronic nose, called the MonoNose, where complex bacterial VOC signals are measured using multiple metal-oxide sensors (22). The MonoNose is a broadly applicable, inexpensive system, which uses real-time VOC pattern recognition and matching of this pattern with a previously identified reference database (**Figure 3**).

In the already mentioned study, eleven different, clinically relevant bacterial species were measured. A total of fifty-two clinically relevant strains were tested, showing diagnostic specificity to be 100% for *Clostridium difficile* to 67% for *Enterobacter cloacae*, with an overall average of 87%. Thanks to the continuous measuring process, reliable identification can be achieved in 4-8 hours. This research was performed on pure cultures and not directly on clinical samples, so an extra culturing step is necessary to identify the potential causative agents. Multiple clinical samples, such as urine, and blood, need to be tested to determine the influence of the sample matrix and the possibilities and drawbacks of direct measurements.

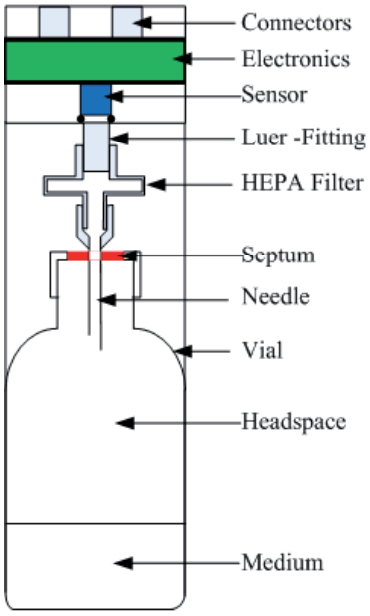

Figure 3. Composition of the MonoNose (Adapted with permission of C-it, Zutphen, The Netherlands).

Possible drawbacks

VOC-based identification of microorganisms has limitations. The available nutrients in the medium will be depleted faster when applying a high inoculum. In this way, there is a need to switch metabolism route, which will change the analysis (time) of the E-nose. Obviously, a higher inoculum will lead to faster identification. The relation between incubation temperature and replication speed is known and will lead to an altered VOC pattern. This pattern depends on the presence and concentration of nutrients in the medium. Furthermore, these influences are organism dependent, which leads to more challenges in creating a reference database.

Conclusion

Above-mentioned factors are crucial for the future of electronic noses for (direct) identification of microorganisms. Direct real-time identification from clinical samples may be a real point-of-care test. An example of the applicability of the electronic nose is a bench-top blood culturing machine; blood cultures can be incubated and analysed next to the patient. Another example can be a surgical bandage with an incorporated nose that gives a signal far before clinical symptoms appear and can be observed by medical staff. A final example may be the direct analysis of expiration gasses from a patient to diagnose pneumonia or tuberculosis.

Raman spectroscopy

Introduction

The Centre for Optical Diagnostics and Therapy (CODT) of the Erasmus MC in Rotterdam develops analytical and therapeutic applications based on Raman spectroscopy. The department cooperates with pathology and urology (healthy and sick tissue differ in molecular composition), dermatology (diagnosis and classification of skin tumours and multiple skin diseases) and medical microbiology (molecular composition of microbial samples, species identification and typing of bacteria) (23-25).

Raman spectroscopy is named after its discoverer, the Indian physicist Chandrasekhara Venkata Raman. In 1928, Raman was the first to find experimental proof for in-elastic scattering of light through matter. For this discovery "*of extraordinary great importance for our knowledge of the structure of molecules*", Raman received the Nobel Prize for physics in 1930.

Principle of Raman spectroscopy

When matter in general or molecules in particular, are radiated with (monochromatic) light, most photons will be scattered with the same, identical wavelength. This process is called elastic scattering. However, a very small fraction of the light will be scattered in-elastically and will, thanks to loss of energy, differ from the incident light. This process is called Raman scattering (**Figure 4**).

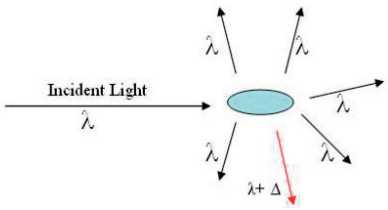

Figure 4. Raman scattering. The incident light λ is being scattered via the molecule, which results in light with the same wavelength (λ), and, in a very small fraction, in a shift in wavelength (λ+Δ).

Atoms in a molecule vibrate around their equilibrium state. When Raman scattering occurs, an incident photon will transfer a small fraction of its energy onto the molecule. This results in elevated vibration(s) and in a photon with a lower energy. This lower energy results in a shift in wavelength, which can be measured by the detector of the Raman spectrometer. The amount of energy needed to ensure molecular vibration of the molecule depends on the mass of the atoms, their chemical bonds, the molecular structure, the micro-environment (such as pH) and the interactions of the molecule with the environment. This all explains the molecular specificity of Raman spectra.

To exactly determine the shift(s) in wavelength between incident and scattered light, monochromatic light is used to create a Raman spectrum of a sample. All molecules of a sample (e.g. a bacterial cell) contribute to the total Raman spectrum, where the intensity of the Raman signal depends on the concentration.

Instrument

Nowadays, a Raman spectroscope is relatively simple and consists of 4 base components: a laser (monochromatic light), a sample place (radiation of the sample), a spectrometer (detection of scattered light) and a computer (analysis of the collected spectra). Since Raman spectroscopy does not use labels or dyes, a highly pure sample is essential. Any kind of contamination contributes to the Raman spectrum and will interfere with (the result of) the analysis.

A high reproducibility is important to maintain the variance within one group smaller than the variance between two groups. Only then two (closely) related samples/bacteria can be distinguished from each other.

Since samples are (almost) always measured versus a background, the composition of this background needs to be reproducible with minimal contribution to the Raman spectrum, since this background will interfere with (the result of) the analysis.

Bacterial typing

Raman research within the CODT has led to the introduction of a commercial typing system, the SpectraCell*IRA* analyzer (SCRA) (River Diagnostics, Rotterdam) in 2009. This system analyses clonal relationship between bacterial strains. The workflow is easy, with little hands-on-time. Shortly: isolates are cultured for 20h on Trypticase Soy Agar (TSA). Biomass is obtained from the agar and suspended in water. This suspension is transferred onto a Microslide. The slide is air-dried and Raman spectra can be measured (**Figure 5**).

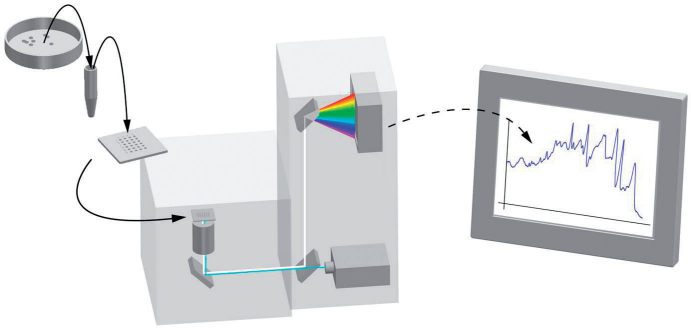

Figure 5. Workflow for the SpectraCellIRA analyzer.

When all measurements are done, the Raman spectra are compared in silico. Subsequently, a similarity matrix is calculated where the relationships between the different samples are displayed.

Possible drawbacks

Despite the enormous potential, there are some limitations to Raman spectroscopy. Raman spectroscopy is a phenotypical method, where minor differences in culture medium, incubation temperature or – time, in theory, can cause differences in Raman spectra. These problems can only be intercepted by using culture media of constant quality and strict incubation times. Extended validation studies are needed to show the influence of the abovementioned factors by using Raman spectroscopy within clinical microbiology. Next to this, there is a lack of standard nomenclature, which gives suboptimal portability; Raman type 1 can be different everywhere.

Conclusion

Raman spectroscopy certainly is highly useful within clinical microbiology thanks to the simplicity, high reproducibility and the high discriminatory power. With a small amount of biomass, it can be easily determined if two (or more) individuals are infected or colonized with the same bacterial strain. Furthermore, due to the possibility of building a database, it can be implemented to generate transmission alerts when a previous found SCRA type is detected again. In theory, this can lead to accurate real-time and continuous, instead of retrospective, monitoring of isolates to prevent health-care associated infections.

Multiplex detection

Multiplex analysis offers significant advantages regarding time, costs, sample requirements and the amount of data that can be generated. Many different techniques are (highly) suitable for multiplex detection. The most important ones are reviewed in the next paragraphs.

Luminex

Different applications of Luminex for the simultaneous detection of multiple respiratory viruses or bacterial pathogens have been reported (26-30). The technique has also been used for other diseases like meningococcal infection and fungal infections (50-51). Luminex technology incorporates flow cytometric methods using custom-designed microbeads that are coloured with slightly different shades of the red spectrum (31). Each coloured bead is labelled with a different fluorescent probe that is specific to each particular target. After multiplex PCR amplification of targets, the PCR products can be incubated with the beads. The bead mixture is sorted by flow cytometry based on the internal bead colours and hybridized samples produce a fluorescent signal (32).

The rapid detection of antibodies against *S. aureus* with Luminex technology showed that antibody levels were associated with the presence of specific genes in infectious *S. aureus* isolates (33). Another study showed that persistent carriage of *S. aureus* is influenced by and associated with genetic variation in host inflammatory response genes (34). Both approaches may be useful in fast screening for (susceptibility) to (methicillin-resistant) *S. aureus* carriage or infection.

Microarray

Microarrays are massively parallel detection platforms that were first used for gene expression studies, but have also been successfully applied for microbial detection and identification (35). The technology has enabled researchers to gain insight into the microbial diversity of human samples and has enabled studies for multi-pathogen infections. In contrast to gene expression studies, the concentrations of targets in analyzed samples for microbial detection are usually much lower, which requires the use of nucleic acid amplification.

The adaptation of microarray technology for diagnostics allows full differentiation of a pathogen from a non-pathogen. This could allow the nucleic acid from a sample to be extracted and be hybridized to a glass slide containing DNA fragments complementary to the genes of interest, unique to the tested pathogen. Rather than individual tests for each pathogen, sample type screening would be able. For example, a microarray slide could be created containing a number of unique gene segments for all pathogens associated with respiratory

tract infections. This would then allow the laboratory to process a single respiratory sample for identification of all pathogens present in that sample in one quick assay.

Another great benefit is that such a gene approach could also employ antibiotic resistance genes, so that antibiotic resistance present in a sample could be detected, which is still a major challenge in adopting molecular diagnostics over culture-based isolation and characterization. Antimicrobial resistance is one of the most important and urgent problems in clinical bacteriology and the use of microarrays has already demonstrated its suitability in general (36-37). For complex resistance problems, such as detection of different Extended Spectrum Beta Lactamase (ESBL) variants or MRSA, DNA microarrays were published (38-39). All these assays, although they need further optimization regarding implementation in routine clinical laboratories, are characterized by a short time-to-result (4-5 hours). Besides the fact that resistance-related information is crucial for the adequate treatment of life-threatening infections, the obtained information can also be used for epidemiological surveillance.

Of course, there are a large number of obstacles before such technology can be employed in a clinical setting. These include the costs and the level of user skill required for the technology. Also the amount of pathogen DNA required to label and then hybridize to the slide to produce a detectable signal needs to be sufficient. This can be overcome by PCR-based amplification of the DNA in the sample, but this is extremely difficult if attempting to amplify hundreds of gene segments for the type of array previously mentioned (40). Most problematic is the fact that still one only finds what one is searching for: arrays always need prior sequence information and are incapable of identifying novel sequence variants. Most likely, the use of microarrays in clinical diagnostics will be overpowered by the introduction of (whole-genome) next generation sequencing.

Sequencing

Another example of multiplex analysis is the use of molecular techniques to determine the nucleotide sequence of 16S ribosomal RNA (rRNA), which was already described in 1980 (41). Where phenotypic identification is subject to variations in morphology and metabolic status, genotypic classification is based on relatively stable and uniform molecular targets. The use of 16S rRNA gene sequences for identification of bacteria has been most successful for a number of reasons. These reasons include the presence of 16S rRNA in all bacteria, that the function of 16S rRNA gene over time has not changed (hence its relatively strong sequence conservation) and that the 16S rRNA gene is large enough (1500 bp) for discrimination between different species (42). Furthermore, 16S rRNA provides the possibility to detect nonviable bacteria and bacteria with special culture requirements.

Naturally, there are challenges associated with 16S rRNA sequencing. The conserved nature of ribosomal genome sequences across the different genera of bacteria and the high sensitivity of PCR easily allows for the generation of false-positive results due to target con-

tamination. There are different conserved regions within the 16S rRNA gene. Therefore there are different primer sequences that can be used for 16S rRNA PCR (43). Despite being broad range primers, it is unlikely that any primer set will amplify all bacteria. Because of difficulties in the interpretation of DNA electropherograms resulting from direct sequencing of clinical polybacterial samples, the use of 16S rRNA sequencing has initially been limited to clinical samples of infections that are predominantly monobacterial. However, Kommedal et al have developed an algorithm for analysis of mixed chromatograms, which makes it possible to apply 16S rRNA sequencing as a diagnostic tool (44-45).

Sequencing is a powerful approach for decoding a number of human infectious diseases. The introduction of next-generation sequencing (NGS) technologies has dramatically reduced sequencing costs and significantly increased the output and throughput, making whole-genome sequencing possible. Although a relatively new technology and only used in research laboratories, whole genome sequencing has the potential to provide an enormous load of diagnostic capabilities as the technology improves and costs becomes reasonable.

Examples are platforms developed by companies such as 454 Genome Sequencer FLX System (GS FLX; Roche Life Sciences, Basel, Switzerland), Genome Analyzer II (GA; Illumina, CA, USA), Sequencing by Oligonucleotide Ligation and Detection (SOLiD; Life Technologies, CA, USA), HeliScope Single Molecule Sequencer (Helicos Biosciences, MA, USA) and Ion Torrent (Life Technologies, CA, USA).

NGS technologies have reduced both cost-per-base and time required to decode an entire genome, making DNA sequencing a cost-effective option for many experimental approaches. Although differing in sequencing chemistries and technical details, all NGS platforms utilize a similar strategy, miniaturization and multiplexing of individual sequencing reactions to overcome the limited power of traditional Sanger sequencing (46-47).

Traditional Sanger sequencing involves creating different sizes of fluorescently labelled PCR products in the same reaction tube. The templates used in this reaction are purified plasmids or amplified DNA fragments. There are four different coloured labels that are specific to one of the four DNA bases. As a result, numerous different-sized products are created which are separated on a capillary electrophoresis instrument by size and detected by fluorescence. The sequence can be compared with database entries for identification of the species of a microorganism or genes indicative for drug resistance. For example, one study describes the use of sequencing for identification of different Mycobacterial species by using several different gene targets (48-49).

The Roche 454 GS FLX System is based on emulsion PCR and pyrophosphatase detection techniques (50-52). Compared with other NGS platforms, the strength of the 454 GS FLX system are its longer sequence reads. The Roche 454 GS FLX can generate more than 1 million individual sequence reads with lengths over 400 bases during a 10 h run. Although its per-base cost is much higher than that of other NGS platforms, the Roche 454 system is best

suited for certain applications, such as *de novo* sequencing of genomes for which long read lengths are essential (50, 53).

The Illumina GA system is the first short-read sequencing platform (46). It uses an array technique to achieve solid-phase DNA amplification. Solid-phase amplification can produce 100-200 million spatially separated template clusters, providing free ends to which a universal sequencing primer can be hybridized to initiate the NGS reaction. This repetitive sequencing process takes approximately 2-3 days to generate 50 million reads, with a read-length of 36 bases. The tedious preparatory steps, however, are being automated as we speak.

The SOLiD system is based on the techniques described by Shendure et al and McKernan et al (54-55). Library construction for the SOLiD system is similar to Roche 454 technology. However, unlike the Roche 454 sequencing approach, the sequencing-by-synthesis in the SOLiD system is based on ligation chemistry.

The HeliScope Genetic Analysis System is the first commercialized single-molecule DNA sequencer. The technology is based on work by Braslavsky et al and relies on the cyclic interrogation of an array of sequencing features (56). Each sequencing cycle consists of the successive addition of polymerase and a different type of dye-labelled nucleotide. The HeliScope instrument is capable of imaging billions of single molecules per run.

Last in line is Ion Torrent, which uses a sequencing strategy similar to the 454, except that hydrogen ions (H^+) are detected (instead of a pyrophosphatase cascade) and standard microchips are used as sequencing chips. Detection of H^+ means that no lasers, cameras or fluorescent dyes are needed. Using common microchip design standards means that low-cost manufacturing can be used.

There are important differences among the abovementioned NGS technologies. For example, the Roche 454 sequencer and the Ion Torrent may be preferable for *de novo* sequencing because of its longer read length (53). The Illumina and Life Technologies platforms are particularly well suited for variant discovery by resequencing genomes because gigantic volumes of high-quality base sequences are produced per run (57). The Helicos platform is well suited for applications that demand quantitative information in RNA-seq or direct RNA sequencing (58-60). **Table 2** provides an overview of the main features of these NGS technologies.

Once the fragment sequences have been determined, they are aligned to a known reference sequence or assembled *de novo*. These technologies can provide gigabytes of sequence information about the whole organism, which has the potential to change the treatment for specific infectious diseases. However, there are a number of challenges that need to be overcome before sequencing can become the predominant diagnostic and epidemiological technology in clinical microbiology.

Cost, ease-of-use and need for technical skilled staff limit this method to be introduced in routine laboratories. There is an urgent need for studies examining the mode and tempo of

Table 2. Overview of next-generation sequencing technologies.

Platform	*NGS chemistry*	*Read length (bases)*	*Run time (days)*	*Yield Mb/run*	*Advantages*	*Disadvantages*	*Applications*	*References*
Roche 454 GS FLX System	Pyrosequencing	~ 400	0.5-1	50	- Longer reads - Short run times - High throughput	- Expensive	- Bacterial genome *de novo* assemblies - Identification of multibacterial samples	(64, 75)
Illumina GA system	Sequencing by synthesis with reversible terminators	35-150	1.5-8	1.000-96.000	- Currently the most widely used system	- Slightly higher costs	- Variant discovery (SNP detection) - Evolution	(76-77)
Life Technologies SOLiD	Sequencing by ligation chemistry	~ 50	3-12	71.000-200.000	- Cheap - High accuracy - Ability to save failed sequencing cycles	- Long run times - Relatively short reads	- Variant discovery	(68-69)
Helicos Bioscience HeliScope	True single molecule sequencing by synthesis	25-55	8	28.000	- Large numbers of reads directly from single molecules	- High error rate	- RNA-seq - RNA sequencing	(79-81, 85)
Ion Torrent	Synthesis with H^+ detection	~ 100	0.25-0.5	100->1.000	- Cheap - Longer reads	- Sample preparation time	- Bacterial genome *de novo* assemblies	(78)

SNP: Single Nucleotide Polymorphism.

genomic evolution in different bacterial lineages and the performance of these technologies when applied in real-life. There is a need for improved user-friendly informatics platforms that allow the average technician to analyze and exploit high-throughput sequencing data. Furthermore, approaches for Single Nucleotide Polymorphism (SNP) detection and validation need to be improved. More informative epidemiological markers need to be identified. The focus should be on improved read lengths to capture longer sequences and, finally, there is a need for better analytical tools and databases.

Towards the end of this decade it is likely that NGS technologies have become a common clinical diagnostic tool and that medical laboratories will directly sequence DNA or RNA extracted from clinical samples. To achieve this, we will need improved approaches to sample preparation. Finally, it still remains to be seen if even the uncovery of full genetic and transcriptional potential can be used to define phenotypes that usually depend on genome-environment interactions as well.

Concluding remarks

We can state that conventional diagnostics still is and will remain present in clinical microbiological laboratories for the coming years. Molecular diagnostics can be implemented as an additional diagnostic tool, but with restrictions.

MALDI-TOF MS is highly suited for high-throughput identification of "common" clinically relevant microorganisms. The electronic nose has the potential to be developed for direct detection and identification of microorganisms, as a real bed-site (point-of-care) test for infectious disease diagnostics. In the field of typing, Raman spectroscopy is a candidate for fast and reliable typing of bacterial strains, in both large and small laboratories.

High-speed multiplex analysis will be the ultimate diagnostic strategy for future diagnostics of infectious diseases, which will ensure more efficient patient management.

Furthermore, the technology push is not over yet! Techniques such as bacterial life-dead assays and various applications of spectroscopy (e.g. infrared, single cell, electrospray ionization) are being explored for their use in clinical diagnostics microbiology as we speak.

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Chapter 10

Summarizing discussion and future perspectives

In this thesis, new diagnostic tests for infectious diseases were explored in addition to applications where conventional culture- and microscopy-based methods have long been and may still be considered as the "gold standard". The following chapter will discuss the previous chapters and a perspective on future developments within the field of diagnostic clinical microbiology will be given.

Introduction

Chapter 2 outlined the microbiological tests that are currently available for the detection of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Legionella* spp. from clinical respiratory tract samples. Historically, atypical etiological agents of respiratory tract infections (RTI) were detected on the basis of microbiological culture or serology. Without exception, sensitivity and specificity of such cultures did not meet criteria of excellence. Therefore, alternatives were sought and a variety of antigen- or antibody mediated tests was developed. Unfortunately, none of these individual tests met the performance criteria to reliably identify these atypical causative infectious agents of RTI in all cases. Moreover, the more recently developed wide variety of nucleic acid amplification tests did not (yet) solve these diagnostic problems as well. In today's clinical microbiology laboratories, no widely accepted gold standard for the detection of *M. pneumoniae*, *C. pneumoniae* and *Legionella* spp. is available. Technicians and physicians have to rely on a combination of test results that, in combination with clinical patient data, may lead to a presumptive identification of these microorganisms at best. Although significant improvements of the diagnostic platforms have been made over the past twenty years, no reliable tool is as yet available. Future developments for identification in clinical diagnostics are still required before the ultimate test for the diagnosis of atypical RTI agents will become available.

Biomarkers

The first part of **Chapter 3** discussed the performance of procalcitonin (PCT)-, neopterin- and soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) levels as potentially suitable biomarkers to discriminate severe *Plasmodium falciparum* disease from non-severe *P. falciparum* disease and non-*P. falciparum* diseae at the initial clinical assessment of travellers with imported malaria. In developed countries such as The Netherlands, fatal cases of malaria are only reported occasionally (1-2), which is in contrast to the situation in regions of high malaria endemicity. Therefore, most clinicians in developed countries encounter problems making a proper diagnosis of malaria.

The quantification of sTREM-1 levels in admission samples of the patient did not result in proper discrimination of severe *P. falciparum* malaria and uncomplicated *P. falciparum* malaria or non-*P. falciparum* malaria. Significantly higher levels of PCT and neopterin were

observed in travellers with severe *P. falciparum* malaria upon hospital admission as compared to travellers with uncomplicated *P. falciparum* malaria or non-*P. falciparum* malaria, respectively. These findings are in agreement with those of several other studies performed in semi-immune malaria patients originating from malaria-endemic regions (3-4). When the ROC curve characteristics of neopterin and procalcitonin were compared to those of plasma lactate, the AUROC of neopterin appeared superior whereas the AUROC of PCT appeared inferior to lactate. This suggests that neopterin may provide the highest accuracy in its diagnostic performance to identify severe *P. falciparum* malaria in this cohort of travellers.

Unfortunately, the applicability of both neopterin and PCT in the initial clinical assessment of patients with severe *P. falciparum* malaria will probably be limited by the poor positive predictive value of both tests, indicating that neither test can serve as a useful tool for the diagnosis of severe *P. falciparum* malaria. On the other hand, the high negative predictive value of both neopterin and procalcitonin suggests that these tests can still be of value by providing an exclusion criterion of severe disease. With either a PCT level of less than 0.9 ng/ml or a neopterin level of less than 7.9 ng/ml in serum on admission as a cut-off point for severe *P. falciparum* malaria, no patient with severe disease would have been denied access to high-level monitoring and intensive treatment.

In conclusion, although neither neopterin nor PCT testing can probably serve as a useful single diagnostic tool for severe *P. falciparum* malaria, the high negative predictive value of both neopterin and PCT may be helpful for a rapid exclusion of severe *P. falciparum* malaria upon admission. This may be valuable – particularly if available as a cheap and rapid diagnostic test - for physicians only occasionally dealing with ill-returned travellers and who need to decide on subsequent oral anti-malarial treatment or a timely referral to a specialized centre for high-level monitoring and intensified parenteral treatment.

The second part of **Chapter 3** focused on the diagnostic application of PCT- and neopterin levels as biomarkers to discriminate between a bacterial and a viral cause of infection. The value of PCT and neopterin in clinical decision making was shown in several studies (5-10), however not in all (11). In our study we found a disappointingly poor diagnostic accuracy for both PCT and neopterin for bacterial and viral causes of fever in ill-returning travellers. In addition, neopterin even had a more accurate performance for bacterial than for viral disease. These findings may be explained by the observation that *S.* Typhi and Paratyphi and *Rickettsia* species are intracellular bacteria and that other intracellular bacteria such as tuberculosis and melioidosis also lead to increased neopterin levels (10).

Of the more traditional leukocyte differential count-based parameters, only presence of leukopenia, lymphocytosis and presence of atypical lymphocytes were suggestive of viral disease. Its use in clinical practice is hampered by its considerable overlap with the findings in the bacterial infections. Interestingly, when empirical antibiotic treatment was withheld on admission on the basis of combined presence of lymphocytosis and/or CRP ≤ 10 mg/l, only 5

of 33 patients with Dengue would receive unnecessary antibiotic treatment but all patients with enteric fever and rickettsial diseases would receive antibiotic treatment; an almost 85% reduction of unnecessary empirical antibiotic treatment. This finding alone would make the use of this simple combination of diagnostics a mandatory procedure for clinical microbiology laboratories.

Since the present findings were based on a relatively small number of selected patients with possibly inherent study limitations such as selection bias and lack of power, the suggested clinical decision rule for empirical antibiotic treatment in returning febrile travellers without leukocytosis on admission needs validation in properly designed prospective studies.

Culture

Chapter 4 described the *in vitro* evaluation of the performance of Granada tube broth (GT) (bioMérieux, Marcy l'Etoile, France) for detection of Group B *Streptococcus* (GBS) in swabs, both direct and after transport. We compared the performance of GT with the currently recommended Amies transport medium (AT).

Direct incubation of GT at 37°C resulted in the highest yield of GBS. No significant differences in growth of GBS were observed between transport of GT at RT (73%) or at 4°C (55%). When GT were subsequently incubated at 37°C, the sensitivity increased to almost 100%.

The limitation of our study was its *in vitro* character. The application of purified isolates of GBS in GT and onto AT may not reflect GBS survival on swabs containing vaginal and/or rectal flora. Nonetheless, we can conclude that GT is a highly sensitive transport and culture medium to detect GBS as the survival rate of GBS was significantly higher in GT as compared to AT.

GT may be especially suited for the transport of swabs from pregnant women, attending general practitioners or midwifery practices, to the laboratory, which may take 2-3 days. Specifically, if a woman has low-density GBS colonization, extended transport times of swab specimens at RT or higher could reduce the culture sensitivity for AT but possibly not for GT.

GT may not be suitable for the direct detection of GBS from clinical samples, as the absence of orange pigment does not conclude in the absence of GBS. Therefore, GT should always be sub-cultured for optimal use. Furthermore, GT should always be incubated at 37°C to improve its sensitivity.

However, the question remains if culture is the most optimal method for detection of GBS since PCR–based assays have fared better in the detection of GBS. One multicenter study demonstrated that a molecular-based assay for GBS colonization during labour was highly sensitive and specific (12). More rapid molecular tests allow for point-of-care testing. GeneXpert GBS (Cepheid Diagnostics, Sunnyvale, CA) is such a rapid, real-time PCR-based assay for the intra-partum detection of GBS colonization, with high sensitivity and specificity (13). The system fully automates DNA extraction and PCR within the assay cartridges and results

are presented in less than 75 minutes. The use of this system could result in a reduction of early-onset GBS disease in neonates, better than culture.

Nucleic acid amplification

Chapter 5 provided an update on molecular diagnostics and typing of methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA is responsible for a large and still growing number of both health-care and community-associated infections, resulting in increased morbidity and excessive healthcare costs. Screening of individuals combined with an aggressive infection control program has become the standard for management of these infections. Rapid screening methods that allow reliable detection of MRSA within hours are now available. The short time-to-result is a clear advantage that has provided a tool for successful infection control strategies. However, every assay should be evaluated against the local MRSA diversity before being introduced in the diagnostic microbiological laboratory. Continuous evolution of SCC*mec*, constrains continuously monitoring of the assay performance and positive results of direct MRSA testing should always be confirmed by a culture method or a second molecular test. For laboratories with high false positivity rates or in regions with low prevalence of MRSA, confirmation is essential.

The quality of molecular diagnostic tests and typing techniques is still under discussion. Adequate internal and external quality control and international standardization for MRSA diagnostics should be improved immediately. To achieve this, both laboratories and manufacturers should be encouraged to participate in EQAs.

Bacterial typing

In **Chapter 6**, we have analyzed a representative selection of an international collection of epidemic MRSA strains, the Harmony collection, originating from 11 different European countries using the DiversiLab System (bioMérieux), pulsed-field gel electrophoresis (PFGE) with S*ma*I macro-restriction analysis and multi-locus sequence typing (MLST). The HARMONY isolates represent all major hospital-acquired MRSA types found in Europe.

The primary aim of this study was to assess the performance and convenience of the DiversiLab system for discrimination of MRSA isolates in a microbiological diagnostic routine setting and to compare evaluation criteria obtained with PFGE and MLST.

DiversiLab results were fully concordant with the CC classification as defined with MLST. The discriminatory power of the DiversiLab system was comparable to that of MLST, while PFGE was the most discriminatory technique.

The reproducibility of the DiversiLab system was excellent (>99%). The feasibility of the DiversiLab system with respect to labour intensity and time-to-result was good. The most labour-intensive step is the extraction, which requires almost half a working day hands-on

time, depending on the number of isolates being tested. Total turn-around-time is one working day with a maximum of 36 samples, which is less than PFGE (3 to 5 days) and MLST (approximately 3 days). Costs of PFGE, MLST and DiversiLab are comparable and are approximately €50 per sample, respectively.

In summary, DiversiLab is a rapid and non labour-intensive technique, but it lacks resolution to differentiate genetically and epidemiologically unique MRSA strains, as is needed for detailed outbreak analysis. Therefore, it may be useful as a library typing system for long-term epidemiological studies.

The primary aim of the study described in **Chapter 7** was to assess the performance of the SpectraCell*RA* system (SCRA; River Diagnostics, Rotterdam, The Netherlands) for typing of an epidemiologically perfectly defined set of MRSA isolates.

Results of SCRA analysis at the isolate level were concordant with the gold standard of epidemiology for 95.6% (108/113). However, when our results were analyzed at the household cluster level, results were concordant for 90.7% (49/54). In other words, when using SCRA in practice, SCRA gives discrepant results in 9% of the small clusters. Analysis of discrepant results using PFGE showed equal results as epidemiological data. When the discrepant households were analyzed for possibilities of exo-household acquisition of MRSA, no possible transmission of another known source or contact with a person with known risk factors were found. This indicates that the discriminatory power of the SCRA system might be too high for adequate outbreak analysis of small clusters.

During transmission and acquisition of MRSA, micro-evolution may take place (14-15). PFGE may not be able to detect this micro-heterogeneity, where Raman spectroscopy does. However, since a household can be considered as a close community, we can assume that transmission and detection have occurred in a short time period. Therefore, it is very likely that the discrepancy between SCRA and the gold standard is not due to micro-evolution of the organism.

Multiple independent measurements of 3 reference isolates and the duplicate measurements of 26 MRSA strains resulted in a reproducibility of 100%. This indicates that SCRA is stable over a longer period of time, which has been published before (16).

Our findings are similar to those found in another study, where the observed concordance between SCRA and the gold standard of epidemiological data and PFGE was 97% (17). Reproducibility in our study was better (100% vs. 95%). In both studies isolates were tested as full biological replicates at different points in the study.

The feasibility of the SCRA system with respect to hands-on-time (~ 3 h) and time-to-result (36-48 h) was good and better than PFGE. Time-to-result may be improved by applying 1 subculture instead of 2 subcultures. In this way, results will be available the next day, what is enormous valuable for prevention control.

Mistyping of strains by using the SCRA system for outbreak analysis is a risk. Isolates epidemiologically assigned to one cluster could now be considered as unique isolates and as a single event each, which has serious consequences for infection prevention measures. Prevention measures to a single event, or 2 separate single events, are different than the response on an outbreak (2 or more MRSA cases belonging to one cluster). For 90-95% of cases a considerable amount of time for implementing prevention measures is gained. For the remaining 5-10%, further analysis will be necessary.

Many studies on typing of MRSA focus on a small set of MRSA isolates and the relevant epidemiological data of the isolates are often (partially) unknown. We had the unique opportunity to use the epidemiological relationships within households as the gold standard, together with PFGE.

Although the high discriminatory power of SCRA may lead to discrepancies, and accompanying consequences, in 5-10% of epidemiologically related MRSA clusters, we conclude that the SpectraCell*RA* system is a highly reproducible, easy-to-use and fast typing platform that can compete with the currently used typing techniques.

Quality control

Chapter 8 focused on the External Quality Assessment (EQA) of molecular diagnostics and genotyping of MRSA. The primary aim of our EQA programmes was to assess the proficiency of laboratories in the molecular detection and characterisation of MRSA strains. We concluded that the molecular detection of MRSA in samples with high CFU counts is reliable, which can and has been implemented in various laboratory settings with confidence. All tests performed equally well. However, we also had to conclude that several of the currently applied tests do not meet the requested quality criteria. The sensitivity of many tests is relatively low and the specificity needs to be improved.

Pre-enrichment of clinical samples leads to concentrations of MRSA exceeding 10^9 CFU/ml, which is higher than the concentrations of MRSA likely to be found in a patient sample, and those in this EQA panel. In the panel, only inactivated cells were present. As a consequence, pre-enrichment was not possible. This may have influenced the results of laboratories that have designed their MRSA assay to be optimal after enrichment.

The incorrect results found in this study underscore the need for improved specificity of molecular MRSA tests. Therefore, positive results should always be confirmed by a culture method or a second molecular test. For laboratories with high false positivity rates or in regions with low prevalence of MRSA such as The Netherlands, confirmation is necessary. Of course, this will cause a delay in results. A correct balance between reliability and speed needs to be found.

We presented the first EQA for typing of MRSA strains. Clear differences in resolution were observed between the datasets, which underscores the need for improved international standardization.

In conclusion, the quality of molecular diagnostic tests still needs improvement and regular quality control and international standardization for MRSA diagnostics and typing should be mandatory for the years to come. To improve the performance and quality of overall molecular diagnostics, both laboratories and manufacturers should be encouraged to participate in EQAs. EQA panels for detection and typing should also be developed for other important (health-care associated) infectious agents, such as vancomycin resistant *enterococci* (VRE) and extended spectrum β-lactamase (ESBL) producing *Enterobacteriaceae*.

Discussion and future perspectives

In **Chapter 9**, an overview was given on new diagnostic tools (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry [MALDI-TOF], electronic nose and Raman spectroscopy) that are currently being developed and implemented for detection and typing of bacteria in clinical laboratories. Next to this, the most promising candidates for multiplex analysis were discussed.

Future perspectives

Current diagnostics of infectious diseases is based on conventional microscopy and culture. Essentially, laboratories can perform simple tests on many clinical samples on daily basis, using inexpensive culture media and simple techniques. This strategy also provides bacterial isolates, which can be further characterized e.g. more detailed identification of the species and analysis of their antibiotic susceptibility profiles and/or epidemiological typing for outbreak analysis. Anti-microbial susceptibility testing (AST) is still performed in a manual or, at most, semi-automated way. These conventional techniques are time-consuming, delaying proper treatment of the patient.

Molecular diagnostics (e.g. real-time PCR) can be of great value for clinical microbiological diagnostics. The combination of excellent sensitivity and specificity, the low contamination risk, the ease-of-use and short time-to-result, has lead to the prominent position of real-time PCR in current diagnostics of infectious diseases. In contrast, there are drawbacks for PCR. There is a chance of false-positive (low specificity, contamination) or false-negative (low sensitivity, variability in the target, inhibition) results.

Point-of-care diagnostics

The last years we have seen an exponential rise in the amount of research and financial support for the development of new diagnostic techniques, resulting in rapid tests for diagnostics of infectious diseases, so called point-of-care (POC) tests. Probably the greatest success for rapid diagnostics to date is the Cepheid GeneXpert system (Cepheid Diagnostics). The GeneXpert is a fully automated, closed system that automates sample preparation, DNA amplification and detection. There are currently 11 commercial GeneXpert tests available, of which the MRSA assay is the most notable and successful one (18). Total assay time for MRSA detection on the GeneXpert is 75 minutes, with a total hands-on-time of approximately 2 minutes. However, despite the encouraging findings of clinical studies, the overall performance of the GeneXpert MRSA assay might be improved. The manufacturer's interpretation is based on the application of a Ct cut-off value of 36 cycles, regardless of the presence or absence of evidence of amplification. This may introduce false-negative results even when tests demonstrate clear evidence of MRSA amplification, but Ct values exceed 36 cycles. Conversely, false-positive results may also occur, where Ct values of <36 are obtained but amplification curves fail to demonstrate an exponential rise. The inclusion of an equivocal result for tests where the amplification data of the MRSA target DNA fail to support the Ct cut-off result may be needed.

What feature and functions should the ideal POC test possess? The primary driver for the development of such a test is shortened time-to-result. The duration of a test should be

maximally one hour, including hands-on-time and analytical time. The device should be portable and the cost per test should be low (± €25 for developed countries, however far less for developing countries!). The device should use a disposable cartridge and no pre-processing of the specimen or user intervention once the assay has been started should be required. The results generated should be clear and easy to understand. The performance characteristics of a POC assay should be comparable with those of the laboratory-performed version. One important consideration is the input volume of the specimen, which can affect the sensitivity. This is especially important for infectious disease testing, where the numbers of pathogen cells may be small. Accuracy and precision within and among devices is another factor that requires consideration. Internal controls that are tested along with the target should be included in every unit and will ensure that the test is performing correctly. The inclusion of external controls should help ensure comparable performance among devices.

The technology should also be available to populations that do not have access to traditional laboratory testing, such as those in developing countries in e.g. Africa.

Automation

In order for rapid diagnostics to be fully accepted in clinical and POC settings, perhaps the major challenge is automation. This will save hands-on-time and allow multiple samples to be analyzed at once. It may reduce contamination of samples, thus reducing false-positive results. Automation will permit more extensive standardization of tests and systems that will open the diagnostic process for operation by less-skilled staff, increasing the number of people capable to run diagnostic analyses.

In addition, automation of laboratory data processing is an important issue as well, since optimal patient management depends on transfer of the result to the clinician. With the introduction of multiple new technologies and equipments in the clinical microbiology laboratory, laboratory information systems (LIS) need to be adapted and expanded. A good LIS is essential to guarantee optimal clinical servicing.

Conclusion

In line with the contrasting advantages and challenges listed in this thesis, the development of POC diagnostics is an area that is a major focus of research to date. Yet, at the same time, very few platforms are currently commercially available. In the next 5-10 years, we will observe a shift in the diagnosis of infectious diseases towards rapid POC diagnostics. Tests developed for research-use-only are currently being reformatted for integration onto novel diagnostic platforms. High-throughput, full automation and short time-to-result will be combined with little sample preparation and a large panel of tests. This will allow diagnosis of infectious

diseases to occur faster with a lesser burden on currently overloaded clinical laboratories, resulting in efficient treatment.

Furthermore, research needs to be expanded to simplify and speed up AST testing, which is still one of the biggest challenges in the clinical microbiological laboratory.

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Chapter 11

Nederlandse samenvatting

René te Witt

Geschiedenis

Het leven is veranderd sinds de Nederlandse botanicus Anthonie van Leeuwenhoek (1632-1723) de diversiteit en het bestaan van de microbiële wereld aantoonde door middel van de ontdekking van de microscoop. Microscopisch bewijs leverde in de 19e eeuw ondersteuning voor de "microbe-theorie". In de jaren 80 van de 19e eeuw stelde Robert Koch zijn postulaten op om te bepalen of een micro-organisme wel of niet de veroorzaker is van een ziekte. Sindsdien is het spectrum aan bacteriën, schimmels, virussen en parasieten exponentieel gegroeid dankzij verbeterde kweektechnieken en de ontwikkeling van geavanceerde microscopische technieken. Echter, de grootste stap voorwaarts sinds Van Leeuwenhoek is de ontdekking van nucleïnezuren in 1871 door Miescher, die heeft geleid tot de ontdekking van DNA als bron van genetische informatie en als basis voor de karakterisatie van een micro-organisme in 1953 door Watson, Crick en Wilkins (1-2).

Diagnostiek van infectieziekten

Vandaag de dag zijn infectieziekten nog steeds een wereldwijd probleem. Infectieziekten kunnen alomtegenwoordig zijn (tuberculose, cholera, malaria) of opkomen als jaarlijkse epidemieën (norovirus, influenza, seizoensverkoudheid). Ze kunnen zich presenteren als incidenteel (vogelgriep, SARS) of worden veroorzaakt door antibioticumresistente pathogenen (methicilline-resistente *Staphylococcus aureus* (MRSA), Extended Spectrum Beta-Lactamase (ESBL) producerende bacteriën, carbapenem-resistente bacteriën). Tot slot kunnen infectieziekten ook voorkomen als pandemieën (AIDS, de recente H1N1 uitbraak).

Huidige diagnostiek van de meeste bacteriële infecties is nog steeds gebaseerd op conventionele microscopie en kweek. Voordelen hiervan zijn de lage kosten, de grote capaciteit en het gebruiksgemak. Op deze manier kunnen laboratoria honderden tot duizend monster per dag verwerken, gebruikmakend van goedkope kweekmedia en simpele technieken. Bovendien levert deze strategie ook bacteriële isolaten op die, indien nodig, verder kunnen worden gekarakteriseerd. Denk hierbij aan antibioticum gevoeligheidsbepalingen (AST) en typering voor uitbraakanalyse.

Echter, er zijn ook nadelen. Kweek is ongevoelig en het duurt lang voor er een definitieve uitslag kan worden gegeven. Versnelling en verbetering van diagnostiek van infectieziekten leidt tot betere en gerichtere behandeling van patiënten. Aan versnelde identificatie wordt momenteel veel onderzoek gedaan, maar het bepalen van antibioticum gevoeligheid gaat nog steeds langzaam.

In dit proefschrift zijn nieuwe mogelijkheden voor diagnostiek onderzocht.

Biomarkers

Koorts bij een patiënt kan zowel een ziekte betekenen die snel verergert en zelfs dodelijk kan zijn (sepsis, meningitis), maar het kan ook een uiting zijn van een triviale infectie (Salmonellosis, griep) of zelfs van een steriele ontsteking (allergie). De arts moet binnen korte tijd beslissen of de infectie (waarschijnlijk) wordt veroorzaakt door een bacterie, virus of parasiet en of de patiënt voor behandeling moet worden opgenomen. Resultaten van routine (microbiologische) laboratoriumtesten geven een eerste indicatie; bijvoorbeeld een verhoogd aantal witte bloedcelen is een aanwijzing voor een bacteriële infectie en een verlaagd aantal witte bloedcellen voor een virale infectie. Helaas kan de arts niet volledig op dit soort bepalingen afgaan, omdat er ook bacteriële infecties zijn die geassocieerd worden met een normaal tot laag aantal witte bloedcellen. Het gebruik van biomarkers zou snelle en betrouwbare detectie van een echte infectie wellicht mogelijk maken.

Diverse studies hebben de relevantie van biomarkers om bacteriële en virale infecties bij opname van een patiënt van elkaar te onderscheiden al beschreven. Procalcitonine zou een goed voorspellende marker zijn voor bacteriële infecties (sepsis, longontsteking) (3-6), waar neopterine voorspellend zou zijn voor virale infecties (7).

In het eerste gedeelte van **hoofdstuk 3** hebben we de mogelijke bruikbaarheid van procalcitonine en neopterine getest om bij opname van de patiënt ernstige malaria te onderscheiden van niet-ernstige malaria. Er zijn significant hogere waarden procalcitonine en neopterine gevonden bij reizigers met ernstige malaria dan bij reizigers met niet-ernstige malaria. Helaas zal de toepasbaarheid van beide testen beperkt zijn doordat beide testen een slechte positief voorspellende waarde hebben en geen van beide testen in staat is om met grote zekerheid de diagnose ernstige malaria te geven. Aan de andere kant suggereert de hoge negatief voorspellende waarde van zowel procalcitonine als neopterine dat beide testen van waarde kunnen zijn bij het uitsluiten van ernstige malaria, zeker wanneer deze test beschikbaar is als een goedkope en snelle diagnostische test. Dit kan artsen die slechts sporadisch hebben te maken met zieke reizigers helpen in het maken van de juiste beslissing om mensen te behandelen met malariapillen of door te verwijzen naar een gespecialiseerd ziekenhuis.

Het tweede gedeelte van **hoofdstuk 3** behandelt de diagnostische relevantie van procalcitonine - en neopterine waardes als biomarkers voor bacteriële of virale infectie bij reizigers die ziek terugkomen uit de (sub)tropen. In deze studie vonden we een teleurstellend lage diagnostische toepasbaarheid voor zowel procalcitonine als neopterine.

Microscopie

Detectie van humane pathogenen door middel van direct microscopisch onderzoek van het klinisch monster is de oudste, maar nog steeds uiterst relevante, diagnostische methode

binnen de medische microbiologie. Veel verschillende, celwand-specifieke kleurmethodes zijn beschikbaar (Gramkleuring, Ziehl-Neelsen etc.), waardoor samen met de vorm en ligging van de bacterie een eerste indicatie kan worden gegeven van de aanwezigheid van een mogelijke pathogeen. Echter, bijna altijd zal de definitieve identificatie worden gegeven op basis van fenotypische (biochemische) eigenschappen van de bacterie na kweek op een geschikt medium.

Kweek

Een conventionele en veelgebruikte diagnostische techniek is kweek op en in diverse vaste en vloeibare media. Deze media kunnen universeel zijn (groei is mogelijk voor de meest voorkomende klinisch relevante bacteriën: b.v. bloedagar, chocolade agar, Müller Hinton bouillon), specifiek zijn (b.v. agar of bouillon speciaal voor Gram-positieve kokken of anaerobe bacteriën) of zelfs speciespecifiek zijn (b.v. MRSA ophopingsmedium, chromogene agars) door middel van het toevoegen van specifieke substraten, groeiremmers en/of bepaalde antibiotica.

Kweekmethoden eisen tijd voordat de bacterie is gegroeid, over het algemeen 24-72 uur. Dit resulteert in een vertraging van gerichtere behandeling van de patiënt. Voor veeleisende of traaggroeiende bacteriën, zoals *Mycobacterium tuberculosis*, wordt deze tijd nog verder verlengd, terwijl bacteriën die helemaal niet groeien op conventionele media en onder conventionele condities, vanzelfsprekend, niet worden gevonden. Bovendien zullen logistieke factoren de bacteriële groei verder beïnvloeden. Monsters moeten naar het laboratorium worden vervoerd en gedurende dit transport kunnen monsters van samenstelling veranderen door bijvoorbeeld veranderingen in temperatuur. Dit kan een dramatisch effect hebben op de bacteriële kwaliteit en kwantiteit.

Een voorbeeld van een bacterie die voornamelijk wordt gedetecteerd door middel van kweek is de Groep B Streptokok (GBS). Gedurende de zwangerschap kan GBS verschillende ernstige ziektes veroorzaken, bij zowel de moeder als het kind (8-9). In de meeste gevallen krijgen neonaten van gekoloniseerde moeders (~35%) GBS tijdens de bevalling (10-12). Om dragerschap van en infectie met GBS vast te stellen zijn er diverse kweektechnieken ontwikkeld. Slechts weinig aandacht is echter besteed aan de mogelijke gevolgen van transportcondities aangaande tijd, temperatuur en medium. Richtlijnen stellen dat GBS isolaten enkele dagen bij kamertemperatuur in leven blijven in Amies transport medium. Wel wordt gedurende 1-4 dagen een vermindering in de opbrengst van GBS gezien van bijna 30%, zeker bij verhoogde temperaturen (13-14). Zelfs wanneer geschikte transport media worden gebruikt is de sensitiviteit van GBS kweek het meest optimaal wanneer het monster voor kweek wordt bewaard bij 4°C en de kweek wordt ingezet binnen 24 uur na monsterafname (8, 13-17).

Een nieuw transport- en ophopingsmedium, de Granada Tube (GT) bouillon (bioMérieux) is recentelijk geïntroduceerd. In dit medium wordt een oranje pigment gevormd in de aanwezigheid van GBS (18-19). In **hoofdstuk 4** wordt de *in vitro* evaluatie van de performance van deze bouillon voor de detectie van GBS beschreven. We hebben deze performance vergeleken met die van het huidige aanbevolen transport medium, Amies transport medium (AT). De gevoeligheid van GT was significant hoger dan die van AT, in het bijzonder voor lage concentraties en verlengde transport/incubatietijden. GT is mogelijk niet bruikbaar voor de directe detectie van GBS van klinische monsters, omdat bij afwezigheid van oranje pigment niet direct geconcludeerd mag worden dat GBS afwezig is. Daarom zal elke GT altijd moeten worden afgeënt voor een optimaal resultaat. Bovendien zullen GT altijd bij 37°C moeten worden geïncubeerd om de gevoeligheid te verhogen.

De vraag is echter of kweek de toekomst heeft bij het zoeken naar GBS. Op PCR gebaseerde methodes zijn gevoeliger voor de detectie van GBS (20). GeneXpert GBS (Cepheid) is een snelle, op real-time PCR gebaseerde assay voor de detectie van GBS kolonisatie, met een hoge sensitiviteit en specificiteit (21). DNA extractie en PCR zijn volledig geautomatiseerd en resultaten zijn binnen 75 minuten beschikbaar.

Serologie

In diagnostische laboratoria worden microscopie en kweek vaak aangevuld met serologische technieken voor (in)directe diagnostiek van infecties door het detecteren van antilichamen of antigenen. Er zijn echter diverse tekortkomingen. Serologieresultaten kunnen onbetrouwbaar zijn als gevolg van kruisreactiviteit. Bovendien, als gevolg van de noodzaak tot het testen van een nieuw monster na 1-4 weken, zijn resultaten vaak te laat beschikbaar om direct invloed te kunnen hebben op de behandeling van de patiënt.

Identificatie en antimicrobiële gevoeligheidsbepalingen

Na kweek van een micro-organisme, is identificatie de volgende stap. De meeste klinische laboratoria gebruiken biochemische en immunologische identificatie testen. Echter, de resultaten hiervan kunnen worden beïnvloed door de testcondities, zoals kweektijd, -temperatuur, - omstandigheden (b.v. met of zonder O_2) en/of samenstelling van het kweekmedium. Geautomatiseerde identificatie - en AST systemen worden tegenwoordig veelgebruikt binnen de klinische bacteriologie. Deze systemen zijn gebaseerd op het gebruik van enzymatische en biochemische eigenschappen van een bacterie voor de identificatie en verdunning voor AST.

Antibioticum resistentie

Penicilline resistentie in *S. aureus* verscheen zeer snel na het klinisch gebruik van penicilline in 1943 en het resistentiemechanisme was de productie van β-lactamase (22). Het eerste geval van MRSA werd gerapporteerd in 1962, slechts 2 jaar na de introductie van methicilline ter behandeling van penicilline resistente *S. aureus*. De originele MRSA stammen circuleerden binnen gezondheidszorginstellingen en konden op deze manier resistent worden tegen een breed scala aan antibiotica. Echter, sinds de jaren 90 van de vorige eeuw is er een stijging waar te nemen in MRSA infecties in voorheen gezonde individuen zonder contact met de gezondheidszorg. Deze MRSA stammen zijn niet ontsnapt uit het ziekenhuis, maar ontstaan in de samenleving (23-25).

Echter, de grootste bedreiging in antimicrobiële resistentie wordt momenteel gevormd door de snelle en globale verspreiding van multiresistente Gramnegatieve bacteriën. Er worden regelmatig nieuwe β-lactamases en andere nieuwe resistentiemechanismen gevonden die gemakkelijk van bacterie naar bacterie kunnen worden overgedragen. Laboratoriumdetectie is moeilijk omdat meerdere resistentiemechanismen aanwezig kunnen zijn en resistentie "verstopt" kan zijn. Het behandelen van ESBL producerende bacteriën met carbapenem heeft geleid tot toenemende resistentie tegen carbapenem en de ontdekking van diverse carbapenemases. De in 2010 ontdekte New Delhi metallo-β-lactamase 1 (NDM-1) en de globale verspreiding hiervan is van grote zorg aangezien er zeer weinig effectieve antibiotica zijn tegen deze zeer resistente bacteriën (26). Bovendien is de detectie van carbapenemases problematisch doordat er verschillende methodes zijn, die verschillende resultaten opleveren. Nucleïnezuur amplificatietechnieken zijn dan ook essentieel om deze resistentiemechanismen te ontdekken.

Nucleïnezuur amplificatie (moleculaire diagnostiek)

Elk species bezit een unieke nucleïnezuur (DNA) vingerafdruk, die kan worden gebruikt als voor de detectie en identificatie van micro-organismen in klinische monsters. Bovendien kan op deze manier ook aanvullende informatie van dat micro-organisme, zoals resistentiegenen en virulentiefactoren, worden gevonden. Duidelijke voordelen ten opzichte van kweek zijn een sneller resultaat en verhoogde sensitiviteit en specificiteit.

Echter, er bestaat een risico op foutpositieve (contaminatie of kruisreactiviteit) en foutnegatieve (ongevoelige test, variabiliteit van het target of remming van de test) uitslagen. Tevens vindt men alleen waarop men test.

Bij het invoeren van dit soort assays dient het hele proces (monsterafname, DNA extractie, amplificatie en interpretatie van de resultaten) uitvoerig gevalideerd te worden. Bovendien dienen positieve - en negatieve controles en remmingcontroles te worden getest. Daarnaast

moet het monster worden voorbehandeld om een goede DNA opbrengst te kunnen garanderen en moeten alle reagentia regelmatig worden getest.

In **hoofdstuk 5** wordt een overzicht gegeven van de laatste stand van zaken ten aanzien van de moleculaire diagnostiek en typeren van MRSA.

Bacterieel typeren

Na detectie en identificatie van (antibioticum resistente) bacteriën, kan het noodzakelijk zijn om deze te identificeren op stamniveau (ook bekend als typeren) om potentiële transmissie vast te stellen. Bovendien is typeren geschikt om (inter)nationale verspreiding van bacteriën aan te tonen. Tegenwoordig zijn er veel verschillende typeertechnieken beschikbaar in het diagnostisch laboratorium.

In **hoofdstuk 6** hebben we een gedeelte van een internationale collectie MRSA stammen afkomstig uit 11 Europese landen (27) geanalyseerd met behulp van het DiversiLab systeem (bioMérieux), pulsed-field gel electroforese (PFGE) en multi-locus sequence typing (MLST). Het doel van deze studie was het bepalen van de performance en het gebruiksgemak van het Diversilab systeem ten opzichte van de huidige gebruikte systemen.

We hebben geconcludeerd dat het Diversilab system een snelle typeertechniek is. De resolutie, nodig voor uitbraakanalyse, is echter onvoldoende om genetisch en epidemiologisch unieke MRSA stammen te onderscheiden. Het Diversilab systeem is waarschijnlijk meer bruikbaar voor lange-termijn studies.

Het doel van de studie in **Hoofdstuk 7** was het bepalen van de performance en het gebruiksgemak van een andere typeertechniek, het SpectraCell systeem (SCRA; River Diagnostics), voor het typeren van MRSA.

Op stamniveau kwamen de gevonden resultaten voor 95.7% overeen met de gouden standaard. Echter, wanneer we onze resultaten analyseerden op het niveau van de huishoudens, waren deze concordant voor 90.7%. Een indicatie dat het discriminerend vermogen van SCRA wellicht te hoog is voor adequate uitbraak analyse.

De reproduceerbaarheid was 100%. Dit wijst erop dat het SCRA systeem over langere tijd stabiel is. Onze resultaten komen overeen met eerdere publicaties (28-29).

De toepasbaarheid van het SCRA systeem met betrekking tot hands-on-time en de doorlooptijd voor het produceren van een uitslag is goed. Zowel de hands-on-time als de doorlooptijd kunnen nog worden verbeterd door te gaan werken met 1 overnachtkweek in plaats van 2 overnachtkweken. Op deze manier zijn resultaten de volgende dag al beschikbaar. Dit levert een enorme tijdswinst op ten opzichte van de huidige typeertechnieken.

Ondanks dat het hoge discriminerende vermogen van SCRA kan leiden tot discrepante uitslagen in 5-10% van epidemiologisch gerelateerde clusters, met bijbehorende consequenties op het gebied van infectiepreventie, concluderen we dat het SpectraCell*RA* system

een uiterst reproduceerbare, gemakkelijk te gebruiken en snelle typeertechniek is, die kan concurreren met de huidige gebruikte technieken.

Kwaliteitscontrole

Voor het uitvoeren van kwalitatief hoogstaande moleculaire diagnostiek is kwaliteitscontrole essentieel. Verschillende laboratoria kunnen verschillende assays gebruiken, met elk hun eigen sensitiviteit en specificiteit. Een manier om hiermee om te gaan is door het verzorgen van externe kwaliteitscontrole programma's (EQA). Deze programma's dienen goed gedefinieerd te zijn en te worden georganiseerd door onafhankelijke instituten.

In **hoofdstuk 8** hebben we de resultaten beschreven van een EQA voor moleculaire diagnostiek van MRSA. We concluderen dat de moleculaire detectie van MRSA in monsters met een hoge concentratie betrouwbaar is. Tevens concluderen we dat de detectie van MRSA in monsters met een lage concentratie onvoldoende is en dat de specificiteit moet worden verhoogd. Ophoping van klinische monsters leidt tot MRSA concentraties boven de 10^9 kolonievormende eenheden per ml, een concentratie hoger dan verwacht mag worden bij een patiënt en veel hoger dan concentraties van dit EQA panel. In het rondgestuurde panel zaten alleen geïnactiveerde stammen, waardoor ophoping niet mogelijk was. Dit zou een mogelijke verklaring kunnen zijn voor de gevonden lage gevoeligheid. De gevonden foute resultaten onderstrepen de noodzaak voor verbetering van de specificiteit van moleculaire MRSA testen.

Om de kwaliteit van moleculaire diagnostiek te verbeteren zullen zowel laboratoria als fabrikanten aangemoedigd moeten worden om deel te nemen aan EQA en zal internationale standaardisatie ingesteld moeten worden. Daarnaast zullen EQA panels ook beschikbaar moeten komen voor detectie van andere belangrijke (resistente) bacteriën, zoals de al eerder genoemde ESBL producerende bacteriën.

Afhankelijk van de technologische en financiële mogelijkheden, zal een klinisch microbiologisch laboratorium de meest optimale diagnostische strategie voor de detectie van microorganismen moeten zoeken door conventionele technieken te combineren met moleculaire diagnostiek. Hierbij zal een balans moeten worden gevonden tussen performance (sensitiviteit, specificiteit) and gebruikersgemak (hands-on-time, kosten, etc.). Uiteindelijk zal dit leiden tot de meest optimale patiëntenzorg.

Discussie en toekomstperspectief

In **hoofdstuk 9** is een overzicht gegeven van nieuwe diagnostische technieken voor de detectie en het typeren van bacteriën in klinische laboratoria (MALDI-TOF, elektronische neus en Raman spectroscopie) die momenteel worden ontwikkeld.

Bovendien zijn in **hoofdstuk 9** de belangrijkste kandidaten voor multiplex analyse (Luminex, microarray en sequencing) bediscussieerd. Snelle multiplex analyse is de ultieme diagnostische strategie. Met betrekking tot Luminex en microarray is het grootste probleem dat beide technieken niet in staat zijn om nieuwe (varianten van) micro-organismen te ontdekken. Het gebruik van beide systemen zal dan ook waarschijnlijk worden verdrongen door de introductie van (totaal genoom) next generation sequencing. Richting het einde van dit decennium is het waarschijnlijk dat next generation sequencing een veelvoorkomend klinisch diagnostische techniek zal zijn, die door laboratoria direct op klinisch materiaal zal worden ingezet.

Toekomstperspectief

Point-of-care diagnostiek

De laatste jaren hebben we een exponentiële stijging gezien in de hoeveelheid onderzoek en financiële ondersteuning voor de ontwikkeling van nieuwe snelle diagnostische testen, de zogeheten point-of-care (POC) testen.

Welke eigenschappen zou een ideale POC test moeten bezitten? De primaire drijfveer voor de ontwikkeling van een dergelijke test is een snel resultaat. De totale tijd totdat het resultaat bekend is, zal, ideaal gezien, maximaal 1 uur zijn. Het apparaat zal draagbaar moeten zijn en de kosten per test moeten laag zijn (± €25 voor de "westerse wereld", maar veel minder voor de "3^{e} wereld"). De uitslagen zullen duidelijk en gemakkelijk te begrijpen moeten zijn. De performance karakteristieken van een POC test zullen vergelijkbaar moeten zijn met die van de laboratoriumversie. Een belangrijke overweging zal het input volume van het monster zijn, wat mogelijk gevolgen heeft voor de gevoeligheid. Nauwkeurigheid en precisie binnen 1 apparaat en tussen meerdere apparaten is een andere overweging. Het gelijktijdig met het monster testen van interne controles zal moeten worden ingevoerd om te zorgen dat elke test correct wordt uitgevoerd. Externe controles zullen moeten worden meegenomen om de performance tussen apparaten te waarborgen. De technologie zal tevens beschikbaar moeten zijn voor bevolkingsgroepen die geen of minder toegang hebben tot traditionele laboratoriumtesten, zoals in ontwikkelingslanden in b.v. Afrika.

Automatisering

Om snellere diagnostiek als POC tests mogelijk te maken is automatisering een van de grootste uitdagingen. Automatisering zal hands-on-time verlagen en maakt het mogelijk om meerdere monsters tegelijk te testen. Bovendien zal automatisering leiden tot uitgebreidere standaardisering, wat ervoor zorgt dat ook minder opgeleid personeel deze POC testen zal kunnen uitvoeren. Op deze manier wordt het aantal mensen dat diagnostische testen kan uitvoeren vergroot.

Automatisering van de resultaatverwerking is ook een belangrijke factor. Hoe sneller een arts een resultaat heeft, hoe adequater en gerichter de patiënt behandeld kan worden. Met de introductie van meerdere nieuwe technieken en apparaten, zullen laboratorium informatie systemen (LIS) hiervoor geschikt moeten worden gemaakt. Een goed LIS is essentieel om optimale klinische zorg te kunnen garanderen.

Conclusie

In overeenstemming met de contrasterende voordelen en nadelen zoals beschreven in dit proefschrift, is de ontwikkeling van POC diagnostiek een speerpunt van huidig onderzoek. Tegelijkertijd zijn er momenteel nog weinig testen commercieel verkrijgbaar. In de komende 5-10 jaar zullen we in de diagnostiek van infectieziekten een verschuiving zien richting snelle POC diagnostiek. Dit zal het mogelijk maken om diagnostiek te versnellen, met efficiëntere behandeling van patiënten als resultaat. Bovendien zal er veel onderzoek nodig zijn om AST te versnellen en vereenvoudigen, wat nog steeds een van de grootste uitdagingen is binnen de klinische microbiologie.

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Appendices

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Curriculum vitae

René te Witt was born in Rotterdam, on December 16, 1975. He passed his secondary school (VWO) exam in 1994 at the Scholengemeenschap Blaise Pascal in Spijkenisse. From 1994 to 1998 he studied Medical Microbiology at the Hogeschool Rotterdam & Omstreken where he graduated for his bachelor degree (Bsc/Ing) on a project studying *Enterococcus faecalis* and *Enterococcus faecium*. In 1998 he started working at the Department of Medical Microbiology and Infection prevention at the Sint Franciscus Gasthuis in Rotterdam. During his nearly 9 years at the SFG he participated in routine microbiological diagnostics and specialized in molecular diagnostics, serology, parasitology and *Mycobacterium tuberculosis*.

In 2007 he moved to the Department of Medical Microbiology and Infectious Diseases, Unit Research & Development at the Erasmus MC in Rotterdam, where he started his PhD project on "Clinical Microbiological Diagnostics 2.0" under the supervision of Prof.dr.dr. A. van Belkum and Dr. W. van Leeuwen.

After obtaining his registration for Medical Microbiological Researcher (Medisch Microbiologisch Onderzoeker; SMBWO), René will start his training for the Medical Molecular Microbiologist (Medisch Moleculair Microbioloog) registration in March 2012.

List of publications

te Witt, R., P.M. Oostvogel, R. Yahiaoui, Y. Wu, A. van Belkum, and A.E. Muller. 2011. In vitro evaluation of the performance of Granada selective enrichment broth for the detection of group B streptococcal colonization. Eur J Clin Microbiol Infect Dis.

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te Witt, R., M.E. van Wolfswinkel, P.L. Petit, J.J. van Hellemond, R. Koelewijn, A. van Belkum, and P.J. van Genderen. 2010. Neopterin and procalcitonin are suitable biomarkers for exclusion of severe *Plasmodium falciparum* disease at the initial clinical assessment of travellers with imported malaria. Malar J 9:255-62.

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Goessens, W.H., P. de Man, J.G. Koeleman, A. Luijendijk, **R. te Witt**, H.P. Endtz, and A. van Belkum. 2005. Comparison of the COBAS AMPLICOR MTB and BDProbeTec ET assays for detection of *Mycobacterium tuberculosis* in respiratory specimens. J Clin Microbiol 43:2563-6.

van Den Braak, N., A. van Belkum, D. Kreft, **R. te Witt**, H.A. Verbrugh, and H.P. Endtz. 1999. The prevalence and clonal expansion of high-level gentamicin-resistant enterococci isolated from blood cultures in a Dutch university hospital. J Antimicrob Chemother 44:795-8.

van den Braak, N., A. van Belkum, **R. te Witt**, H.A. Verbrugh, and H.P. Endtz. 1998. Glycopeptide resistance amongst *Staphylococcus* spp. J Antimicrob Chemother 42:673-5.

PhD Portfolio

Name PhD student: René te Witt
Erasmus MC Department: Medical Microbiology and Infectious Diseases
PhD period: September 2007 – December 2011
Promotor: Prof.dr.dr. Alex van Belkum
Co-promotor: Dr. Willem B. van Leeuwen

PhD training	*Year*	*ECTS*
In-depth courses		
• Quality and troubleshooting with real-time PCR	2008	1.1
• Molecular Diagnostics	2008	0.6
• Molecular Microbiology of Infectious Diseases	2008	0.6
• Biomedical Research Techniques	2008	0.1
• Epidemiology	2009	0.1
• Primer and probe design	2010	0.6
• Molecular Typing Methods	2011	0.8
• "Succesvol gebruik van moleculaire diagnostiek"	2011	1.4
Seminars and workshops		
• Department Journal Clubs	2007-2011	4
• Department Research Meetings	2007-2011	4
• PhD Day Erasmus MC	2008, 2011	1
• Research Day Dept. MMID	2008-2010	1
National and international conferences		
• EMMD, Scheveningen	2007	0.6
• ECCMID, Barcelona, Spain	2008	1
• ASM, Philadelphia, USA	2009	1
• EMMD, Scheveningen	2009	0.6
• ECCMID, Vienna, Austria	2010	1
• Scientific Spring Meeting NVMM, Papendal	2010	1
• IMMEM, Wernigerode, Germany	2010	1
• EMMD, Scheveningen	2011	1
Presentations and lecturing		
• Poster presentation, ECCMID, Barcelona	2008	0.5
• Poster presentation, ASM, Philadelphia	2009	0.5
• Poster presentation (2x), EMMD, Scheveningen	2009	1

• Poster presentation, Mol Med Day, Rotterdam	2009	0.5
• Lecture, medical students, Rotterdam	2010, 2011	0.5
• Oral presentation (2x), ECCMID, Vienna	2010	1
• Poster presentation, ECCMID, Vienna	2010	0.5
• Oral presentation, bioMérieux symposium, Kaatsheuvel	2010	0.5
• Oral presentation, IMMEM, Wernigerode	2010	0.5
• Lecture (3x), Hogeschool Leiden, Leiden	2010	0.5
• Oral presentation, STAR laboratory symposium, Rotterdam	2010	0.5
• Oral presentation, HET instrument, Amsterdam	2010	0.5
• Oral presentation, Mol Med Day, Rotterdam	2011	0.5
• Oral presentation, NVML symposium, Ede	2011	0.5
• Poster presentation, EMMD, Scheveningen	2011	0.5
• Lecture, Hogeschool Leiden, Leiden	2011	0.5
• Oral presentations, Dpt. MMID, Rotterdam	2008-2011	2

Scientific meetings

• WMDI meeting, Utrecht	2007-2011	3
• Mol Med Day, Rotterdam	2009	0.3
• Roche symposium, Utrecht	2009	0.3
• Microbial typing symposium, Utrecht	2009	0.3
• Mol Med Day, Rotterdam	2011	0.3
• "2e Nederlandse Infectieziektendag", Zeist	2011	0.3
• Cepheid symposium, Leiden	2011	0.3
• BD symposium, Eindhoven	2011	0.3

Supervision of students

• Supervision of Bachelor of Science students	2007-2011	34
• Supervision of medical students Dermatology	2009, 2011	4
• Supervision of medical students "Vaardigheidsonderwijs"	2008, 2010, 2011	3

Other activities

• 3 months "Internship" molecular diagnostics, MMID	2009	12