A Systematic Approach to Elucidate Causes of Gastroenteritis Outbreaks of Suspected Viral Etiology

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A Systematic Approach to Elucidate Causes of Gastroenteritis Outbreaks of Suspected Viral Etiology

Een systematisch onderzoek naar oorzaken van gastro-enteritis uitbraken verdacht van virale etiologie

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Chapter 1

General Introduction

Introduction.

The main objective of this thesis was to investigate the etiology of outbreaks of viral gastroenteritis that remained without diagnosis after testing for common viral pathogens causing gastroenteritis, e.g. noroviruses, rotaviruses, sapoviruses, adenoviruses, and astroviruses. No causative pathogen could be detected in over 10% of these outbreaks. Viral gastroenteritis is about the most frequent disease in humans and this unexplained fraction concerns about 150000 cases in the Netherlands each year (41). Here, an overview of the state of the art of the literature on viral gastroenteritis is provided.

Gastroenteritis.

Gastroenteritis, which is also known as stomach flu, is an inflammation of the gastrointestinal tract that can be caused by a wide range of infectious and non-infectious causes. The infectious causes are a broad range of viruses, bacteria, and parasites. Gastroenteritis is defined by its clinical picture, which is mainly characterized by diarrhea and vomiting. Based on the clinical picture alone, it is not possible to distinguish between infectious and non-infectious causes. Here, we focus on the infectious causes only.

Epidemic and sporadic acute viral gastroenteritis (GE), is one of the most common diseases in humans in all age groups worldwide causing substantial morbidity and mortality (1, 23, 169). The estimated incidence is highest in children under the age of 5 years, and more than 700 million cases per year of acute diarrhea occur in this age group (23, 169). Annually, 3-5 million deaths occur as a result of diarrheal disease, placing these in the top five causes of death worldwide, with the majority again in children under age of 5 years in developing countries (1, 23, 37). The majority of these infections are of viral origin. In developed countries the mortality due to gastroenteritis is limited, as a result of better living conditions and improved hygiene and sanitation, which limits the spread of gastroenteritis viruses, and adequate and timely treatment. As a result, gastroenteritis episodes in these countries typically are considered mild and self-limiting. However, due to the high incidence, the morbidity of this disease remains high, and diarrheal disease remains an important reason for hospitalization, particularly in children (1, 23, 37, 169).

History of viral gastroenteritis.

Epidemic non-bacterial gastroenteritis or epidemic vomiting disease has been described first in 1929 and was named “winter vomiting disease” (177), describing the seasonality and one of the symptoms of the disease. It could not be attributed to known bacterial or parasitic pathogens, and the etiological agent was identified many years later. The breakthrough was the discovery of virus particles in human-passaged fecal material derived from an outbreak of acute gastroenteritis at an elementary school in 1968 in Norwalk, Ohio, USA (79). Teachers and students had symptoms of acute gastroenteritis, both vomiting and diarrhea, and attempts to identify a pathogen failed. In 1972, Kapikian and co-workers observed viral particles in stool samples of volunteers that had been infected with purified stool samples from a patient from the Norwalk outbreak (4, 79, 147). Particles of 27 to 32 nm were measured and the agent was named Norwalk virus. These were the first viruses associated with gastroenteritis. Similarly, a year later, Bishop et al. identified rotaviruses in the duodenal mucosa of children with gastroenteritis (17), and these are now recognized as major pathogen in childhood diarrhea. In 1975 astroviruses (98) and enteric adenoviruses (115) were identified in the feces of children with acute diarrhea and were added to the list of viruses causing gastroenteritis (Figure 1).

Definitions of gastroenteritis.

Gastroenteritis is defined by its clinical picture, but there is no internationally accepted case definition. The WHO defines diarrhea as three or more loose stools in 24 hours, but this does not include vomiting, which is a common symptom of viral gastroenteritis. Persons with viral gastroenteritis may have fever, headache, abdominal pain, and cramps, along with vomiting and frequent and watery diarrhea. The condition is usually characterized by acute onset, and lasts 1 to 6 days. The disease is generally mild and self-limiting although dehydration may develop, especially with rotavirus infections.

For the diagnosis of viral gastroenteritis, and particularly noroviruses, a definition to recognize outbreaks has been proposed and successfully used with the following criteria (81) 1) absence of bacterial and parasitic pathogens in stool samples 2) vomiting in more than 50% of the affected cases; 3) mean (or median) duration of illness from 12 to 60 hours, and 4) mean (or median) incubation period of 24 to 48 hours.

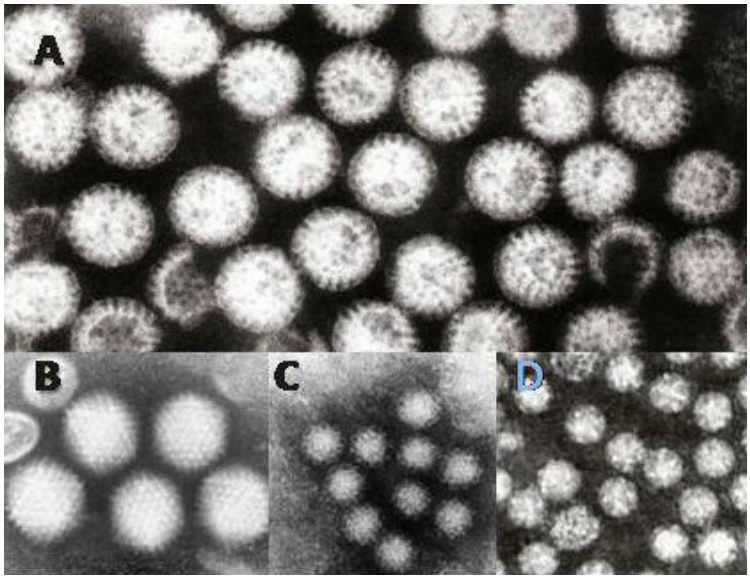

FIGURE 1. Electron micrographs of viruses that cause gastroenteritis in humans. The virus particles are shown at the same magnification (approximately x 200,000) to allow size comparison. A = rotavirus, B = adenovirus, C = norovirus and D = astrovirus. Picture adapted from Wikipedia (http://en.wikipedia.org/wiki/Gastroenteritis)

DESCRIPTION OF THE VIRAL AGENTS CAUSING GASTROENTERITIS

Human caliciviruses: noroviruses and sapoviruses.

Taxonomy and morpohology

Noroviruses and sapoviruses are the two out of five genera of the family *Caliciviridae*.

Noroviruses and sapoviruses contain viruses that cause infections in humans (Figure 2). Furthermore, noroviruses have also been detected in porcine, bovine, murine, feline, ovine, pigs, cattle, canine and other species (45, 100, 101, 105-107, 109, 122, 127, 165, 170), and sapoviruses in swine, clams and oysters (65, 121, 164, 178). In humans, noroviruses and sapoviruses cause gastroenteritis, and will be described here in more detail.

The other genera of the family *Caliciviridae* are *Lagovirus*, *Vesivirus*, and *Nebovirus* encompassing viruses infecting rabbits, and brown hares (lagoviruses), sea lions, swine, cats, dogs, fish, seals, other marine animals, cattle and primates (vesiviruses), and cattle (Nebovirus) (15, 26, 38, 61, 103, 128, 135, 137, 140, 166, 179). Another potential genus, called Recovirus, in the *Caliciviridae* family has been described comprising viruses detected in rhesus macaques (57, 58). The animal *Caliciviridae* viruses can cause a range of different clinical syndromes in animals, including oral lesions, systemic disease with hemorrhagic syndromes, upper respiratory tract infections and other.

The virions of human caliciviruses are composed of a single structural capsid protein, with icosahedral symmetry, with characteristic existence of 32 cup-shaped depressions, situated on the axes of the icosahedrons. Its Latin designation, calyx, meaning a cup, is from where the family name is derived (Table 1) (10, 70, 169). The genome is a positive-sense, single-stranded RNA of 7.5 kb with two or three open reading frames (ORFs). In noroviruses, ORF1 encodes for non-structural proteins, ORF2 for the structural proteins, and ORF3 encodes a protein for which the function is unknown (10, 149, 169). In the sapovirus genome, ORF1 encodes the non-structural and structural proteins, ORF2 is a small protein of unknown function and the significance of ORF3 is still uncertain (95).

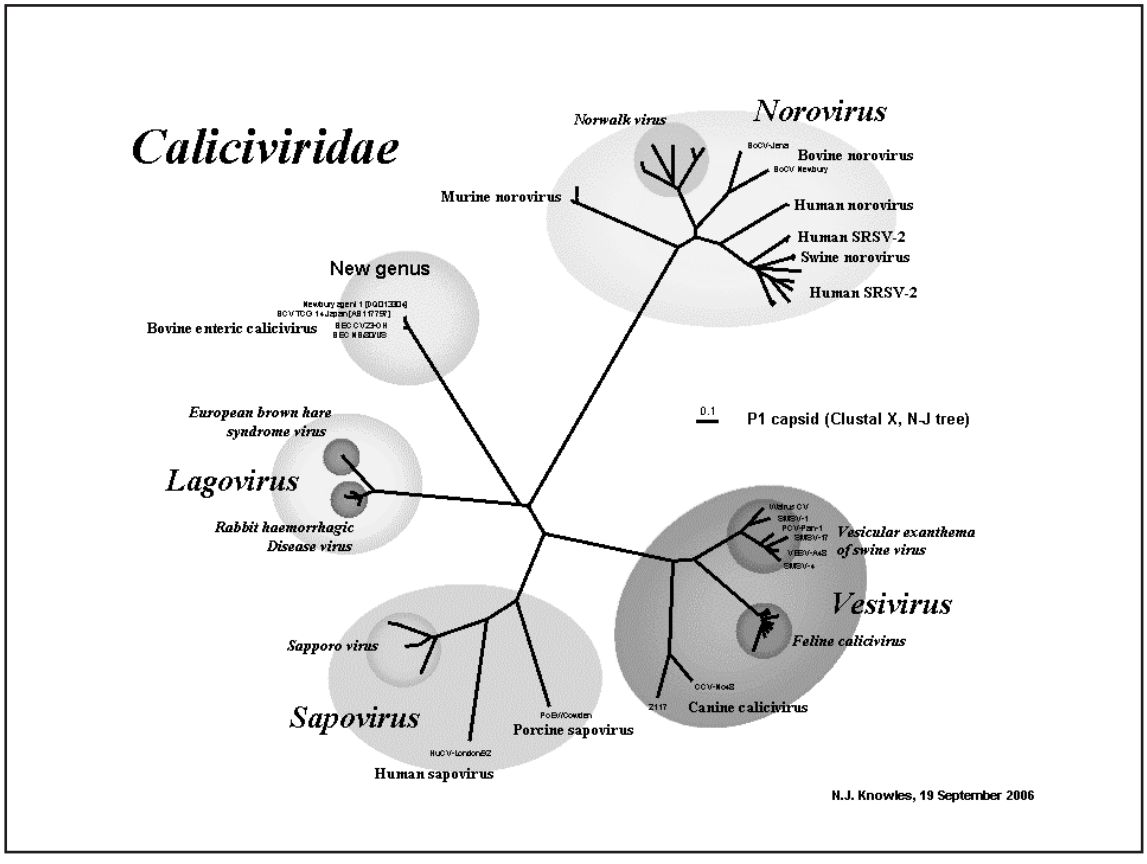

FIGURE 2. Neighbor-joining tree of family *Caliciviridae* based on capsid gene sequences.
Picture adapted from www.caliciviridae.com (N.J. Knowles). New genus has been named Nebovirus.

Classification

Noroviruses and sapoviruses are genetically diverse and can be divided in genogroups.

Noroviruses are divided in five genogroups, of which genogroup I, II and IV infect humans, while genogroup III only infects cattle and genogroup V mice. The genogroups have been characterized based on DNA sequences of PCR products from the RNA polymerase region in ORF1 (9), while sequencing of the capsid gene (ORF 2) has provided further strain discrimination and recognition of additional genotypes (56, 64, 161).

Sapoviruses are divided in five genogroups (GI to GV), where genogroup III has been found to infect porcine species, and GI, GII, GIV, and GV are known to infect humans (59, 148). Based on sequence analysis sapoviruses are genetically more similar to the members of Lagoviruses than to those in the *Norovirus* genus.

Epidemiology

Noroviruses are the most common cause of acute gastroenteritis (42), and notorious for causing outbreaks. They are responsible for about 70% of all outbreaks in developed countries. In the Netherlands, noroviruses accounted for 78% of the outbreaks in a twelve-year study (152). Often when a new strain is detected an increase of the norovirus outbreaks is noted (150). Infections with the new strain can result in outbreaks with a high attack rate that infects thousands of people. Sapovirus infections are less common and known to cause disease primarily in young children, usually under 5 years of age (66, 67, 73). Although sapovirus associated diarrhea is generally mild, severe cases can occur (142). Recently sapoviruses have been reported as an occasional cause of outbreaks in hospitals and other health care facilities (74, 174).

Clinical manifestations

Noroviruses are extremely infectious and therefore able to cause large outbreaks. The infection dose is low, as 10 -100 particles are enough to cause an infection (33, 34, 156). After ingestion, within 12 to 48 hours, nausea, vomiting and diarrhea may be seen as symptoms. Stool samples contain high numbers of particles, and shedding may be for at least two weeks, often longer (37). Dehydration is a common complication that can affect young children, elderly, and immunodeficient patients (96, 143). Illness by noroviruses has a highattack rate, which results in high rates of transmission. The illness is usually mild and self-limiting, although sapoviruses can cause severe diarrhea (80, 172). Furthermore, some settings are more prone to outbreaks, like nursing homes and hospitals, where relatively more susceptible individuals (immunocompromised) are present (35, 44, 157).

Asymptomatic calicivirus infections are common in the general population (42, 143). Immunity to the infecting strain may develop, but is short-lived, strain specific, and does not provide protection for future infections. Since noroviruses are genetically variable, people are likely to be infected many times during their lifetime (75, 131, 132, 172). Seroprevalence at year 1 for norovirus genogroup II.4 was 18% and 43%, IgA and IgG, respectively. For genogroup I.1 noroviruses seroprevalence was lower at 2% and 19% respectively (138).

Distribution and transmission

Noroviruses are now the most widely recognized viral agents associated with outbreaks of non-bacterial gastroenteritis. Noroviruses are transmitted through the fecal-oral route, the oral-oral route (vomiting), directly by consumption of contaminated food or water or through environmental transmission. The most common route of transmission, person-to-person transmission, is facilitated by the low infectious dose of 10-100 virus particles that can cause an infection (32, 168). Foodborne transmission plays an important role as well, and infected food handlers can be responsible for fast spread of a virus (19, 21, 43). Furthermore, several waterborne outbreaks have been reported (7, 18). Outbreaks caused by noroviruses commonly occur in closed communities such as residential homes, schools, camps, hospitals, cruise ships, and the places where food and water is shared. Furthermore, many outbreaks have a combined transmission, viruses are introduced by food and subsequently spread through the closed setting by person to person transmission.

Sapoviruses cause gastroenteritis among children and adults, but most often are found in children. Therefore it is most likely that transmission happens primarily from person to person and/or by the fecal-oral route.

Rotavirus.

Taxonomy and morphology

Rotaviruses are classified in the genus *Rotavirus* and belong to family *Reoviridae*, a large family that consists of nine genera (104). Rotaviruses can cause disease in humans and domestic animals. Human and animal strains are usually distinct, but some strains are closely related to animal strains, and cross-species infection can occur.

Rotaviruses have a non-enveloped, isocahedral structure and a diameter of 60 to 80 nm. Particles have a wheel-like appearance, which was used in the nomenclature (in latin rota means wheel). Rotaviruses have a genome of 16 to 27 kb (Table 1) (169). The capsid consists of a double protein layer. The outer one is composed of proteins VP7 and VP4, and the inside layer consists mainly of VP6. The core encloses the genome that consists of 11 segments double stranded RNA. These RNA's encode six viral structural proteins, VP1, 2, 3, 4, 6 and 7, and six nonstructural proteins (NSP) 1 to 6 (11, 84, 169).

Classification

Based on antigenic properties of VP6, rotaviruses are classified into seven groups, named A to G. Groups A, B, and C all contain human pathogens. Within these groups, subgroups I and II are distinguished, and classification into serotypes has been done on the antigenic differences in the proteins of the outer capsid, VP7 and VP4. For rotavirus group A there are 15 G types, based on VP7 antigens, and 20 P types, based on VP4 antigens (8, 55, 136, 169).

Epidemiology

Rotaviruses cause almost one million deaths per year worldwide. The disease is most severe in children under 5 years, especially in developing countries, where it accounts for 2 million hospitalizations and up to 600000 deaths a year (130). Rotavirus is a significant cause of hospitalizations in developed countries (36).Most infections are caused by rotaviruses of group A, and are sporadic, while rotavirus group B has been identified in epidemic outbreaks of severe diarrhea in adults, and in symptomatic infections in children (5, 146).

Group C rotaviruses cause sporadic infections, but severe illness can occur in co-infection with group A viruses (28-31, 145). Of the rotavirus group A serotypes, at least 10 G serotypes and 8 P genotypes cause human infections. G1-4 are most common types found worldwide, and P[4] and P[8] are most common types in association with these serotypes. G1P[8], G2P[4], G3P[8], and G4P[8] are the most common combinations found to cause disease globally (49, 82, 83, 89, 93, 151), although in recent years strains with G9 surface proteins have emerged (91, 102).

Clinical manifestation

Most rotavirus infections occur during the winter months, and in children from 6 months to 2 years. The disease can be severe and cause dehydration. The incubation period after ingestion of the virus particles takes 2 to 6 days, followed by 1 to 4 days of diarrhea. Clinical symptoms include fever for 2 to 3 days and vomiting, which is followed by non-bloody diarrhea. Virus is shed in feces for 5 to 7 days, usually in extreme high numbers. In adults, the symptoms of rotavirus infection can also involve nausea, anorexia, fever, headache, malaise, and vomiting (8, 37).

Infection by rotaviruses is mostly asymptomatic during the neonatal period, due to maternal antibodies or by the attenuated nature of certain strains that are frequently detected in symptomatic and asymptomatic infections in neonates (70). Seroprevalence at 1 year of age was 39% and 29 % for IgG and IgA, respectively and breastfeeding during the first three months of life resulted in lower IgG seropositivity (35%) compared to 49% when not breastfed (138).

Distribution and transmission

Rotavirus infections occur worldwide, and disease can occur in all age groups, although it is generally considered to be a mild infection in adults, due to immunity developed through previous infection with the same serotype of virus (endemic viruses) (37). However infections with a new genotype can cause more severe infections (91, 102).

Rotaviruses spread from person to person by fecal-oral transmission. Because rotaviruses most often infect children, the major route of transmission is person-to-person through care-givers and the parents that take care of infected children. Rotavirus has occasionally been associated with food and waterborne outbreaks (37).

Enteric adenoviruses.

Taxonomy and morphology

Human adenoviruses belong to the family of *Adenoviridae* and are classified into two genera *Mastadenovirus*, infecting mammals, and *Aviadenovirus*, infecting birds. There are more than 100 members of the *Adenoviridae* that are isolated from humans and animals. Adenoviruses measure 80 to 110 nm, have no envelope, have a linear double stranded DNA genome, and particles with icosahedral symmetry (Table 1). Their genome has a size of 28-45 kilo base pairs (kbp) (22, 169).

Classification

At least 51 serotypes, which are divided in six subgenera (A to F) have been described in humans (37, 40, 169). The majority of adenoviruses that cause gastro-enteritis belong to genogroup F serotypes 40 and 41. Occasionally other serotypes have been implicated in acute diarrhea (serotype 1, 2, 5, and 6 of subgenus C and serotypes 31, 12, and 18 of subgenus A) (37, 40, 169).

Epidemiology

Adenoviruses are important cause of gastroenteritis in children under four years (6, 20, 85). About 15

% of all childhood diarrhea has been caused by adenoviruses (37) . By the age of 3 years most children have neutralizing anti-adenovirus 40/41, suggesting that most children have been infected by adenoviruses 40 and 41. Approximately half of the children have positive adenovirus titers, as a result that serologic diagnosis is less useful in a clinical setting (21, 37, 70).

Clinical manifestation
The illness caused by enteric adenoviruses is mild and self-limiting. They can cause infections throughout the year, and mostly in children. In most cases of clinical infection, the symptoms are relatively mild. The incubation period of adenoviruses is eight to ten days and diarrhea can last three to eleven days. Illness can be severe in immunodeficient patients, and infection with adenoviruses 40 and 41 can cause chronic diarrhea (20).

Distribution and transmission
Adenoviruses are widespread in nature, and infect birds and mammals. They commonly cause respiratory infections, but are also involved in gastroenteritis, particularly, enteric adenoviruses (6, 20). The transmission route of enteric adenoviruses occurs via fecal-oral route, and they can be transmitted from person-to-person (direct contact) or via environmental and respiratory routes (6, 20).

Astroviruses.
Taxonomy and morphology
The family *Astroviridae* is divided in two genera, named *Avastrovirus* (containing avian viruses), and *Mamastrovirus* consisting of human, feline, mink, ovine, porcine, sea lion, bat and other viruses (112). Astroviruses are non-enveloped viruses with a diameter of 28-35 nm. The particles appear as a five or a six pointed star . Astroviruses have a positive sense single-stranded RNA with a genome of 6.8-8 kb that contains three open reading frames ORF1a and ORF1b, and ORF2 encodes the capsid (Table 1) (27, 37, 70, 163, 169).

Classification
Astroviruses are classified into genotypes based on the nucleotide sequence of ORF2 (126). This has a good correlation with astrovirus serotypes, which are based on the reactivity of the capsid proteins with monoclonal antibodies and cross neutralization assays. There are eight geno/serotypes. Serotypes 1 and 2 are most often detected in diarrheal diseases (60, 126, 169).

Epidemiology
Human astroviruses are distributed worldwide, and typically infect the same age groups as rotaviruses. They can cause outbreaks of infection in adults, but more often they cause single cases of gastroenteritis in children, and cause mild illness. They are often involved in mixed infections, especially with rotaviruses (63, 111).

Clinical manifestation
The main characteristic of human astrovirus infection is self-limiting gastroenteritis. Astrovirus diarrhea is short lasting and less severe than that caused by other viruses. Symptoms include diarrhea, fever, nausea, with occasional vomiting and lasts, normally, only 2-3 days. Viruses can be excreted in feces for up to 14 days. Children under the age of one year are most often infected, and have more severe disease than adults. Although astroviruses cause mild infection in adults, in immunocompromised patients this can be more severe and excretion in feces can be even longer. Astrovirus type 3 can cause more severe diarrhea, and symptoms may be more prevalent in immunodeficient patients. Asymptomatic excretion of astroviruses can occur in 5-20% of neonates and young children (25, 37, 53).

Distribution and transmission
Astroviruses are distributed worldwide and have been isolated from birds, cats, dogs, pigs, sheep, cow and man. Transmission of astroviruses mostly occurs via the fecal-oral route via food, water, and person-to

TABLE 1. Characteristic of the viral agents causing gastroenteritis

Virus	Family	Nucleic Acid Type	Morphology/ symmetry	Size of virion (nm)	Cultivable	Genome Size (kb)	Main symptoms	Duration	Incubation time	Risk groups
Norovirus	Caliciviridae	(+) ssRNA	Icosahedral	28-35	N	7.4-7.7	Diarrhea, vomiting	1-2 days	1 day	All
Sapovirus	Caliciviridae	(+) ssRNA	Icosahedral	28-35	N	7.4-7.7	Diarrhea, vomiting			All
Rotavirus	Reoviridae	dsRNA	Icosahedral	60-80	Y	16-27	Watery diarrhea, fever, vomiting	3-9 days	1-2 days	Children
Adenovirus	Adenoviridae	dsDNA	Icosahedral	70-90	Y	28-45	Watery diarrhea, fever	1-2 weeks	3-7 days	Children
Astrovirus	Astroviridae	(+) ssRNA	Icosahedral	28-30	Y	7-8	Diarrhea, fever	2-3 days	3-4 days	Children
Coronavirus	Coronaviridae	(+) ssRNA	Helical	80-220	Y	20-30	Cold, diarrhea, vomiting	7 days	2-5 days	Children and adults with chronic bronchitis
Torovirus	Coronaviridae	(+) ssRNA	Helical	100-150	Y	20-25	Diarrhea, vomiting			Children
Picobirnavirus	Birnaviridae	(+) ssRNA	Icosahedral	35	Y	5.8-6.4	Diarrhea, nausea			
Enterovirus	Picornaviridae	(+) ssRNA	Icosahedral	28-30	Y	7.2-8.4	Wide range of symptoms	3- 10 days	3-7 days	Children
Aichi virus	Picornaviridae	(+) ssRNA	Icosahedral	27-30	Y	7-9	Diarrhea, vomiting	3- 10 days	3-7 days	Children
Parechovirus	Picornaviridae	(+) ssRNA	Icosahedral	27-30	Y	7.5	Mild respiratory complaints, encephalitis, myocarditis	3- 10 days	3-7 days	Children
Saffold virus	Picornaviridae	(+) ssRNA	Icosahedral	27-30	Y	7.5	Diarrhea	3- 10 days	3-7 days	Children

person contact. Asymptomatic excretion can occur in neonates and young children and is a significant cause of infection, especially in nurseries, child daycare centers, and hospitals (20, 33, 34).

OTHER PATHOGENS ASSOCIATED WITH GASTROENTERITIS

Other viruses found in feces of humans include coronaviruses, toroviruses, picobirnaviruses, enteroviruses, Aichi viruses and parechoviruses (Table 1). The ability of these viruses to cause gastroenteritis in humans is still unproven, but will briefly be discussed.

Coronaviridae

Coronaviruses are 80 to 220 nm, pleomorphic, enveloped positive-stranded RNA viruses that belong to the family *Coronaviridae*. Although "corona-like" particles have been isolated from human feces, their etiologic role has in human gastroenteritis not been established yet (62).

Toroviruses are 100 to 150 nm, enveloped, positive-sense, single-stranded RNA viruses belonging to genus Torovirus in the *Coronaviridae* family. They were first detected in 1979, and were named Breda virus (86-88, 171). They have a characteristic pleomorphic appearance with club-shaped projection extending from the capsid. They are known to cause gastroenteritis in animals, and although they have been associated with acute diarrhea in children, and have been detected in the fecal samples of patients with gastroenteritis (86, 87), their role in human gastroenteritis is still obscure.

Picobirnaviruses

Picobirnaviruses are small viruses of 35 nm from the family *Birnaviridae*. They are positive-sense, single-stranded RNA viruses. These viruses cause gastroenteritis in a range of domestic animals. They have also been detected in humans with and without diarrhea, but have only been associated with gastroenteritis in human gastroenteritis in immunocompromised patients. Their role as a true pathogen in unknown, although they have been detected in humans (12, 159).

Picornaviridae

Viruses from the *Picornaviridae* family have also been associated with gastroenteritis. Different species from this family have been reported to cause diarrhea in humans and have also been detected in patients with diarrhea as well as healthy individuals e.g, enteroviruses, Aichi virus, human parechoviruses.

Enteroviruses are 28-30 nm, non-enveloped, positive sense, single-stranded RNA viruses with icosahedral symmetry and a genome of 7.2-8.4 kb. These viruses cause a range of diseases, including viral meningitis and poliomyelitis. They are mainly spread by the fecal-oral route, and excreted in feces but do not generally cause gastroenteritis. Food-borne outbreaks associated with coxsackieviruses and echoviruses have been reported (69, 99, 133).

Aichi virus, a member of the *Kobuvirus* genus of the *Picornaviridae* family, is a positive-sense, single-stranded RNA virus with a genome of 8,2 kb and a poly(A) tail. Aichi viruses have been identified in only few cases. They are often associated with oyster related outbreaks (77, 92, 173, 175).

Human parechoviruses are single-stranded, positive-sense RNA viruses in the *Parechovirus* genus within the large family of *Picornaviridae*. HPeV1 infections have generally been associated with mild gastrointestinal and respiratory infections (13, 71, 76, 129).

MOLECULAR DETECTION TECHNIQUES

During the past decades there has been a substantial development and application of molecular detection techniques in the diagnosis of infectious diseases. The PCR based systems can assist identification of target pathogens directly from clinical samples, without need for culture, and have been useful in rapid identification of uncultivable and fastidious organisms.

Molecular diagnostic determinants.

The major determinants for an appropriate molecular diagnostic method are the specificity, clinical sensitivity, technical sensitivity (detection limit), and positive and negative predictive values.

The specificity shows if the chosen method can correctly identify and separate the negative cases, or cases that do not meet the given criteria, so it shows the ability of the method to specifically identify the targeted pathogens.

The clinical sensitivity shows if the chosen method correctly identifies the positive cases, while technical sensitivity (detection limit) measures the minimum of input signal is required to produce a specified output signal, which correlates with the minimum number of target pathogens necessary to obtain reproducible results.

The positive predictive value is the proportion of patients with correctly diagnosed positive test results, so it shows the probability that a positive result actually indicate the underlying disease. The negative predictive value is the proportion of patients with negative test results that are correctly diagnosed.For the proper diagnosis these parameters are important requirements, and together they assure to only detect the pathogens that are aimed to be detected in a very few number of copies.

Polymerase chain reaction.

Polymerase chain reaction (PCR) is a widely used technique in molecular biology because of its high sensitivity, specificity and user-friendly nature. Invented by Cetus scientist Kary Mullis in 1983 (116-119), PCR is the best-developed and most widely used method of nucleic acid amplification. The PCR is based on the ability of thermostable DNA polymerase, which copies a strand of DNA by elongation of complementary strands by amplification of a specific region of the target DNA initiated by a pair of chemically synthesized short oligonucleotides (primers). The basic technique of PCR includes repeated cycles of amplifying selected nucleic acid sequences (116-119). It is a cycling reaction that results in an exponential amplification, producing a vast amount of DNA at the end of the procedure (72). Each cycle consists of three steps: (a) a DNA denaturation step at high temp (90-98°C), in which the double strands of the target DNA are separated; (b) a primer annealing step, performed at a lower temperature (40-65°C), in which primers anneal to their complementary target sequences; and (c) an extension reaction step at approx 72-75°C in which DNA polymerase extends the sequences between the primers. At the end of each cycle that consists of the above three steps, the quantities of PCR products are theoretically doubled, resulting in an exponential amplification, producing vast amount of DNA at the end of the procedure (72). Generally, performance of 30 to 50 thermal cycles results in an exponential increase in the total number of DNA copies synthesized (51, 167). During the PCR reaction, the primers and dNTPs will be built in to the newly synthesized DNA strands. If the reaction consumes the available chemicals, the amplification will also stop (78).

Reverse transcriptase (RT)-PCR

For amplification of RNA targets RT-PCR was developed. During this process, RNA targets are first converted to complementary DNA (cDNA) by RT, and then amplified by PCR. RT-PCR has played an important role in diagnosing RNA-containing virus infections (144). The conventional enzymes used for reverse transcription cannot tolerate high temperatures, and therefore limit the wide application of the method in clinical diagnosis. The thermostable DNA polymerase derived from other organisms have efficient reverse transcription activity and therefore can be used in detection of RNA targets without the need for a separate RT step (120, 176). The higher reaction temperature increases stringency of primer hybridization and avoids the possible RNA secondary structure, making the reaction more specific and efficient than previous protocols that used avian myeloblastosis virus RT.

Nested PCR

Nested PCR assays use two sets of amplification primers and are used to increase sensitivity (detect smaller quantities of target) (52, 68). One set of primers is used for the first round of amplification, which consists of 15 to 30 cycles. The amplification products of the first reaction are then subjected to a second round of amplification with another set of primers that are specific for an internal sequence that was amplified by

the first primer pair (68, 141). Sensitivity of the nested PCR is high due to the dual amplification process. A major disadvantage of the nested-amplification protocol is the high risk of contamination during transfer of the first-round amplification products to a second reaction tube (52), which is why its use for diagnostic purposes should be discouraged.

Multiplex PCR

Multiplex PCR is an amplification reaction with two or more sets of primer pairs specific for different targets in the same tube allowing amplification of more than one target DNA sequence in a specimen (153). Primers for multiplex reactions should be designed to have similar annealing temperatures, requiring extensive experimental testing. Multiplex PCR is often used in diagnostic settings, detecting multiple pathogens and an internal control from a single specimen (141, 153). Quantitative competitive PCR is a variation of multiplex PCR used for quantification of the amount of target DNA or RNA in a specimen (134).

Broad-range and generic PCR

For development of a broad-range and generic PCR assays conserved sequences within phylogenetically informative genetic targets are used for diagnosis. Broad-range rRNA PCR techniques offer the possibility of rapid bacterial identification through use of a single pair of primers targeting bacterial small-subunit (16S) rRNA or DNA (113, 123). For viruses, there are no universal conserved elements, but conserved motifs within a family or genus may be used as targets. Generic assays use degenerate primers for amplification. Degenerate primers are a mix of primers with similar sequences allowing mismatches with multiplex sequences within a sample. For highly diverse RNA viruses, such as noroviruses, this may increase the sensitivity of detection for diagnostic purposes. Broad-range and generic PCR approaches have identified several novel, fastidious, or uncultivated pathogens directly from clinical specimens and are used to amplify divergent DNA/RNA sequences (139). The major obstacles to implementation of broad-range PCR are background contamination and reduced specificity.

The use of PCR detection assays

The PCR detection assays can be separated into generic and specific assays, identifying a genomically related pathogens or only one species. Although the sensitivity and specificity of the PCR detection assays are high, the multiplexing capability of PCR assays is limited. It is possible to use more primer pairs in one reaction resulting in simultaneous amplification of different pathogens, but the accumulation of by-products is much higher with a risk of false diagnosis. The multiplex PCR systems usually have lower sensitivity and the development of the assay needs more extensive optimization. The different lengths and melting temperatures of the primers, and the susceptibility of the oligo-nucleotides forming secondary structures in the reaction also affect the efficiency of the assay.

Identification of the amplified products by the gel-based detection is a long procedure with high risk of contamination and carryover and consequently false positive results. By the improvement of fluorescent chemistries, various real-time PCR methodologies were developed. These techniques proved to be very useful because of the increased speed, the quantitative measurements, and the reduced risks for false positive results caused by contamination (24, 97). They are called real-time PCR because they allow to actually view the increase in the amount of DNA as it is amplified. By introduction of real-time PCR assays, the PCR techniques have become more sensitive, and faster. Therefore, it is a technique that is used increasingly. Several real-time PCR chemistries exist (14, 46), like the SYBR Green chemistry that uses a non-specific fluorescent dye that binds to double-stranded DNA, but the most popular chemistry used is the TaqMan chemistry. Taqman chemistry uses a dual-labeled fluorogenic probe, a probe with a reporter and a quencher, and non-labeled primers. The principle of Taqman probe is based on the exonuclease activity of the polymerase. The intact probe cannot produce fluorescent signals because the reporter and quencher molecules are close to each other, but during the PCR, the polymerase degrades the annealed proportion of the TaqMan probe. When there is no distance between the quencher and the reporter molecules, the fluorophore produces a fluorescent signal, therefore the detected fluorescence is proportional to the amount of DNA generated during the reaction (Figure 3). Subsequently, the machines that are used for real-time PCRs were made applicable for simultaneous use of more clinical samples and with five channels that

absorb colors, which means that more specific probes can be used in one reaction. Using real-time PCR with virus-specific-primer and probe combinations in multiplex assays, the detection of a maximum of five different viruses in one test has become feasible. Additionally, real-time assays are semi-quantitative, i.e., a lower crossing threshold (Ct) value for the same virus indicates that the amount of viral RNA present in the original samples was higher.

While multiplexing PCR assays have advantages, the accumulation of undesired products and the overlapping fluorescent spectra can decrease the sensitivity and elongate the optimization time.

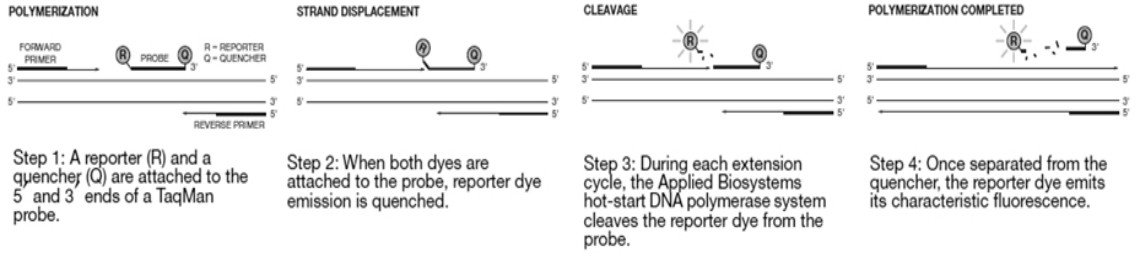

FIGURE 3. Illustration of TaqMan probe based chemistry (Picture adapted from Real-Time PCR Systems, Applied Biosystems).

DETECTION OF GASTROENTERITIS VIRUSES

Detection of gastroenteritis viruses has historically been based on direct visualization of virus particles using electron microscopy (Table 2). This is a technique, that is still useful for detection of the viruses, but now is limited to few reference laboratories. Cell culture techniques are not useful for routine diagnostic purposes, since the propagation of human caliciviruses is not yet possible, and this technique is not often used for other viruses, as the most common viruses causing gastroenteritis are shed to high titers in stool of infected persons. This allows much faster direct antigen detection, methods that are standard for rotaviruses, astroviruses and adenoviruses detection. For noroviruses, first generation antigen detection assays have been developed and although their specificity and sensitivity is lower than that of the molecular assays these assays are popular because they are easy to use.
The recent introduction of molecular techniques improved the diagnosis of gastroenteritis viruses and they are increasingly used in routine diagnostic laboratories. These techniques are more sensitive than any other techniques previously used for detection of viral pathogens causing gastroenteritis. Molecular techniques, followed by sequencing, are also used for typing of the viruses detected, e.g. genotyping of noroviruses, rotaviruses, sapoviruses, which enables linking of outbreaks or finding a common source of infection. Detection techniques used for detection of common gastroenteritis viruses will be explained in detail later in the text.

TABLE 2. Properties of test that are used to measure the presence of virus or viral infection (adapted from Foodborne viruses: an emerging problem by Erwin Duizer and Marion Koopmans, International Journal of Food Microbiology, Volume 90, Issue 1, 1 January 2004, Pages 23-41).

Principle of assay	Example	Infectivity test	Approximate detection limit (particles per gram)
Visualization of particles	EM	NO	10^{5-6}
Detection of viral protein	ELISA, latex test	NO	10^{5}
Detection of genome	Probe identification	NO	10^{4}
Detection of genome	RT-PCR	NO	10^{1-3}
Screen for effect on living cells	Cell culture isolation (where possible)	YES	10^{0-1}
Measurement of exposure	Antibody assays	YES	Window of detection varies by type of antibody. IgM indicates recent infection.

Diagnosis of Human caliciviruses: noroviruses and sapoviruses.
Prior to development of molecular methods, little was known about the caliciviruses due to their difficult identification by EM. Also, attempts to culture human caliciviruses during the last decades failed (50, 54). The development of reverse transcriptase polymerase chain reactions (RT-PCR) has greatly enhanced the identification of these viruses. Although caliciviruses have a great genetic diversity, which complicated their detection by use of molecular techniques, these techniques did help to clarify the role of noroviruses in gastroenteritis outbreaks. There are numerous norovirus detection assays, however none of these assays has been able to detect all strains known. However, there are assays that are more sensitive and have a broader detection range, and are therefore able to detect more different strains (162).
Serological and immunological detection of human caliciviruses has been hampered by lack of a culture system. Recently, EIA assays for routine detection of noroviruses, which use recombinant norovirus capsid proteins, have become commercially available. These noninfectious proteins are immunogenic, and therefore suitable for antiserum production (54). However, EIA assays are not able to detect all norovirus strains, have limited sensitivity and specificity, and are therefore not the most suitable detection method for noroviruses. They can be used for detection of noroviruses in a gastroenteritis outbreak where specimens are plentiful, but risk for false positives exists (47, 48).
Advantages of EIA testing over PCR based assays include simplicity, and speed, as a rapid EIA assays can give a result within 15 minutes. EIAs can be problematic in detection of variants and emerging genotypes, and if outbreak samples are negative when EIA assay is used, screening by PCR is recommended.
Sapoviruses, as noroviruses, have not been cultivated *in vitro* yet, and have not been as intensively studied as noroviruses. For detection and identification molecular techniques are mostly used (64, 160, 162).

Diagnosis of rotaviruses, adenoviruses and astroviruses.
For diagnosis of rotavirus infections, antigen detection in fecal specimens is the most widely used method. These enzyme immunoassays have proven to be more sensitive and highly specific when compared to visualization by electron microscopy. There are several rapid assays for rotavirus infection diagnosis including latex agglutination (LAT), enzyme immunoassay (EIA) and immunochromatographic (ICG) methods. Although the LAT assays are rapid and easy to perform, they show low sensitivity. EIA and ICG assays have a good specificity and sensitivity and are often used for diagnosis (39, 94).
At present, for identification and genotyping of rotaviruses PCR techniques are used as well. This method is more sensitive and less time consuming than the other previously used methods, microscopy and EIA assays. Reverse transcriptase PCR (RT-PCR) has been shown to increase the rate of detection of rotavirus in clinical specimens from patients with AGE 10 to 20% over that by EIA (154, 158).

Diagnosis of adenovirus infection is usually done by EIAs. For adenoviruses, EIA assays detect common group antigens or specific adenovirus type 40 and 41.These assays are sufficiently sensitive for the diagnosis of adenovirus-associated diarrhea. Recently more sensitive and faster PCR techniques are used to identify specific enteric adenovirus types 40 and 41 (2, 3, 16, 90, 114, 125, 155).

Virus detection of astroviruses in stool samples historically was mainly done by use of EM. However, the diagnosis of astroviruses has been difficult, since only about 10% of particles have the typical “astrovirus” star. EIA techniques are also available for detection of astroviruses, based on monoclonal antibodies against this virus. The recently marketed EIA have a good sensitivity and specificity when compared to EM and PCR. However lately, also for this virus more sensitive and faster PCR techniques have become routine for identification (16, 108, 110, 124, 126).

History of detection of viral gastroenteritis in the Netherlands.
In the Netherlands, viral gastroenteritis surveillance was started in 1994 when the routinely used technique for detection of viruses in stool samples was EM, since then new methods have successfully been implemented (Figure 4).

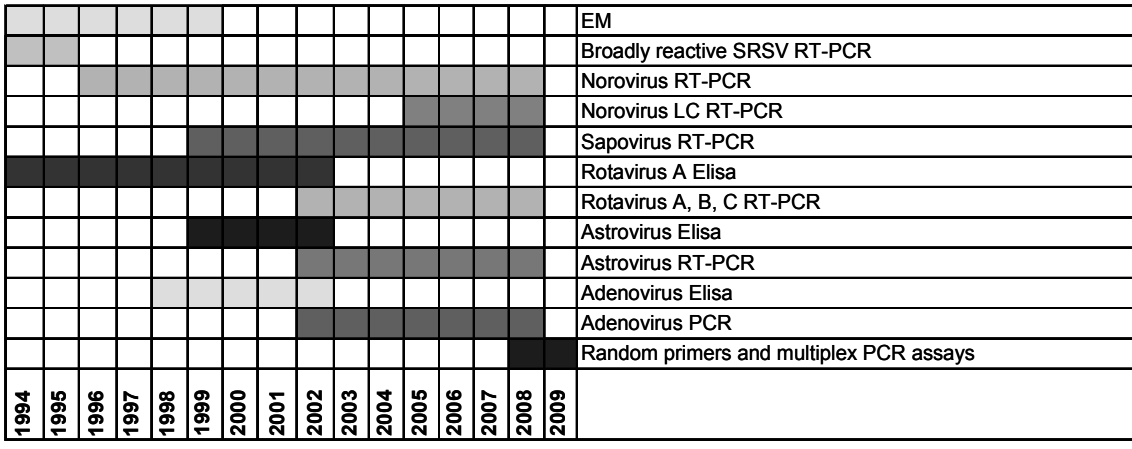

FIGURE 4. Methods used for detection of viruses in stool samples from viral gastroenteritis outbreaks.

OUTLINE OF THESIS: aim, objectives and approach

The main objective of this thesis was to investigate the etiology of unexplained viral gastroenteritis outbreaks, gathered through viral gastroenteritis surveillance, as following specific objectives within:

1) To determine the role of viruses in outbreaks of acute gastroenteritis in the Netherlands.
2) To develop (generic) detection methods for viruses causing gastroenteritis.
3) To use these new techniques to screen samples from patients with unexplained etiology of gastroenteritis.
4) To study their role as causes of outbreaks of gastroenteritis.
5) To provide reference testing and scientific guidance for laboratories that perform routine diagnostic testing for viral gastroenteritis.

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Chapter 2

Etiological role of viruses in outbreaks of acute gastroenteritis in the Netherlands from 1994 through 2005

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ABSTRACT

Acute gastroenteritis is one of the most common diseases worldwide. In developed countries, viruses, particularly noroviruses, are recognized as the leading cause. In The Netherlands, the surveillance of gastroenteritis outbreaks with suspected viral etiologies (as determined by Kaplan criteria) was established by the National Institute for Public Health and the Environment in 1994. This paper presents an overview of viral gastroenteritis outbreaks reported from 1994 through 2005. A minimum epidemiological data set consisting of the associated setting(s), the probable transmission mode, the date of the first illness and the date of sampling, the number of persons affected, and the number of hospitalizations was requested for each reported outbreak. Stool samples were tested for the presence of norovirus, sapovirus, rotavirus, astrovirus, adenovirus, and Aichi virus by electron microscopy, enzyme-linked immunosorbent assay, and/or reverse transcription-PCR. A total of 6,707 stool samples from 941 gastroenteritis outbreaks were investigated. Noroviruses were detected as the causative agent in 735 (78.1%) of the outbreaks, and rotaviruses, adenoviruses, and astroviruses were found to be responsible for 46 (4.9%), 9 (1.0%), and 5 (0.5%) outbreaks, respectively. Among the gastroenteritis outbreaks in which a mode of transmission was identified, most outbreaks (38.1%) were associated with person-to-person transmission, and the majority (54.9%) of the outbreaks investigated were reported by residential institutions. Since 2002, the total number of outbreaks reported and the number of unexplained outbreaks have increased. Furthermore, the number of rotavirus associated outbreaks has increased, especially in nursing homes. Despite thorough testing, 115 (12.2%) outbreaks suspected of having viral etiologies remain unexplained. Increases in numbers of reported outbreaks may indicate undefined changes in the criteria for reporting or the emergence of new pathogens.

INTRODUCTION

Diarrheal diseases are a leading cause of morbidity and mortality worldwide (6) and occur most often in young children in nonindustrialized countries (4, 27, 30). A large proportion of these infections are viral in nature. In industrialized countries, viral gastroenteritis (GE) is one of the most common diseases in all age groups (15, 26). Symptoms may include vomiting and diarrhea, often with secondary infections occurring in contacts of patients. Based on these properties, outbreaks have been defined as probable viral outbreaks when vomiting and/or diarrhea occurs in more than 50% of affected persons, the incubation period is 24 to 48 h, the duration of illness is 12 to 60 h, and routine bacterial cultures of stool samples test negative (17). Members of the genus *Norovirus* in the family *Caliciviridae* have been identified as the most common viral cause of acute GE in humans (2, 11, 15, 36). Noroviruses (NoV) affect people of all ages; are a major cause of outbreaks as well as sporadic cases; are transmitted through many routes, such as fecally contaminated food and water, the environment, and person-to-person contact; and are difficult to control (15). NoV-related outbreaks occur in association with a wide range of settings, including residential institutions, hospitals, restaurants, schools, and others (2, 20, 21). Members of the genus *Sapovirus*, also in the *Caliciviridae* family, have been found in humans and are mainly associated with pediatric acute GE (9, 10, 11).

Other viruses that are clearly associated with diarrhea and vomiting are group A rotaviruses (RV), astroviruses (AsV), and enteric adenoviruses (AdV) (1, 7, 14). These viruses affect mostly children in the first few years of life and have been described as common causes of GE in countries with great differences in the levels of hygiene and the qualities of water, food, or sanitation (15). Their etiologic importance in outbreaks of acute GE is less well described. Group A RV is the major cause of severe GE in infants and young children worldwide and is occasionally reported as the cause of outbreaks and hospitalization (7, 29, 35, 46). In addition to the major pathogens RV and NoV, other newly described enteric viruses have been added to the list of possible causes of outbreaks of GE in the past decade. AdV, AsV, and sapoviruses (SaV) are also associated with sporadic cases and occasional outbreaks of GE associated with such settings as day care centers, schools, and nursing homes for the elderly (25, 37).

In 1998, Aichi virus (AV) was described as a cause of oyster-associated GE in Japan (44). More recently, AV was detected in GE outbreaks in Germany, South America, and France (28; K. Balay et al., personal communication). The routes of transmission of these viruses are classified as person-to-person spread or food-borne, waterborne, or environmental transmission (19).

The systematic surveillance of outbreaks of GE with possible viral etiologies was started in 1994 at the National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu [RIVM]) in The Netherlands. Since then, the diagnostic routine has expanded as novel detection methods have become available, resulting in the broad panel of assays used to date. This paper provides an overview of the etiological contributions of known viral pathogens to the GE outbreaks reported to the RIVM in The Netherlands from 1994 through 2005, by which time new and improved detection methods were in use.

MATERIALS AND METHODS

Outbreak surveillance.

In 1994, The Netherlands initiated a surveillance system for outbreaks of GE with suspected viral etiologies (41). For this surveillance, regional health services (Geneeskundige en Gezondheidsdienst) were requested to collect fecal samples from people involved in outbreaks of illness reported to these organizations. The case definition used was based on the criteria of Kaplan et al. (17). Where possible, a minimum epidemiological data set consisting of the associated setting, the probable transmission mode, the date of the first illness and the date of sampling, the number of persons affected, and the number of hospitalizations was requested (38). Samples were sent to the RIVM by regular overnight mail for routine testing and were stored at 4°C.

Routine for detection.

The routine for detection evolved over time. Initially, routine evaluation was done by electron microscopy, which was replaced by molecular detection testing (reverse transcription-PCR [RT-PCR]) for the presence

of NoV in 1996 (41). Since the majority of outbreaks proved to be caused by NoV, since 1996 initial routine testing has been done for these viruses only. Typically, when all specimens from an outbreak tested negative for NoV, the samples were assayed for the presence of SaV, AsV, AdV, and RV. In total, specimens from 373 outbreaks were tested for all viruses; these were the outbreaks with few NoV-positive samples, day care center outbreaks, and outbreaks in which this testing was specifically requested.

Specific detection methods.
The presence of NoV was detected by RT-PCR. NoV detection methods were adjusted over time based on an increasing understanding of the level of diversity of NoV and the consequences of NoV diversity for detection (8, 39, 41, 42). In 2004, LightCycler (LC) RT-PCRs for NoV genogroups I and II (GGI and GGII) were developed. Since 1999, SaV have been detected with an assay developed in 1999 (40). For the detection of RV group A, AdV, and AsV, enzyme-linked immunosorbent assays were used up until 2002. In 2002, PCR-based methods for the detection of each pathogen were implemented. The RV PCR method detects RV of groups A to C (E. de Bruin et al., unpublished data). The primers for RV PCR are targeted to the RNA-dependent RNA polymerase of rotaviruses. The primers were derived from multiple sequence alignments of all available (partial) VP1 sequences from rotaviruses of groups A, B, and C. They were designed to be able to detect rotaviruses from all three groups. Since rotaviruses have double-stranded RNA, both primers were used in the RT step to increase the chance for the detection of RV. For the molecular detection of AdV (32) and AsV (31), previously described primers have been used with adjustments in the amplification protocols (see below). Additionally, specimens from all unexplained outbreaks have been tested for AV (44, 45).

Retrospective analysis of unexplained outbreaks by using new and improved detection methods.
Since the methods for the detection of enteric viruses have improved over the period of surveillance, it was decided that specimens from previous outbreaks that had tested negative should be retested by present standards (see below). NoV genogroup I and II assays were performed on specimens from all unexplained outbreaks occurring before 2005. The outbreak specimens that tested negative before 2003 were retested using the improved PCR-based assays for RV groups A to C, AdV, and AsV.

RNA extraction.
Stool homogenates (10%, wt/vol) in minimal essential medium with Hanks salts and 0.1 mg/ml gentamicin were mixed by vortexing and clarified by centrifugation (1,300 × *g*) for 20 min at 3,000 rpm. The supernatant was used for RNA extraction. For the testing of specimens from unexplained outbreaks, two RNA isolation methods were used. The first method was described by Boom et al. (3) and was used until 2002. The second was the MagNA Pure LC total nucleic acid isolation kit using the total nucleic acid external lysis protocol according to the recommendations of the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). This protocol was introduced into the routine following side-by-side comparison of the methods used in 21 outbreaks corresponding to 150 samples since 2002. The MagNA Pure extraction method was also used to reanalyze the negative outbreak specimens with the improved methods of detection of NoV, SaV, RV, AdV, and AsV described here.

RT-PCR protocols for NoV, RV, AsV, and AdV detection.
Reverse transcription of 2.5 μl of the extracted RNA was done for 60 min at 42°C after annealing with RT sense primers (Table 1). For the detection of RV groups A, B, and C, two primers, one for each strand, were used in the RT step. RT primers were used at 50 pmol in 15 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates (dNTPs), and 5 U of avian myeloblastosis virus reverse transcriptase (Promega, Leiden, The Netherlands).

TABLE 1. Sequences, polarities, targets, and amplicon sizes corresponding to the primers and probes used for routine surveillance of viral GE outbreaks.

Virus or probe	Target	Primer	Polarity	Sequence (5′ to 3′)f	Nucleotide position	Amplicon size (bp)
Norovirus GGI	Capsid	NV1LCF	+	CARGCCATGTTYCGYTGGATG	5279-5299a	98
Norovirus GGI	Capsid	NV1LCR	−	CCTTAGACGCCATCATCATTTAC	5354-5376a	
Norovirus GGI probe	Capsid	NV1LCpr		VIC-TGGACAGGAGAYCGCRATCT-TAMRA	5321-5340a	
Norovirus GGII	Capsid	NV2LCF	+	GARYCIATGTTYAGRTGGATG	5000-5020b	95
Norovirus GGII	Capsid	NV2LCR	−	TCGACGCCATCTTCATTCAC	5075-5094b	
Norovirus GGII probe	Capsid	NV2LCpr		FAM-TGGGAGGGSGATCGCRATCT-TAMRA	5042-5061b	
Rotavirus	VP1, core protein	ABC F	+	TAYACIGAYGTITCICARTGGGA	1578-1600c	385
Rotavirus	VP1, core protein	ABC R	−	GTAGTTGTCGTCICCRTCIAC	1896-1916c	
Astrovirus	Capsid	AC′1	+	ATGGCTAGCAAGTCTGACAAG	4326-4346d	231
Astrovirus	Capsid	AC230	−	GGTTTTGGTCCTGTGACACC	4542-4561d	
Adenovirus	Hexon protein	HEXAA1885	+	GCCGCAGTGGTCTTACATGCACAT	17663-17686e	308
Adenovirus	Hexon protein	HEXAA1913	−	CAGCACGCCGCGGATGTCAAAGT	17941-17963e	

[a]Norwalk virus complete coding sequence; accession number M87661.
[b]Norwalk-like virus strain GIFU′99 complete genome; accession number AB084071.
[c]Human rotavirus C VP1 gene for structural protein VP1 from genomic RNA; accession number AJ304859.
[d]Human astrovirus type 8 complete genome; accession number AF260508.
[e]Human adenovirus F complete genome; accession number L19443.
[f]TAMRA, 6-carboxytetramethylrhodamine; FAM, 6-carboxyfluorescein.

For NoV genogroups I and II, 2 µl of the reverse transcription mix was added to 18 µl of an LC PCR mix containing 2 µl of DNA master hybridization probes (Roche Diagnostics GmbH, Mannheim, Germany), 4 pmol of hybridization probe, 6 pmol of primer, 4.3 mM $MgCl_2$, 0.16 µl of TaqStart antibody (end concentration, 8.8×10^{-3} µg/µl; Clontech Laboratories, Inc.), and 11.84 µl of H_2O. Samples were denatured for 1 min at 95°C; subjected to 45 (GGI) or 50 (GGII) amplification cycles of 95°C for 0.1 s, 50°C (GGI) or 43°C (GGII) for 5 s, and 72°C for 10 s with fluorescence acquisition in a single mode; and cooled at 40°C for 30 s. Detection was performed at 560 and 530 nm for NoV GGI and GGII amplification products, respectively. PCRs were performed with the LightCycler apparatus (Roche Diagnostics GmbH, Mannheim, Germany).

For RV and AsV detection, 5 µl of the reverse transcription mix was added to 45 µl of a PCR mix containing 10 mM Tris-HCl (pH 9.2), 50 mM KCl, 0.2 mM dNTPs, 2.5 U of AmpliTaq, and 1.5 mM $MgCl_2$ (RV mix) or 2 mM $MgCl_2$ and 0.3 pmol of AsV PCR primer (AsV mix) (31) (Table 1). Samples were denatured for 3 min at 94°C and subjected to 40 cycles of 94°C for 60 s and 45°C for 90 s for RV and 40 cycles of 55°C for 90 s and 72°C for 60 s for AsV.

For the detection of AdV, 5 µl of the extracted DNA was added to 45 µl of a PCR mix containing 10 mM Tris-HCl (pH 9.2), 0.2 mM dNTPs, 3.5 mM $MgCl_2$, 5 U of avian myeloblastosis virus, 10 pmol of both AdV primers (32) (Table 1), and 35.6 µl of H_2O. Samples were denatured for 3 min at 94°C and subjected to 40 cycles of 94°C for 60 s, 45°C for 90 s, and 72°C for 60 s. AdV and AsV were detected by PCR and amplicon size analysis after gel electrophoresis, followed by confirmation by sequencing if a positive signal was detected. RV was confirmed and typed by using reverse line blotting or sequencing.

Assays were validated by using known positive samples and samples from outbreaks caused by bacterial pathogens or other enteric viruses as positive and negative controls, respectively. In total, 8 AsV serotypes (serotypes 1 through 8), 9 RV group A strains (69 M, B223, DS1, EDIM, G9P6, K8, NCDV, SA11, ST3, and WA), and 39 AdV strains (representative of human adenovirus groups A to F) from virus cultures were used in the evaluation. For RT-PCR validation of NoV detection, we used previously characterized genogroup I (26) and genogroup II (14) strains kept in aliquots as clarified 10% fecal suspensions at −80°C.

Sequencing.

Samples that were positive for viral RNA were confirmed by sequencing using a fluorescence-labeled dideoxynucleotide technology from Applied Biosystems (Foster City, CA). Sequence reactions were analyzed on an ABI 3700 automated sequencer. The sequences obtained were assembled using Seqman and Editseq software (DNAStar, Konstanz, Germany).

RESULTS

Outbreaks.

Stool samples (6,707) from 941 GE-associated outbreaks out of the total of 1,025 outbreaks reported to the RIVM during the study period were investigated. Through routine surveillance, specimens from 719 (76.4%) of the outbreaks had tested positive for NoV (Table 2). Based on a review of the descriptive epidemiology, 18 of the 941 outbreaks were excluded from the analysis because they were likely to be caused by other pathogens (influenza virus [two outbreaks]; *Salmonella*, *Campylobacter*, and *Shigella* species [eight, five, and two outbreaks, respectively]; and *Clostridium perfringens* [one outbreak]). After the first round of testing, samples from 41 (4.4%), 7 (0.7%), and 5 (0.5%) of the outbreaks were found to be positive for RV, AdV type 40/41, and AsV, respectively.

Reanalysis of the remaining 140 unexplained outbreaks with the new and improved methods described in Materials and Methods clarified an additional 25 outbreaks (Table 2). Of these 25 outbreaks, 16 were found to be caused by NoV, 5 by RV, 2 by AdV type 40/41, and 2 by two or more viruses. SaV and AV were not detected.

Transmission mode.
The transmission mode was reported for 359 (38.1%) of the outbreaks (Table 3). Person-to-person spread was the most commonly identified mode of transmission, followed by food-borne transmission. Eighteen (39.1%) RV-related outbreaks were reported to be transmitted by personal contact. In addition, outbreaks caused by AsV, AdV, and mixed pathogens were also reported to be spread by personal contact (Table 3).

Settings.
The settings associated with 95.2% of the outbreaks were reported. Table 4 shows the settings corresponding to the pathogens causing GE. Residential institutions (55.7%), hospitals (17.8%), restaurants (8.8%), and day care centers (6.0%) were the settings associated with the most outbreaks. Unexplained as well as NoV-caused outbreaks were linked to all of the above-mentioned settings. RV group A was initially found mostly in GE outbreak samples from children, but in the last few years it was increasingly detected in outbreak samples from other patients, including elderly people in residential institutions. Overall, more than 58% of all RV-caused outbreaks occurred in residential institutions. Most outbreaks in which multiple pathogens were involved (61.5%) were associated with day care centres.

Seasonality.
Most NoV-related outbreaks (80.3%) occurred from October through March, with a peak in December (172 outbreaks). The RV-associated outbreaks also took place predominantly in the winter months but with a slightly different seasonality than those caused by NoV. The RV GE season started in December and continued through April (12 outbreaks), when most RV GE outbreaks occurred. Only a few outbreaks were caused by AdV and AsV, and both of these viruses caused the most outbreaks in March, 55.6% (five of nine outbreaks) and 60% (three of five), respectively.

Unexplained outbreaks.
The number of unexplained outbreaks increased after 1994, concomitant with the total number of reported GE outbreaks (Table 5). Unexplained outbreaks for which months of occurrence were reported took place throughout the year, but 59.1% occurred during the winter season (October to March). Most unexplained outbreaks were found in residential institutions (Table 4). A high number were also linked to hospitals, restaurants, cafés, pubs, bars, and day care centers. The unexplained outbreaks were reported throughout the year without evident peaks; 75 of 115 unexplained outbreaks involved unknown modes of transmission.

TABLE 2. Pathogens as causes of suspected viral gastroenteritis[a]

Pathogen(s)	No. (%) of outbreaks attributed to the indicated pathogen before additional testing	No. (%) of outbreaks attributed to the indicated pathogen after additional testing	No. of people affected in outbreaks	No. of people tested in outbreaks (% of people tested among people infected)	% Of non-NoV GE outbreaks caused by the indicated pathogen (n = 206)
Norovirus	719 (76.4)	735 (719 + 16)b (78.1)	14,063	5,481 (39.0)	NA
Rotavirus	41 (4.4)	46 (41 + 5)b (4.9)	873	270 (30.9)	22.3
Adenovirus	7 (0.7)	9 (7 + 2)b (1.0)	157	44 (28.0)	4.4
Astrovirus	5 (0.5)	5 (0.5)	31	26 (83.9)	2.4
Sapovirus	0 (0)	0 (0)			0
Aichi virus	0 (0)	0 (0)			0
Mix	11 (1.2)	13 (11 + 2)b (1.4)	192	81 (42.2)	6.3
Other pathogens	18	18	403	119 (29.5)	8.7
Unknown	140 (14.9)	115 (12.2)	1,400	686 (49.0)	55.8
Total	941 (100)	941 (100)	17,119	6,707 (39.2)	100

[a]Numbers of outbreaks attributed to the indicated pathogens before and after retesting using state-of-the-art methods are shown, along with percentages of the total number of GE outbreaks investigated. Numbers and percentages of people tested out of people infected are according to data reported to the National Institute for Public Health and the Environment. NA, not applicable.
[b]Among the outbreaks initially attributed to unknown pathogens, 16 were later found to be caused by noroviruses, 5 were found to be caused by rotaviruses, 2 were attributed to adenoviruses, and 2 were determined to be caused by a mixture of pathogens.

TABLE 3. Modes of transmission associated with outbreaks of GE

Mode(s) of transmission	No. (%) of outbreaks caused by:						Total no. (%) of outbreaks
	Norovirus	Rotavirus	Adenovirus	Astrovirus	Mixed pathogens	Unknown pathogen(s)	
Unknowna	447 (60.8)	27 (58.7)	8 (88.9)	4 (80.0)	8 (61.5)	75 (65.2)	569 (61.6)
Person to person	236 (32.1)	18 (39.1)	1 (11.1)	1 (20.0)	5 (38.5)	20 (17.4)	281 (30.4)
Food borne	48 (6.6)	1 (2.2)				17 (14.8)	66 (7.2)
Waterborne	3 (0.4)					2 (1.7)	5 (0.5)
Food borne, person to person	1 (0.1)					1 (0.9)	2 (0.2)
Total	735 (100)	46 (100)	9 (100)	5 (100)	13 (100)	115 (100)	923 (100)

[a]If the mode of transmission was reported as “Unknown” or not reported, it was classified as unknown.

TABLE 4. Reported settings associated with outbreaks of GE

Setting	No. (%) of outbreaks caused by						Total no. (%) of outbreaks
	Norovirus	Rotavirus	Adenovirus	Astrovirus	Mixed pathogens	Unknown pathogen(s)	
Residential institution	441 (60.0)	27 (58.7)	4 (44.4)	2 (40.0)	3 (23.1)	37 (32.2)	514 (55.7)
Hospital	133 (18.1)	6 (13.1)	2 (22.2)	1 (20.0)	1 (7.7)	21 (18.3)	164 (17.8)
Restaurant, café, pub, or bar	60 (8.2)				1 (7.7)	20 (17.4)	81 (8.8)
Day care center	24 (3.3)	10 (21.7)	3 (33.3)		8 (61.5)	10 (8.7)	55 (6.0)
School	12 (1.6)	1 (2.17)		1 (20.0)		5 (4.3)	19 (2.1)
Hotel or guest house	6 (0.8)						6 (0.6)
Aircraft, ship, train, or bus	6 (0.8)					1 (0.9)	7 (0.7)
Private house	3 (0.4)					3 (2.6)	6 (0.6)
Shop or retailer	1 (0.1)						1 (0.1)
Swimming pool	1 (0.1)					2 (1.7)	3 (0.3)
Institution	3 (0.4)						3 (0.3)
Other	16 (2.2)					4 (3.5)	20 (2.2)
Unknown	29 (3.9)	2 (4.3)		1 (20.0)		12 (10.4)	44 (4.8)
Total	735 (100)	46 (100)	9 (100)	5 (100)	13 (100)	115 (100)	923 (100)

TABLE 5. Pathogens found as causes of suspected viral GE outbreaks from 1994 to 2006 by using routine detection methods[a]

Pathogen(s)	No. of suspected viral GE outbreaks caused by the indicated pathogen(s) in:												Total no. of outbreaks caused by the indicated pathogen from 1994 to 2006
	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	
Adenovirus			1	1					6		1		9
Astrovirus				1		1		1	1	1			5
Rotavirus	1		1	1		3	4	5	5	4	8	14	46
Mix			3	1		2		1	1	1	3	1	13
Unknown		2	2	6	6	4	2	12	24	23	10	24	115
Other pathogens		1			1				10		4	2	18
Norovirus	9	30	59	20	13	31	41	73	195	46	133	85	735
Subtotal of non-NoV viruses	1	0	5	4	0	6	4	7	13	6	12	15	73
Total	10	33	66	30	20	41	47	92	242	75	159	126	941

[a]Outbreaks were reported to the National Institute of Health and the Environment, The Netherlands. Other pathogens identified as causes include influenza virus; Salmonella, Campylobacter, and Shigella species; and Clostridium perfringens.

DISCUSSION

To our knowledge, this is the first description of a long-term outbreak surveillance system in which a broad detection panel has been used to test for presently known enteric viruses. This testing included retrospective testing of samples from unexplained outbreaks by using new and improved methods.

Since the start of the GE surveillance in 1994, the number of reported outbreaks has increased, mainly due to rising numbers of outbreaks caused by NoV and RV and unexplained outbreaks. This increase may be due to the emergence of more-virulent strains, as has been suggested in the case of NoV (24), or to the introduction of strains unfamiliar to people in The Netherlands. The highest proportions of NoV GGII.4 strains were reported in years with high numbers of outbreaks (33). In the epidemic seasons of 1995 to 1996, 2002 to 2003, and 2004 to 2005, GGII.4 viruses caused 82, 83, and 89%, respectively, of all NoV GE outbreaks (33). The numbers of reported RV-associated outbreaks, especially the number of those occurring in residential institutions, have increased, which may also indicate that in The Netherlands RV infection is emerging among the elderly although it is usually found in association with illness in children (7, 29, 35, 46). The etiological importance of RV as a cause of illness in adults and the elderly may have been missed due to referral biases in diagnostic requests, as RV infection in adults has been described previously (10, 11, 13, 16). Whether or not there has been a true increase in RV-associated outbreaks among the elderly needs to be investigated in more detail.

Another explanation for this increase may be that health care workers have become more aware of problems caused by NoV and the availability of (free) diagnostics at the RIVM for use in viral GE outbreaks.

NoV was found to be the causative agent in 78.1% of the investigated GE outbreaks reported to the RIVM in the period from 1994 to the end of 2005. This prevalence was also found in other studies and confirms that NoV is the leading cause of GE in outbreaks in The Netherlands as well as in other countries (18, 24). By using new methods, even more NoV-associated outbreaks were identified. Remarkably, a considerable number of newly detected NoV could not be typed by using the standard sequencing detection procedure. For sequencing, a block PCR assay which is less sensitive than the LC PCR assay was used, and this may be a reason for not being able to type some additionally found NoV. Another explanation may be the detection of other NoV variants that are not detected by the sequencing primers.

After testing for other viral pathogens causing GE, such as AdV, AsV, SaV, RV, and AV, the etiologies of an additional 73 outbreaks were resolved.

AV and SaV were not found as causative agents of GE outbreaks in The Netherlands. So far, one outbreak caused by AV in Germany has been reported (28) and three AV-related outbreaks and four single cases of AV GE (identified among 457 stool specimens) in France have been reported (K. Balay et al., personal communication). Since we have not found any AV-associated outbreaks, it is possible that there have been no outbreaks caused by AV in The Netherlands. AV is seldom found and occurs usually in mixed infections, suggesting that its etiological role remains to be established (K. Balay et al., personal communication). In our study, most outbreak samples were not tested further for other pathogens if NoV was detected, so mixed infections with NoV and AV were possibly missed. Besides, only 64 (6.8%) of the GE outbreaks studied occurred in association with day care centres, indicating that GE outbreaks among children are likely to be underreported. SaV-associated outbreaks in The Netherlands were also not detected, although the virus was found in sporadic cases of GE in children (9, 10, 11). Again, underrepresentation of GE cases in children and young adults in this study may influence the overall number of SaV-related outbreaks recognized in The Netherlands. Day care centres and schools should be approached for active reporting of the outbreaks to obtain a broader picture of viral GE outbreaks occurring in these age groups. It is also possible that the person-to-person transmission of SaV does not occur as easily as that of NoV, thereby truly causing fewer outbreaks.

One of the criteria defining viral GE outbreaks is that bacterial pathogens must be excluded as causative agents. Nevertheless, bacterial pathogens that are commonly recognized as causes of GE (*Salmonella*, *Shigella*, and *Campylobacter* species and *Clostridium perfringens*) were found to be the causative agents in 1.6% of all reported outbreaks. Since this error was observed mostly in recent years, it probably reflects a changing practice: since viruses have been recognized as the number one cause of GE outbreaks for several years now, specimens are no longer always screened for bacterial pathogens. On the other hand, it is possible that the

Kaplan criteria are not followed correctly, resulting in the submission of samples from nonviral outbreaks. Residential institutions and hospitals reported the most outbreaks over the years. The reporting of outbreaks in these settings is obligatory, unlike that of outbreaks in other settings like private houses, day care centres, and schools. Consequently, outbreaks in settings other than residential institutions are likely to be underreported.

In this study, samples from all unexplained outbreaks were retested with new and improved methods. This retesting identified the causative agents of an additional 25 outbreaks, meaning that the improved methods are more sensitive and have a wider scope than those used before. Nevertheless, a small proportion of viral GE outbreaks, 115 (12.2%), remained unexplained. These outbreaks may have been caused by other viruses associated with GE, such as toroviruses (12, 15, 22, 23, 43), picobirnaviruses (15, 43), and enteroviruses (5, 15), for which the outbreak samples have not yet been tested or by other emerging pathogens.

In summary, this paper gives a comprehensive overview of viral GE in The Netherlands from 1994 till the end of 2005, with NoV identified as the leading cause. Outbreaks that happened in settings other than residential institutions or hospitals are likely to be underreported. To determine the importance of viruses as causes of GE outbreaks among children, targeted surveillance is needed for day care centers, schools, and other institutions. Using new and improved detection assays, we have resolved the causes behind 17.9% of the previously unexplained outbreaks. The remaining outbreaks from which all samples tested negative will be studied further by using new molecular methods and classical virology, such as electron microscopy (34) and culture.

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Chapter 3

Novel approach for detection of enteric viruses to enable syndrome surveillance of acute viral gastroenterotis

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ABSTRACT

Acute gastroenteritis is one of the most common diseases worldwide, with viruses, particularly noroviruses, being the leading cause in developed countries. In The Netherlands, systematic surveillance of gastroenteritis outbreaks of suspected viral etiology was established by the National Institute for Public Health and the Environment in 1994. Since 2002, the total number of outbreaks reported has been increasing, and with that comes the need for sensitive assays that can be performed quickly. In addition, the diagnostic demand changed so that now the proportion of samples from hospitals is higher and there is a need for patient-based test results. In order to target the diagnosis of acute gastroenteritis, we reviewed our data on outbreaks of gastroenteritis and the prevalence of individual viruses to provide a priority list of viruses for which samples should be evaluated. Random primers were used to replace the separate specific primers for each virus used in the reverse transcription steps. The individual PCR assays were replaced by multiplex PCR assays. We employed a two-step method in which in the first step we screened for the most common causes of viral gastroenteritis, noroviruses of genogroup II and rotaviruses of group A, with equine arteritis virus used as the internal control. Subsequently, in the second step, two parallel PCR assays were developed for the detection of noroviruses of genogroup I and equine arteritis virus in one run and adenoviruses, sapoviruses, and astroviruses in the other run. The specificities of the assays were calculated to be 92.5% for the assay for noroviruses of genogroup I and 100% for the assays for all other viruses, the detection limits were equal for all viruses, and the turnaround time was reduced to 1 day compared to the at least 3 days required for the methods used previously. This approach allows the targeted, rapid, and cost-effective elucidation of the causes of acute gastroenteritis outbreaks.

INTRODUCTION

Viral gastroenteritis is one of the most common diseases in humans in all age groups worldwide (11, 13), with noroviruses (NoV) and rotaviruses (RVs) of group A being the leading cause (4, 8, 20). Other viruses that are associated with viral gastroenteritis are sapoviruses (SaVs), group B and C RVs, astroviruses (AsVs), enteric adenoviruses (AdVs), and Aichi virus (AV) (1, 6-8, 15, 25, 26).
In recent decades, the methods of virus detection have shifted away from conventional labor-intensive techniques, like cell culture and electron microscopy, and less sensitive techniques, like immunodiagnostic assays (5, 22, 23), toward real-time reverse transcription (RT)-PCR assays with specific primers. Current procedures for the diagnosis of acute gastroenteritis in individual patients are based on viral RNA or DNA detection by conventional PCR assays and specific real-time RT-PCR assays. To optimize the use of sometimes limited quantities of patient materials, random primers can be used in the RT step required for the detection of RNA viruses (9, 19, 21). The cDNA formed in such a reaction can be used in PCR assays specific for different viruses (9, 19, 21). The detection of several different viruses can be accelerated and simplified by use of multiplex real-time PCR assays with multiple primer pairs in a single tube, making it possible to amplify multiple target sequences (9, 15, 18, 19, 21). The use of random primers and multiplex PCR assays in combination reduces reagent and labor costs, as well as the turnaround time. Furthermore, the use of an internal control facilitates the detection of inhibition of the RNA extraction and/or PCR and thus permits the diagnosis of sporadic cases of acute gastroenteritis. In this study, we present a scheme for the detection of enteric viruses to enable syndrome surveillance of acute viral gastroenteritis.

MATERIALS AND METHODS

Rationale of method development.

Since we started our routine evaluation of reported outbreaks of gastroenteritis, the diagnostic demand has shifted from mainly requests from nursing homes to increasing requests from hospitals. With that, the etiological fraction of different viruses has changed. While NoVs are the most common etiological agents detected in nursing home outbreaks, a broader range of pathogens might be found in hospitals and other settings, such as child day care settings. Therefore, we reviewed our data on outbreaks of gastroenteritis and the prevalence of individual viruses in the typical patient groups to provide a priority list of viruses for evaluation. That list was then used to design the combinations of assays described below.

RNA extraction.

Stool homogenates (10%, wt/vol) were prepared in minimum essential medium with Hanks salts and 0.1 mg/ml gentamicin, mixed by vortexing, and clarified by centrifugation at 3,000 rpm (1,300 × *g*) for 20 min. A volume of 200 µl of the supernatant was used for RNA isolation. As internal control consisting of 20 µl of 10^7 PFU/ml of equine arteritis virus (EAV; kindly provided by E. C. J. Claas, Leiden University Medical Center, Leiden, The Netherlands) was added. The RNA was extracted with a MagNAPure LightCycler total nucleic acid isolation kit by the total NA (nucleic acid) external lysis protocol and eluted in a volume of 50 µl, according to the recommendations of the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

RT with random primers.

Random primers were used in the RT reaction. For annealing, 1 µl (50 µg/ml) of random primers (Promega, Madison, WI), 1 µl of total deoxynucleotide triphosphates (10 mM), 6 µl H_2O, and 5 µl of the extracted nucleic acids were heated for 2 min at 95°C, followed by cooling on ice for at least 2 min. In total, 7 µl of the RT mixture, which contained 4 µl of 5× first-strand buffer (Invitrogen, Carlsbad, CA), 1 µl of dithiothreitol (0.1 M; Invitrogen), 1 µl of RNAseOUT (Invitrogen), and 1 µl of Superscript III RT (200 U/µl; Invitrogen), was added and the mixture was incubated for 5 to 10 min at room temperature. The sample was subsequently incubated for 60 min at 50°C, followed by inactivation of the enzyme at 95°C for 2 min. The tubes were cooled and subsequently centrifuged to collect all reaction fluid.

RT with specific primers.
RT with primers specific for each virus was performed in order to compare the sensitivities, specificities, and detection limits obtained with these primers to those obtained with random primers, as described previously (19, 20, 27).

PCR amplification.
Two microliters of the RT mixture with random primers was added to 18 µl of a LightCycler PCR mixture containing 4 µl of TaqMan master solution (Roche Diagnostics GmbH), 0.1 µl (each) forward and reverse primers (100 pmol/µl), and 1 µl probe (5 pmol/µl) and was filled to 18 µl with H_2O. For reactions for AsV and NoV genotype II (GII), 0.5 µl probe (2.5 pmol/µl) was used (20, 26) (Table 1). The PCR amplifications were performed on a LightCycler 480 apparatus (Roche Diagnostics GmbH). Samples were denatured for 10 min at 95°C and subjected to amplification of 55 cycles at 95°C for 10 s, 52°C for 20 s, and 72°C for 10 s with fluorescence acquisition in the single mode. Detection was performed with multiple-color-detection compensation at 530, 560, and 610 nm.
Two microliters of the RT mixture with the specific primers was added to 18 µl of a LightCycler PCR mixture containing 2 µl of the hybridization probe Mastermix solution (Roche Diagnostics GmbH), the forward primer and probe specific for each virus (Table 1), $MgCl_2$, TaqStart antibody (5 U/µl; Clontech Laboratories, Inc.), and H_2O, as described previously (20, 22, 25, 26). The assays used for the diagnosis of acute gastroenteritis, which use specific primers, were described previously (12, 20, 22, 25-27).

Sensitivities, broadness, and specificities of the assays.
The sensitivities of the assays were assessed for different strains of each virus species for both the monoplex and the multiplex PCR assays. The sensitivities and broadness of the PCR assays for NoV GI and NoV GII were determined by using runoff RNA transcripts from recombinant plasmids containing the appropriate target sequences of all currently known genotypes of GI, GII, and GIV (H. Vennema et al., unpublished data). Serial 10-fold dilutions (up to a dilution of 10E−8) of each transcript of every recombinant plasmid were used. For the assay for the detection of RV, we used RV strains WA, ST3 (G4P2A), SA11 (G3P5B), G9P6, and NCDV (G6P6); for the assay for the detection of AdV, we used AdV types 40 and 41; for the assay for the detection of SaV, we used SaV strains GI.2 (Parkville), GII.2 (London), GI.3 (Stockholm), and GI.1 (Sapporo). The broadness of the assay for the detection of AsV was determined with cell culture supernatants containing AsV serotypes 1 to 8. The sensitivity of the assay for the detection of AV was determined with AV strain A846/88 (kindly provided by Teruo Yamashita). In total, 67 cell cultures and clinical isolates were used for further validation, e.g., for the determination of assay specificities.

Detection limits of the assays.
The detection limits of the assays were determined and compared by testing serial 10-fold dilutions of single and dual positive samples by use of specific and random primers and monoplex and multiplex PCR assays (Table 1). The efficiencies of the RT PCRs were determined by using the threshold cycle (C_T) values for serial 10-fold dilutions of each virus.

Clinical samples.
For this study, fecal samples from cases involved in outbreaks which were reported to the National Institute for Public Health and the Environment as a part of ongoing surveillance of viral gastroenteritis outbreaks were used in the analyses. In total, 150 fecal samples from patients with acute gastroenteritis were tested by the newly developed multiplex PCR assay (this study) and the currently used PCR assays (20). These 150 samples originated from 28 outbreaks, of which 18 were caused by NoVs, 2 were caused by RVs, 3 were caused by SaVs, and 5 were of an unexplained etiology, and from 23 single cases of gastroenteritis. Of these outbreaks, 16 were in a hospital (95 samples), 8 were in a residential institution (29 samples), 1 was at a day care center (3 samples), and 4 were in other settings (a hotel, a wedding, a rehabilitation clinic, and single cases; 23 samples).

TABLE 1. Primers and probes, corresponding concentrations, and labels used for syndrome surveillance of viral gastroenteritis.

Virus	Primers and probes	Direction[a]	Sequence (reference or source)	Concentration (pmol) in PCR	Label[b] (detection wavelength)
NoV GII	NV2LCR	REV	TCGACGCCATCTTCATTCAC (20)	10	
NoV GII	NV2LCF	FOR	GARYCIATGTTYAGRTGGATG (20)	10	
NoV GII	NV2LCpr		TGGGAGGGSGATCGCRATCT (20)	2.5	YY-BHQ1 (560 nm)
RV	RVNSP3R	REV	GGTCACATAACGCCCCTA (16)	10	
RV	RVNSP3F	FOR	ACCCTCTATGAGCACAATA (16)	10	
RV	RotaNSP3P1		CTAACACTGTCAAAAACCTAA (16)	5	FAM-TAMRA (530 nm)
EAV	EAV 2049F2	FOR	CTTGTGCTCAATTTACTGG (19; modified for this study)	10	
EAV	EAV 2147R2	REV	GGCAGAGGTACAGAATAG (19; modified for this study)	10	
EAV	EAV 2102P		TGCAGCTTATGTTCCTTGC (19; modified for this study)	5	Texas Red-BHQ2 (610 nm)
NoV GI	NV1LCF	FOR	CARGCCATGTTYCGYTGGATG (20)	10	
NoV GI	NV1LCR	REV	CCTTAGACGCCATCATCATTTAC (20)	10	
NoV GI	NV1LCpr		TGGACAGGAGAYCGCRATCT (20)	5	FAM-BHQ1 (530 nm)
AdV	HEXAA 1885	FOR	GCCGCAGTGGTCTTACATGCACAT (20)	10	
AdV	HEXAA 1885a	FOR	GCCCCARTGGGCRTACATGCACAT (this study)	10	
AdV	HEXAA 1913	REV	CAGCACGCCGCGGATGTCAAAGT (20)	10	
AdV	HEXAA 1913a	REV	ARCACICCICGRATGTCAAAG (this study)	10	
AdV	HEXAA LCpr		TGGTGCAGTTYGCCCG (this study)	5	FAM-BHQ1 (530 nm)
AsV	AC230	REV	GGTTTTGGTCCTGTGACACC (20)	10	
AsV	AC'1	FOR	ATGGCTAGCAAGTCTGACAAG (20)	10	
AsV	AC LCpr		CAACGTGTCCGTAAMATTGTCA (this study)	2.5	YY-BHQ1 (560 nm)
SaV	SVLCF	FOR	GAYCWGGCYCTCGCCACCT (27; modified for this study)	10	
SaV	SVLCR	REV	GCCCTCCATYTCAAACACTA (27; modified for this study)	10	
SaV	SVLCP		TGYACCACCTATRAACCAVG (27; modified for this study)	5	Texas Red-BHQ2 (610 nm)

[a]FOR, forward; REV, reverse.
[b]YY, Yakima yellow; BHQ, black hole quencher; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

RESULTS

Rationale of method development.

Data from our laboratory on outbreaks of gastroenteritis and literature on the prevalence of individual viruses in typical patient groups were reviewed (Tables 2 and 3). On the basis of that review, we decided to develop a first-line assay targeting the top two etiological agents, i.e., NoVs of GII and RVs of group A, in a single run (multiplex PCR assay 1). This would be the method of choice for clinical settings and laboratories with a public health mission, since these pathogens cause most viral gastroenteritis outbreaks. Additional multiplex assays for NoV GI and EAV (multiplex PCR assay 2), AdVs, AsVs, and SaVs (multiplex PCR assay 3) were then chosen on the basis of the increasing demand for the testing of samples from individual patients (hospitalized patients) for these viruses. Multiplex PCR assays 2 and 3 can be used in parallel, since they share the same cycling conditions. The third-level diagnostic assay covered rare etiological agents, i.e., AVs. Figure 1 outlines the steps used in the assays.

TABLE 2. Settings reported to be associated with gastroenteritis outbreaks from 1994 to October 2008[a]

Setting	No. (%) of outbreaks			
	Total, 1994-2005	2006	2007	2008
Residential institution	514 (55.7)	140 (51.1)	200 (48.6)	103 (47.2)
Hospital	164 (17.8)	83 (30.3)	166 (40.3)	97 (44.5)
Restaurant, café, pub, bar	81 (8.8)	6 (2.2)	6 (1.5)	1 (0.5)
Day care center	55 (6.0)	10 (3.6)	12 (2.7)	7 (3.2)
School	19 (2.1)	6 (2.2)	4 (1.0)	
Aircraft, ship, train, bus, coach	7 (0.7)	8 (2.9)	2 (0.5)	
Private house	6 (0.6)	1 (0.4)	2 (0.5)	
Otherb	33 (3.6)	10 (3.6)	6 (1.5)	2 (0.9)
Unknown	44 (4.8)	10 (3.6)	14 (3.4)	8 (3.7)
Total	923 (100)	274 (100)	411 (100)	218 (100)

[a]Data for 1994 to 2005 are from a previous study (20).
[b]A wedding, office, a police office, a summer camp, a monastery, a hotel, and a rehabilitation center.

TABLE 3. Reported settings associated gastroenteritis outbreaks caused by NoVs, RVs, AdVs, AsVs, SaVs, mixed viruses, and unexplained sources from 1994 to October 2008[a]

Virus	No. (%) of outbreaks					
	Residential institution	Hospital	Day care center	Restaurant, café, pub, bar	Other[b]	Total
NoV	786 (82.1)	397 (77.8)	41 (49.3)	71 (75.5)	130 (71.4)	1,425 (78.0)
RV	59 (6.2)	22 (4.3)	13 (15.7)		4 (2.2)	98 (5.4)
AdV	4 (0.4)	2 (0.4)	4 (4.8)			10 (0.5)
AsV	2 (0.3)	1 (0.2)			2 (1.1)	5 (0.3)
SaV	8 (0.8)	5 (1.0)	1 (1.2)		1 (0.5)	15 (0.8)
Mixed	4 (0.4)	3 (0.6)	12 (14.5)	1 (1.1)	1 (0.5)	21 (1.2)
Unexplained	94 (9.8)	80 (15.7)	12 (14.5)	22 (23.4)	44 (24.2)	252 (13.8)
Total	957 (52.4)	510 (27.9)	83 (4.5)	94 (5.1)	182 (10.0)	1,826 (100)

[a]Data are from a previous study (20).
[b]A school; private house; aircraft, ship, train, bus; wedding; office; police office; summer camp; monastery; hotel; rehabilitation center; and unknown.

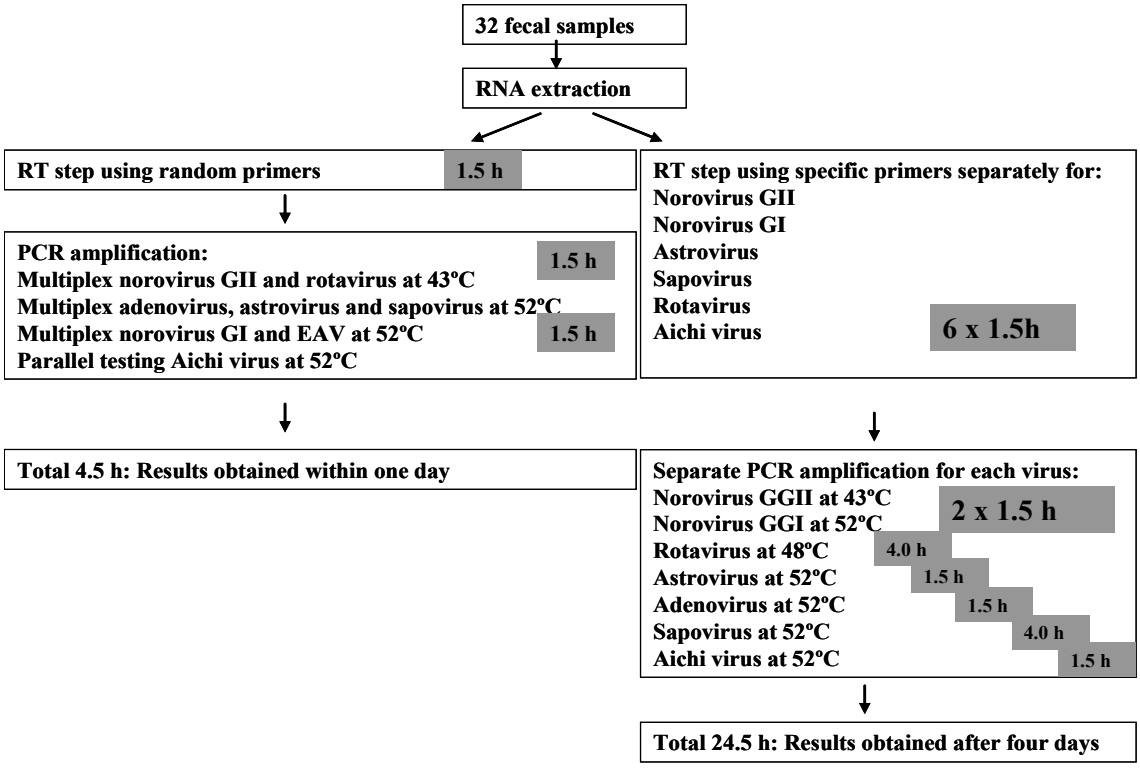

FIGURE 1. Comparison of diagnostic systems that use random primers and multiplex PCR assays (left) and specific primers and separate PCR assays (right) for the detection of viruses in 32 clinical samples. h, time (in hours) needed for procedure.

Sensitivities, broadness, and specificities of the assays.
Each pair of primers and the accompanying probe used in this study (Table 1) yielded PCR products when the positive controls were tested in all assay formats (random or specific primers in the RT step and/or the monoplex or multiplex PCR assays).
The specificities of the assays were tested with a panel of viruses, as described in Materials and Methods. Only assays (monoplex and multiplex PCRs) for the detection of NoV GI cross-reacted with other NoVs, NoV GII types II.2, II.3, II.10, and IIb and NoV GIV. If this cross-reactivity was used for calculation of the specificity of the assay for NoV GI, the specificity would be 92.5%. None of the other multiplex or monoplex assays amplified any of the other viral genomes used for evaluation, and the specificities of all other assays were calculated to be 100%.

Detection limits.
Comparison of the detection limits was first done by using serial dilutions and protocols for cDNA synthesis with specific and random primers, followed by the monoplex PCR assay. Use of the random primers did not decrease the detection limit of any of the assays; in fact, the detection limits of the PCR assays for SaV, AdV, AsV, and NoV GI were improved. The detection limits of the assays for the other viruses remained similar. The detection limits of the original test formats (specific primers in the RT reaction and monoplex PCR assays) and the new methods (random primers in the RT reaction and multiplex PCR assays) were compared. Tenfold dilutions of the nucleic acids of single viruses and nucleic acids from mixtures of viruses (e.g., mixtures of nucleic acids of NoV GII, RV, and EAV; AdV, AsV, and SaV; and NoV GI and EAV) were used, as presented in Table 4. The average C_T values (averages from two or more determinations) of the PCRs with 1/10 dilutions of these templates are presented in Table 4. The C_T values of 1/10 dilutions of GII NoVs, RVs, and AsVs (when single viruses were used) were higher in the multiplex assays than in the monoplex assays; however, when serial dilutions were tested, the detection limits remained the same (for AsVs, however, the detection limits were higher). The C_T values were determined and were used to

calculate the coefficient of determination (R^2), which indicates the efficiency of every PCR assay (Table 4 and Fig. 2). The efficiencies were high for the assays for all viruses except RV, for which the efficiency was calculated to be lower for the multiplex assay (0.8859) than for the monoplex assay (0.9827) (Table 4); however, this is not a problem, because the detection limits remained similar.

Clinical samples.

In total, 150 fecal specimens were tested in parallel by both approaches. Eighty-six fecal specimens were positive for a viral pathogen when they were tested in the multiplex assays, while 54 fecal samples were positive for a viral pathogen when the monoplex assays were used (Table 5). Two fecal specimens were positive only by the monoplex assays, and 34 were positive only by the multiplex assays. By using EAV as an internal control in the multiplex PCR assays, inhibition of the reaction was measured; no inhibition was detected for any of the fecal samples.

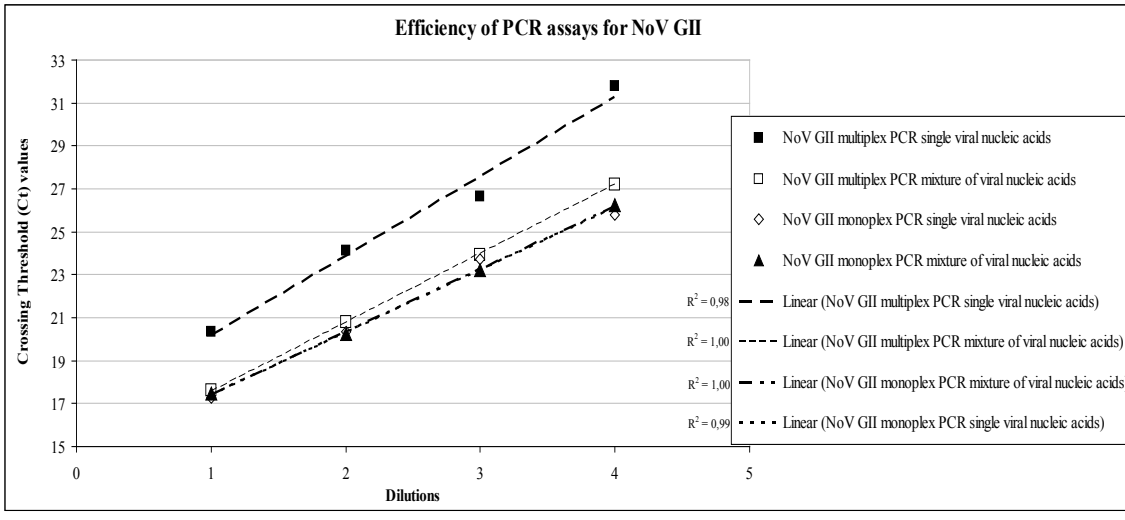

FIGURE 2. Efficiencies of PCR assays for detection of NoV GII. The multiplex PCR assays with nucleic acids from single viruses and mixtures of viruses had efficiencies of 0.98 and 1.00 CT value units, respectively. The monoplex PCR assays with nucleic acids from single viruses and mixtures of viruses had efficiencies of 0.99 and 1.00 CT value units, respectively.

TABLE 4. Calculated efficiencies of each multiplex or monoplex PCR assay with nucleic acids from single viruses and mixtures of viruses.

PCR type and nucleic acid source(s)	Efficiency (R2 value) of PCR assay for:							
	NoV GII	RV	EAV	NoV GI	EAV	SaV	AsV	AdV
Multiplex PCR								
NoV GII, RV, EAV	1.00	0.99	1.00					
NoV GII, RV	1.00	0.99						
NoV GII, EAV	1.00		1.00					
RV, EAV		1.00	0.98					
NoV GII	0.98							
RV		0.89						
EAV			0.9975					
NoV GI, EAV				0.99	0.99			
NoV GI				0.99				
EAV					0.99			
AsV, SaV, AdV					1.00	0.99	0.97	
AsV, SaV					1.00	1.00		
AdV, SaV					1.00		0.99	
AsV, AdV						0.98	0.99	
SaV						0.99		
AsV							0.98	
AdV								1.00
Monoplex PCR								
NoV GII, RV, EAV	1.00	0.96	0.99					
NoV GII, RV	0.96	0.99						
NoV GII, EAV	1.00		0.99					
RV, EAV		1.00	0.99					
NoV GII	0.91							
RV		0.98						
EAV			1.00					
NoV GI, EAV				1.00	0.99			
NoV GI				1.00				
EAV					0.99			
AsV, SaV, AdV						0.99	0.99	0.97
AsV, SaV						1.00	1.00	
AdV, SaV						1.00		0.99
AsV, AdV							0.99	0.99
SaV						1.00		
AsV							0.99	
AdV								0.98

TABLE 5. Clinical samples found to be positive by monoplex and multiplex PCR assay

Virus	No. (%) of samples positive by:		No. (%) different
	Monoplex PCR assays	Multiplex PCR assays	
NoV GII	42 (28)	67 (44.7)	25 (16.7)
NoV GI	0 (0.0)	2 (1.3)	2 (1.3)
RV	6 (4)	8 (5.3)	2 (1.3)
SaV	4 (2.7)	7 (4.7)	3 (2)
AdV	1 (0.7)	1 (0.7)	0 (0)
AsV	1 (0.7)	1 (0.7)	0 (0)
Total for positive samples	54 (36)	86 (57.3)	32 (21.3)
Total for negative samples	96 (64)	64 (42.7)	32 (21.3)
Total for all samples	150 (100)	150 (100)	

DISCUSSION

In this report, we present a scheme for the syndrome detection of acute viral gastroenteritis which was developed to meet the changing demand for virus detection from the perspective of a reference laboratory center. With the increasing awareness of NoV outbreaks, diagnostic methods are being implemented in routine local and regional medical microbiological laboratories. As a consequence, the pattern of referral for diagnostic evaluation at our national reference laboratory is changing, and a broader diagnostic package is more often being requested.

The most common request is for the evaluation of clinical samples for determination of the etiology of outbreaks of acute gastroenteritis, for which a rapid diagnosis is required in order to be useful to guide infection control policies. Therefore, with the changing demand, we also sought to decrease the turnaround time. One modification that we used was to change the initial step in the RT step by producing cDNA using random primers (2, 14, 17). The use of random primers in a single RT step reaction replaced the six individual RT step reactions with specific primers and lowered our reagent costs by 23% for the testing of one patient sample for the presence of all viruses. Similarly, the cost of labor was reduced by an estimated 58%.

A crucial step to be taken when essential assay conditions are changed is validation with clinical samples. This is particularly important, since we use these assays and their results as the basis for laboratory-based surveillance of trends in the prevalence of gastroenteritis viruses. Even relatively small differences in methods may fundamentally change the performance of the assay, as has been observed in a multicenter comparative evaluation of NoV detection and typing methods (2, 17, 24). Published studies rarely provide a thorough evaluation in this respect, making the decision to implement such novel assays difficult. Our study showed that the use of the random primers in the RT step and multiplex PCR assays for the panels of enteric viruses did not lower the efficiency or detection limits of most of the PCR assays. Only the PCR for RV had a lower efficiency. An explanation for this could be that RV is a double-stranded RNA virus for which denaturation requires more stringent conditions, resulting in less efficient annealing (10, 21). However, as the detection limits were not affected, we decided that this slight decrease in efficiency of the PCR for RV was acceptable. Remarkably, once the multiplex PCR assays were implemented in the clinical setting, we found that more viruses causing acute viral gastroenteritis were detected by the multiplex PCR assays than by the previously used monoplex assays but with the same specificity, showing a clear improvement in the diagnostic yield (20). A possible explanation for this lies in the increased efficiency of cDNA production by the use of random primers. All the viruses targeted in these assays have a substantial degree of genetic variability. Given this diversity, the choice of primers for use for cDNA synthesis and PCR, by definition, requires a compromise between the broadness of that assay and specificity. The original RT primers had several ambiguities to allow broad-range detection, which may have decreased the cDNA yield (3). The results presented above also show the importance of validation of the assay with clinical samples, as the difference in yield was not expected on the basis of the results of the technical validation.

In summary, this paper describes an approach for the syndrome detection of acute viral gastroenteritis. The results obtained showed that this method is highly sensitive and specific for the differentiation of viruses. The detection of all these viruses is rapid, with a turnaround time of 1 day, whereas at least 3 days is required when the previous methods are used. This approach leads to reductions in labor and reagent costs, which allows high-throughput testing for pathogens that cause viral gastroenteritis.

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Chapter 4

A new generic real-time reverse transcription polymerase chain reaction assay for vesiviruses; vesiviruses were not detected in human samples

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ABSTRACT

Different viruses belonging to the genus *Vesivirus* infect a broad range of animals, and cause gastroenteritis, vesicular skin lesions, hemorrhagic disease, respiratory diseases and other conditions. A recent report on *Vesivirus* viremia, as detected by PCR, in samples from patients with hepatitis of unknown etiology in the USA suggested a zoonotic potential for vesiviruses. These results have not been confirmed by another laboratory. In order to do so, a generic PCR assay on the RNA polymerase region was developed, and validated with RNA from 69 different *Vesivirus* species. Except SMSV serotype-8, all species tested were detected, including the ones that were suggested to be involved in zoonotic transmission in the USA (SMSV serotype-5).
The generic *Vesivirus* assay was used on RNA extracted from serum samples from patients with hepatitis, stool samples from patients with gastroenteritis, throat-swab specimens of patients with rash illnesses, throat-swab and nose-swabs of patients with acute respiratory diseases, and cell cultures with cytopathologic effect from enterovirus surveillance in which no pathogen was found. None were found positive. In this study a generic *Vesivirus* assay was developed and it was concluded that vesiviruses are an unlikely cause of common illnesses in humans in the Netherlands.

INTRODUCTION

The *Caliciviridae* are a family of positive-sense single-stranded RNA viruses comprising both human and animal pathogens (Clarke and Lambden, 1997). The four genera of the *Caliciviridae*, i.e.*Norovirus*, *Sapovirus*, *Lagovirus* and *Vesivirus*, have been classified on the basis of their genome organization and structure (Green et al., 2000). A fifth genus has been suggested following discovery of a bovine enteric calicivirus, which is genetically most similar to sapoviruses and lagoviruses, based on genomic organization of two open reading frames (ORF's) (Chen et al., 2006; Liu et al., 1999; Smiley et al., 2002; Sugieda et al., 1998). Noroviruses and sapoviruses are common causes of acute viral gastroenteritis in humans (Chen et al., 2006; Liu et al., 1999; Smiley et al., 2002; Sugieda et al., 1998), and are also found in swine, cattle, mice and possibly companion animals (cats and dogs) (Guo et al., 1999; Wang et al., 2005). Viruses in the genus *Lagovirus* infect lagomorphs (rabbits and brown hares) and the viruses in the genus *Vesivirus* are animal pathogens with a clearly broader host range (Radford et al., 2004).
Vesiviruses infect many different animal species, and cause a range of diseases (Smith et al., 1977). Infections with vesiviruses in humans have been reported to cause vesicular exanthema on hands after a laboratory incident, and on the face of a field biologist working with marine mammals. The strain recovered from the laboratory worker was related most closely to San Miguel sea lion virus (SMSV) serotype-5, which has several natural hosts (Chen et al., 2006; Smith et al., 1998a,b). The zoonotic potential of some *Vesivirus* strains has further been suggested by reports of SMSV antibodies in humans (Chen et al., 2006; Smith et al., 1998a,b). A high prevalence of anti-*Vesivirus* antibody together with *Vesivirus* viremia was observed among patients with hepatitis of unknown etiology in the USA (Smith et al., 2006). These findings indicate a potential for *Vesivirus* infection, and possibly illness, in humans. It was suggested that vesiviruses, with their broad host range, might manifest themselves by causing hepatitis and possibly other diseases in humans.
In the Netherlands studies are conducted currently to decrease the diagnostic gap that exists for several illnesses. Samples from hepatitis patients that have been tested negative for hepatitis A, B, C viruses, Cytomegalovirus, and Epstein Barr virus were collected. From this cohort, over 90% remains unexplained after testing for hepatitis E (Herremans et al., 2007;Waar et al., 2005). Furthermore, in outbreaks of acute gastroenteritis, 12% remains unexplained after elaborate testing (Svraka et al., 2007). In samples of respiratory diseases, 50% remains unexplained (Heijnen et al., 1999), about 12% of samples of unexplained rash illnesses (van Binnendijk et al., 2003), and 5% of the isolates from enterovirus surveillance (van der Sanden et al., 2008) currently remain without any pathogen being detected. To determine if vesiviruses have caused infections in humans in the Netherlands a generic RT-PCR for vesiviruses is described and the results of this assay on the samples described above are reported.

MATERIALS AND METHODS

Design of synthetic oligonucleotide primers.

In order to develop a *Vesivirus* generic PCR a complete search of GenBank was done and 300 *Vesivirus* entries were identified. For the primer development 37 complete genome and partial polymerase gene sequences of vesiviruses were used. These sequences were imported as GenBank files into BioEdit Sequence Alignment Editor and aligned using the ClustalW multiple alignment tool, which is included in BioEdit software. The following *Vesivirus* genome sequences were used: Feline calicivirus (FCV) isolates (AY560118, AY560117, AY560116, AY560115, AY560114, AY560113 and AF098932) and strains (NC 001481, D31836, DQ424892, L40021, AF479590, M86379 and AF109465); Canine calicivirus (CaCV) (NC 004542, AB070225 and AF053720); Skunk calicivirus (U14668, U14670, U14672 and U18743); Vesicular exanthema of swine virus (VESV) strains (NC 002551, AF091736 and U76874); Rabbit vesivirus (AJ866991); various SMSV serotypes (U15301, U52094, U52093, U52089, U52087, U52088 and U52090); Walrus calicivirus (NC 004541); Reptile calicivirus (U52092); Mink calicivirus (AF338405); Cetacean calicivirus (U52091); Primate calicivirus Pan-1 (U52086). Subsequently, conserved genomic regions of different *Vesivirus* species were selected and primers were developed (Table 1).

TABLE 1. Primer sequences, and their position on the genomes. Expected product sizes of PCR products are indicated in base pairs (bp). ND, not done.

Primer			Primer BR1	Vesi FOR	Vesican FOR	CaCV FOR
	Nucleotide position		4294–4311a	4466–4485a	4484–4501a	5095–5114b
		Sequence (5'–3')	CTGGGGWTGYGAYGTTGG	GTTGACTAYTCNAARTGGGA	GACTCNACCCAACCNCCA	GTGGTGTGTCCTTCAAAAC
FCO YGDD	4778–4795a	TACGGGGATGATGGTGTC	501 bp	329 bp	311 bp	ND
VVrev	4778–4795a	TACGGRGATGATGGTGTC	501 bp	329 bp	311 bp	ND
YGDD	4778–4795a	TATGGTGATGATGAGATT	501 bp	329 bp	311 bp	ND
Vesi REV	4778–4800a	TACGGCGACGACGGNGTNTACAT	506 bp	334 bp	316 bp	ND
SAN1 REV	4781–4800a	GGYGACGACGGTGTCTACAT	506 bp	ND	ND	ND
SAN2 REV	4778–4795a	GGYGACGACGGTGTCTACAT	501 bp	ND	ND	ND
CaCV REV	5298–5317b	TTTGTACTTTCTGTTATGCC	1023 bp	851 bp	833 bp	

Reverse transcription and PCR amplification.
This generic assay is based on SybrGreen chemistry and uses a set of primers targeting the polymerase region. The concentrations and the conditions as described below were used for the most optimal primer set and were the same throughout. Annealing with the reverse primer was done using 1.5 µL (50 µM) of the reverse primer, 5 µL H2O, and 2.5 µL of the extracted RNA for 2min at 94 ºC followed by cooling for at least 2 min. Six microliters of reverse transcription mix containing 1.5 µL of 10× PCR buffer 10mM Tris–HCl (pH 8.3), 1.8 µL of MgCl2 concentration of 25mM, 1.5 µL of dNTPs (10mM each), 0.5 µL of 10U AMVRT (Promega, Leiden, the Netherlands) were added and incubated for 60min at 42 ºC. Two microliters of the RT-mix were added to 18 µL of a LightCycler (LC) PCR-mix containing 2 µL of the DNA SybrGreen Mastermix Solution (Roche Diagnostics GmbH, Mannheim, Germany), 0.12 µL forward primer with concentration of 50 µM, 2.6 µL of MgCl2 concentration of 25mM, 0.16 µL TaqStartTM Antibody (5 U/ µL) (Clontech Laboratories Inc.), and 13.12 µL H2O.
The PCR amplifications were performed on a LightCycler apparatus (Roche Diagnostics GmbH, Mannheim, Germany).Samples were denatured for 1 min at 95 ºC, subjected to 40 amplification cycles with 0.1 s denaturing at 95 ºC, 55 ºC annealing for 5 s, and 72 ºC elongation for 20 s with fluorescence acquisition in single mode. The first-derivative melting curve analysis was performed by heating the mixture to 95 ºC for 0.1 s and then cooling to 55 ºC for 5 s and heating back to 95 ºC at increments of 0.5 ºC. Detection was performed at 530 nm. Identification of PCR products of vesiviruses was done using the first-derivative melting curve analysis. Additionally, the PCR products were run on a 2% agarose-gel in order to define product size, and visualized using SybrSafe solution according to manufacturer's instructions.

Evaluation of the generic Vesivirus assay.
Detection limit of the assay
Feline calicivirus strain F9 (from the collection of Faculty of Veterinary Medicine, Virology Department, Utrecht University) and Canine calicivirus strain no. 48 (kindly provided by Dr. M. Mochizuki, Laboratory of Clinical Microbiology, Tsukuba Central Laboratories, Kyoritsu Seiyaku Corporation, Japan) were chosen to evaluate the detection limit of the real-time PCR. Tenfold dilutions from 100 to 10^{-6} were made from both virus suspensions. Each dilution was subjected to reverse transcription, PCR amplification, and the first-derivative melting curve analysis in order to determine the detection limit. Additionally, PCR products of the dilution series were analyzed on a 2% agarose-gel.

Sensitivity and specificity of the assay
Sensitivity of the assay was evaluated against a diverse range of *Vesivirus* strains (Table 2). This includes strains with various origins of FCVs, e.g. 20 FCV isolates from across the UK (kindly provided by Dr. K. Coyne, University of Liverpool Veterinary Teaching Hospital, United Kingdom), 10 field isolates from Italy, and FCV 2280 (kindly provided by Dr. V. Martella, University of Bari, Bari, Italy), 5 FCV field isolates from across the Netherlands (Faculty of Veterinary Medicine, Virology Department, Utrecht University), 3 strains of FCV F9 (University of Utrecht, the Netherlands, Dr. S. Reid, and Dr. V. Martella), 1 strain of CaCV no. 48 (kindly provided by Dr. M. Mochizuki, Laboratory of Clinical Microbiology, Tsukuba Central Laboratories, Kyoritsu Seiyaku Corporation, Japan). Other *Vesivirus* isolates (kindly provided by Dr. S. Reid; Institute for Animal Health, Surrey, United Kingdom) were SMSVs serotypes 1–13 and 11 VESV strains (A48, C52, D53, E54, G55, I55, J56, B1, B51, F55, H54 and K54), and other VESV strains such as primate (isolated from a gorilla), Cetacean (dolphin) and Skunk CV, and Bovine Tillamook virus (BCV Bos-1) strain (Reid et al., 2007). Specificity was evaluated using clinical samples containing different NoV genogroups I (GI.1, GI.2 WR, GI.2 SOV, GI.3, GI.4, GI.5, GI.6, GI.7 and GI.10), II (GII.1, GII.2, GII.3, GII.4, GII.6, GII.7 and GII.10) and IV, 4 clinical samples of SaV (genotypes GI.1, GI.2, GI.3 and GII.1), clinical isolates from astrovirus types 1–8, adenovirus types 40 and 41, 34 clinical serum samples of HEV, and 15 samples from HAV infected patients, clinical samples of Influenza viruses A and B, rhinoviruses, corona viruses OC43, 229E, and NL63, RSV A and B, *human metapneumovirus* (hMPV), *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, clinical isolates of 10 *human parvoviruses B19*, 14 rubella viruses, 5 measles viruses, 3 mumps viruses, and 5 enteroviruses (Human enterovirus 71, coxsackieviruses B3 and A9, echoviruses 9 and 30). *Rotavirus* isolates (WA, DS1, K8, NCDV, 69M, SA11, ST3, B223), and strains

G10P[11], G9P6, G9P[23]) were used, and Aichi virus strain A846/88 (Yamashita et al., 2000).

TABLE 2. *Vesivirus* strains used in this study.

Virus	Strain/isolate	Year of original isolation	Country or region(s) of origin
VESV	VESV A48	1948	USA
VESV	VESV B1–34	1934	USA
VESV	VESV B51	1951	USA
VESV	VESV C52	1952	USA
VESV	VESV D53	1953	USA
VESV	VESV E54	1954	USA
VESV	VESV F55	1955	USA
VESV	VESV H54	1954	USA
VESV	VESV G55	1955	USA
VESV	VESV I55	1956	USA
VESV	VESV J56	1954	USA
VESV	VESV K54	1972	USA
SMSV	SMSV-1	1972	USA
SMSV	SMSV-2	1972 USA	USA
SMSV	SMSV-3	NK	USA
SMSV	SMSV-4	1973	USA
SMSV	SMSV-5	NK	USA
SMSV	SMSV-6	1975	USA
SMSV	SMSV-7	1976	USA
SMSV	SMSV-8	1975	USA
SMSV	SMSV-9	1975	USA
SMSV	SMSV-10	1977	USA
SMSV	SMSV-11	1977	USA
SMSV	SMSV-12	1977	USA
SMSV	SMSV-13	1984	USA
Primate CV	Primate CV	1978	USA
Cetacean CV	Cetacean CV	NK	USA
Skunk CV	Skunk CV	NK	USA
Bovine CV (Bos-1)	Bovine CV (Tillamook)	1981	USA
CaCV strain no. 48	CaCV	NK	JP
FCV F9	FCV	NK	NL
FCV F9	FCV	1984	NK
FCV F9	FCV	2000	IT
FCV 2280	FCV	2000	IT
FCV (10 isolates)	FCV	2000–2005	IT
FCV (20 isolates)	FCV	2005–2006	UK
FCV (5 isolates)	FCV	NK	NL

VESV, Vesicular exanthema swine viruses; SMSV, San Miguel Sea Lion virus; CV, Calicivirus; FCV, Feline Calici Virus; CaCV, Canine Calici virus; NK, not known;

SA, United States of America;JP, Japan; NL, the Netherlands;IT, Italy;UK, United Kingdom.

Spiking of fecal samples with CaCV and FCV isolates
Fecal samples, in which no viruses were detected, were spiked with CaCV strain no. 48 and FCV F9 strain. Five hundred microliters of the stool homogenates were spiked with 500µL of 10-fold dilutions of CaCV no. 48 strain (1.6×107 TCID50/mL) and FCV F9 strain (1.6×106 TCID50/mL). Tenfold dilutions were added to the stool homogenates prior to centrifugation and RNA extraction was performed on 210µL of the supernatant, with MagNAPure LC Total Nucleic Acid Isolation kit using the Total NA External Lysis protocol and eluted in a volume of 50µL according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany). Five microliters of the extracted RNA were subjected to amplification. Melting curve analysis of PCR products was performed. Subsequently, the PCR products were visualized on agarose-gel.

Sequencing
PCR products were purified from gel using the QIAgen PCR purification kit according to the manufacturer's instructions and confirmed by sequencing using a fluorescence-labeled dideoxynucloetide technology from Applied Biosystems (Applied Biosystems, Foster City, USA). Sequence reactions were analyzed on an ABI 3700 automated sequencer. The sequences obtained were assembled using Seqman and Editseq software (DNAStar, Konstanz, Germany).

Sample panels.
In order to determine if vesiviruses have caused infections in humans in the Netherlands, the following patient samples were collected systematically and tested.

Samples from patients with acute clinical hepatitis of unknown etiology
A panel of 412 serum samples was used, collected from patients with acute hepatitis and sent to the RIVM in 2005 and 2006. The sera were serologically negative for hepatitis A, B, C, and E viruses, Cytomegalovirus, Epstein Barr virus and the available volumes exceeded 500 µL (Herremans et al., 2007; Waar et al., 2005). The serum samples had been stored at −20 ºC prior to testing. RNA was extracted from these samples with MagNAPure LC Total Nucleic Acid Isolation kit using the Total NA External Lysis protocol and eluted in a volume of 50 µL according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany).

Samples from gastroenteritis outbreaks with unexplained etiology
In order to narrow the diagnostic gap in the viral gastroenteritis surveillance all unexplained outbreaks collected from1994 through 2006 (Svraka et al., 2007) were tested. In total, 605 fecal samples from 153 unexplained outbreaks of gastroenteritis were tested for the presence of vesiviruses.

Samples from patients with unexplained rash illnesses
The RNA isolated from throat-swabs of 131 patients with unexplained rash illnesses was used. These samples had previously been tested using PCR for Human parvovirus B19, rubella virus, and measles virus (van Binnendijk et al., 2003) and were negative using these assays. The RNA samples had been stored at −70 ºC prior to testing for vesiviruses. Throat-swab specimens from patients with rash illnesses were collected in viral transport medium. Cells from the swabs were sedimented (10min of centrifugation at 350×*g*), and 20% of the cells were reconstituted in 200 µL of PBS, from this RNA was extracted using the High Pure Viral Nucleic Acid RNA isolation kit (Roche Diagnostics) according to manufacturer's recommendations, and modified by adding poly[A] to the lysis buffer (0.04mg/mL) to improve the recovery of specific RNA (van Binnendijk et al., 2003).

Samples from patients with unexplained respiratory disease
In total, 286 RNA samples extracted from combined throat swab and nose-swab samples from patients with acute respiratory diseases of unknown etiology from 2006 were used. These samples previously tested negative for Influenza viruses A and B, rhinoviruses, enteroviruses, coronaviruses, adenoviruses, respiratory syncytial viruses (RSV) A and B, *human metapneumovirus*, *C. pneumoniae*, and *M. pneumoniae*.

These clinical samples were spiked with a known amount of Equine arteritis virus (EAV). This internal control was co-extracted and co-amplified in the reaction (Scheltinga et al., 2005). A 10−4 dilution of EAV was used for spiking of patient material. Inhibition was not detected. The RNA samples of unexplained respiratory diseases had been stored at −70 ºC prior to testing. The combined nose-swabs and throat-swabs (200 µL) from patients with respiratory illnesses were added to 300 µL lysis buffer. The RNA of all these samples was extracted with MagNAPure LC Total Nucleic Acid Isolation kit using the Total NA External Lysis protocol and eluted in a volume of 50 µL according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany).

Stool culture isolates of unknown etiology from enterovirus surveillance
Enterovirus surveillance in the Netherlands involves screening of stool samples from children with systemic viral infection, varying from meningitis to gastrointestinal disorders (van der Sanden et al., 2008). One of the objectives of this surveillance is to exclude poliovirus. These samples were cultured previously on tertiary monkey kidney (tMK) cell lines and tested negative for enteroviruses, parechoviruses, and adenoviruses by PCR. The culture isolates had been stored at −70 ºC prior to RNA extraction. The RNA of 29 cell cultures collected between 2000 and 2006 was extracted with MagNAPure LC Total Nucleic Acid Isolation kit using the Total NA External Lysis protocol and eluted in a volume of 50 µL according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany).

RESULTS

Design of synthetic oligonucleotide primers.
A global alignment of 37 *Vesivirus* genome sequences was used for development of *Vesivirus* generic primers. In total, four forward and seven reverse primers were developed (Table 1) on the polymerase region. These primers were used in different combinations in order to select the most optimal primer pair. This was done using serial 10-fold dilutions of CaCV no. 48 and FCV F9. The combination of the primers BR-1 5'-CTGGGGWTGYGAYGTTGG-3' and VVrev 5'- GACACCATCATCRCCGTA-3' generated a 501 base pairs (bp) PCR product and was found to be the most optimal, when assessing at the melting temperatures and bands on an agarose-gel of the dilution series, for the detection of the two strains tested (Figs. 1 and 2 and Table 1).

Evaluation of the generic Vesivirus assay.
Detection limit of the assay
The detection limit of the generic assay was determined using serial 10-fold dilutions of CaCV no. 48 and FCV F9 stocks with respective titers of 1.6×107 and 1.6×106 TCID50/mL. A PCR positive signal was obtained at 10−4 dilution for FCV F9, with melting temperatures (ºC) of 89.04 for undiluted FCV F9, 88.89 for 10−1, 88.72 for 10−2, 88.39 for 10−3, and 88.54 for 10−4 FCV F9. For CaCV no. 48 a signal at 10−4 dilution was observed when the first-derivative melting curve analysis was performed. This isolate had melting curve temperatures (ºC) of 86.01 for 100 CaCV no. 48, 86.09 for 10−1, 86.18 for 10−2, 86.43 for 10−3, and 86.14 for 10−4 CaCV no. 48. Detection limits were determined at 1.6×103 TCID50/mL for CaCV no. 48 and 1.6×102 TCID50/mL for FCV F9.
The dilution series were visualized on 2% agarose-gel in order to compare the results with the melting curve analysis. A PCR positive signal on agarose-gel was observed at 10−4 dilution for both isolates.

Sensitivity and specificity of the assay
The sensitivity of the assay was tested using different *Vesivirus* species, as described in Section 2.3.2. All of the isolates generated a PCR product and the melting temperatures were determined. All *Vesivirus* isolates were also visualized on an agarose-gel, and all serotype-8. The PCR products were sequenced to confirm specificity of the methods. All SMSV, VESV, CaCV, and FCV isolates were identified correctly.
The specificity of this assay was tested using a panel of viruses as described in Section 2. This assay yielded products with several viruses, however the melting temperatures of PCR products of noroviruses, adenoviruses, astroviruses, Aichi virus, HEVs, HAVs, enteroviruses, Influenza virus A and B, rhinoviruses,

coronaviruses, adenoviruses, RSV A and B, hMPV, *C. pneumoniae*, and *M. pneumoniae* did not match the melting temperatures of vesiviruses. These products were also analyzed on an agarose-gel and no PCR product was visible. The amplicons/products of two clinical *Sapovirus* samples and a *Rotavirus* 69M positive sample had a melting temperature of 87.94 and 86.37 ºC approached that of the FCV and CaCV products, respectively. On agarose-gel these three samples yielded three visible PCR products, which differed in size from the *Vesivirus* amplicon. The products were sequenced and confirmed as one *Sapovirus*, one bacterial sequence with a highest homology of 88% with *Pectinatus frisingensis* and a *Rotavirus* product. The sequence of the product of the *Rotavirus* 69M containing sample confirmed the presence of *Rotavirus* RNA when the sequence obtained was subjected to BLAST. Furthermore, viruses belonging to 12 different norovirus genogroups developed a PCR product on agarose-gel. The melting temperature of these viruses did not match those of vesiviruses, however the samples were sequenced for confirmation and matched to noroviruses. The specificity calculated (after melting curve and gel electrophoresis analysis) for the generic *Vesivirus* assay for detection of vesiviruses was 90%.

Spiking of fecal samples with CaCV and FCV isolates
RNA loss in extraction and possible inhibition by fecal components of the RT-PCR were measured in fecal samples spiked with 10-fold dilutions of CaCV no. 48 and FCV F9 isolates. CaCV and FCV dilutions were detected down to 0.8×103 TCID50/mL.

Sample panels.
To determine if vesiviruses have caused infections in humans in the Netherlands, samples collected from patients with five different clinical syndromes for which common pathogens had been ruled out, were tested (n = 1493). None of the samples from unexplained etiologies yielded a specific product in the *Vesivirus* generic assay. None of the products formed were compatible with *Vesivirus* characteristics, based on melting curve analysis or expected product size of 501 bp by agarose-gel analysis. However, 244 clinical samples did generate a non-specific PCR product around 300 bp, as described below. PCR of 99 clinical samples from patients with gastroenteritis outbreaks of unexplained etiology generated a non-specific PCR product around 300 bp. Sequencing of these bands did not yield *Vesivirus* sequences when subjected to BLAST, but sapoviruses (n = 1), Bacteroides spp. (n = 19) and the other products did not yield a sequence. The *Sapovirus* positive sample had two bands on gel, of which the upper band was approximately 500 bp. This sample was confirmed as *Sapovirus* using a *Sapovirus*-specific assay (Svraka et al., 2007). From the samples of patients with unexplained rash illnesses 16 generated a product of 300 bp. When sequenced, these products matched *Staphylococcus aureus*. One hundred and thirty-six samples (46%) from patients with unexplained respiratory disease generated non-specific products of 300 bp, of these samples 40 were sequenced and all of them matched *S. aureus*.
Three stool culture isolates of unknown etiology from enterovirus surveillance generated a PCR product of 300 bp, when visualized on agarose-gel. The sequences of these products yielded a mammalian orthoreovirus 1.

DISCUSSION

In this study a sensitive broad range real-time reverse transcriptase *Vesivirus* PCR assay was developed. The primer set of BR-1 and VV-rev targets is a highly conserved motif in the RNA polymerase region.
The sensitivity of the assay was evaluated using 69 various *Vesivirus* strains. It successfully detected all available *Vesivirus* strains, except SMSV serotype-8. It was reported previously that SMSV serotype-8 may be different antigenically and antigenetically from other marine calicivirus serotypes and this type was found to be negative in other PCR assays. In contrast with previously published *Vesivirus* assays, SMSVserotype-12was detected by our assay (Reid et al., 1999, 2007; Seal et al., 1995). The specificity of the primers was tested using a wide variety of viruses. None of the samples generated a product of 501 bp using the *Vesivirus* assay. Several non-specific products of 300 bp were obtained for sapoviruses. However, 244 non-specific products of approximately 300 bp were detected in clinical samples, what necessitated in sequencing of 10.6% (158 of 1493) of clinical samples, in order to exclude vesiviruses. SybrGreen was used

because development of a generic assay was aimed, which would be able to detect all (or almost all) *Vesivirus* isolates. It was not possible to design a generic probe, which would be able to detect all vesiviruses. Development of different probes for different strains of *Vesivirus* genus could be an option for this and will be considered.
Competition in clinical samples between *Vesivirus* RNA and large amounts of viral or bacterial nucleic acids from other pathogens was assessed by spiking of the fecal samples with cultured CaCV and FCV strains. Although no noticeable competition was noticed, large amounts of other nucleic acid could interfere with the detection of vesiviruses, if those are present in much lower amounts. However, presence of other caliciviruses, like NoVs and SaVs, was excluded before testing with the *Vesivirus* generic assay and therefore little competition is expected.

Inhibition is not expected since no inhibition was detected in respiratory clinical samples that were spiked with EAV as internal control. Furthermore, EAV was included in 533 fecal samples to measure the inhibition. These samples were tested for common gastroenteritis viral pathogens, in none of these samples inhibition was measured (Svraka et al., manuscript in preparation).
Syndromic detection of acute viral gastroenteritis by use of random primers and internally controlled multiplex real-time PCR assays). Vesiviruses are known as animal pathogens, and infect a broad range of species causing different clinical syndromes. In addition, cross-species infections have been documented for several vesiviruses (Smith et al., 1998a). Recently, Smith and co-workers detected *Vesivirus* RNA by PCR in 9.8% (11 of 112) of serum samples from a group of blood donors in the USA, suggesting that such infections are widespread. Ten of the RNA positive serum samples originated from blood donors with elevated blood liver alanine aminotransferase levels. Smith et al. (2006) also found a high seroprevalence of anti-*Vesivirus* antibodies in patients with acute hepatitis of unknown etiology (5 of 26), and the highest seroprevalence was detected in patients with acute hepatitis associated with blood transfusion and dialysis (7 of 15). Furthermore, VESV and SMSV have been described in swine, sea lions, and other animals in the USA (O'Hara et al., 1998; Sawyer, 1976; Smith and Akers, 1976; Smith and Latham, 1978; Smith et al., 1977). This may imply that these vesiviruses are endemic on the Northern American continent. In contrast, no reports on any vesivirus infection in either animals or humans in Europe have ever been published. In view of these findings, it was decided to assess the possibility of vesiviruses as causes of illness in the Netherlands. Therefore, unexplained hepatitis serum samples, stool samples of unexplained etiologies from patients with gastroenteritis, throat-swab specimens of patients with rash illnesses, throat-swab and nose-swabs of patients with acute respiratory diseases, and unexplained cultures from enterovirus surveillance were tested. No evidence of any *Vesivirus* involvement was found in these samples.
Since the study described in this paper does not include serological testing, but is limited to detection of viral RNA in patient specimens, it cannot be concluded with certainty that *Vesivirus* infections do not play a role in the Netherlands. However, since we have tested a larger number of acute hepatitis patients with PCR than Smith and co-workers (n = 412 versus 112), with a *Vesivirus* assay with a detection limit of 1.6×103 TCID50/mL or better, detection of a *Vesivirus* species as causative pathogen in patients with acute hepatitis would have been very likely.
In summary, a rapid and sensitive real-time reverse transcriptase PCR assay for the broad detection of vesiviruses was developed successfully. This generic assay was validated using RNA from 69 *Vesivirus* strains and was shown to detect all strains of the *Vesivirus* genus, except SMSV serotype-8. *Vesivirus* RNA was not found in any of the clinical samples tested. Therefore, it can be concluded that vesiviruses are an unlikely cause of acute hepatitis and gastroenteritis, rash illnesses, respiratory and intestinal diseases in humans in the Netherlands.

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Chapter 5

Epidemiology and genotype analysis of emerging sapovirus associated infections across Europe

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ABSTRACT

Sapoviruses belong to the *Caliciviridae* family and can cause gastroenteritis in humans and swine. Despite extensive testing, human sapoviruses have only been found in sporadic cases and in one mixed outbreak in children between 1994-2007 in the Netherlands. Here we describe a change in sapovirus epidemiology in the Netherlands, resulting in sapovirus outbreaks and infections in adults. From November 2007 to January 2009, 478 outbreaks of acute gastroenteritis were reported to the National Institute for Public Health and the Environment in the Netherlands as a part of ongoing surveillance. Sapoviruses were found to be the most likely cause of nineteen outbreaks (4%).

During the same two-year period, sapovirus infections were reported in Sweden, Slovenia and Hungary. In the Netherlands further characterization of outbreak strains showed that twelve (63%) sapovirus outbreaks were caused by genotype I.2 viruses. Most patients were adults older than 60 years (range 1-100). Phylogenetic analysis using all presently available SaV sequences showed high homology between genotype I.2 strains detected in different geographical regions (Sweden, Slovenia, Taiwan, Japan, and Russia) since 2007.These first reported outbreaks of sapovirus infections in adults in the Netherlands were remarkable. Detection of an identical genotype in many samples might suggest that these viruses could have the same origin, and since the infection is spreading fast, prevalence of sapovirus infection may be increasing. The incidence of sapovirus infections in these countries suggests that a substantial part of Europe is affected by this virus.

INTRODUCTION

Sapoviruses (SaVs) and noroviruses (NoVs) are human caliciviruses (HuCV) that are recognized as a major cause of acute gastroenteritis (GE) worldwide (7, 19, 33). Human NoV and SaV strains cannot be reliably cultivated in vitro, and currently reverse transcription-PCR (RT-PCR) is the most widely used method for their detection. NoVs and SaVs differ in their epidemiology and host range. NoV infections are common in all age groups and are responsible for about 80% of all acute GE outbreaks (29). On the other hand, SaV infections are less common and known to cause disease primarily in children, usually under the age of five (10, 11, 13, 24).

However, recently, SaVs have been reported as an occasional cause of outbreaks inhospitals and other healthcare facilities. The age groups affected in these facilities has ranged from young adults to elderly, suggesting less age restriction to illness than previously thought (8, 14, 15, 19, 22, 34). Although SaV associated diarrhea is generally mild, severe cases can occur (25).

SaV has a single-stranded positive-sense RNA genome of approximately 7.3 -7.5kb,containing two to three open reading frames that encode non-structural proteins, a capsid protein (ORF 1), and a putative protein with an unknown function (ORF 2 and3) (9). Based on complete capsid gene sequences, SaV strains are divided in five genogroups (GI to GV), where genogroup III has been found to infect porcine species, whereas GI, GII, GIV, and GV are known to infect humans (4, 26).

In the Netherlands, a GE outbreak surveillance program has been implemented since 1994 to monitor outbreaks and their potential causative agents, including SaV (29). In this study we describe the emergence of SaV as a cause of outbreaks of acute GE in the Netherlands. In addition, we analyzed SaV-positive samples from GE outbreaks in Sweden, Slovenia and Hungary (23). Emergence of sapoviruses may be explained by the genetic variability of SaV strains. The evolutionary dynamics of SaV strains were investigated using the BEAST program package (2) to assess if the increased number of SaV infections is a result of an increased incidence or an artifact of a new, more sensitive detection methods.

MATERIAL AND METHODS

Reporting of outbreaks of acute gastroenteritis in the Netherlands.

All the stool samples used for this study were obtained from outbreak cases reported to the National Institute for Public Health and the Environment (RIVM) through the ongoing surveillance of viral GE outbreaks. The outbreak surveillance has been operational in the Netherlands since 1994 and provides a systematic overview of all notified outbreaks (29). From November 1st 2007 to January 1st 2009, 478 GE outbreaks (3233 samples), were reported to the surveillance program and tested for the presence of noroviruses, rotaviruses, enteric adenoviruses, astroviruses and SaVs by a combination of multiplex RT-PCR assays (3, 28-30). Outbreaks were attributed to one of the pathogens mentioned above depending on the diagnostic yield as a proportion of samples tested (3). To evaluate if changes observed in SaV incidence were not due to modifications in the detection protocols used, outbreak samples from 2006 and 2007 were retested.

Sequencing of the sapovirus positive samples.

Detection of sapoviruses was preformed as described by Svraka et al. (30). This is a multiplex RT-PCR that uses random primers and is sapovirus specific, it proved to be more sensitive than previously used sapovirus PCR assays, as described by Svraka et al. (30). Sequencing of partial capsid genes of SaVs was performed using primers previously described by Yan et al. (35). The RT-reaction step was slightly modified and performed using random primers and Superscript III (30). PCR amplification was performed as described by Svraka et al. (30) and adding 0.1 µL forward and reverse primers [100 pmol/µL] in the PCR mixture (Table 1) (35).

Sequence analysis.

The BioNumerics software (Applied Maths BVBA, Sint-Martens-Latem, Belgium) was used for alignment of sequence data and clustering for genotype assignment. The different isolates used in this study were assigned to specific genogroups and subgenogroups according to nomenclature described in Fields Virology (6).

Nine reference strains (U65427, U73124, AF194182, AF435814, AY289803, AY289804, AF435812, U95645, AF182760) were included in the analysis to assign the genotypes of the clinical samples used in this study. Phylogenetic trees were generated using the neighbor-joining method in the TREECON software, version 1.3b (32). Statistical confidence of the trees was obtained by bootstrap analysis using 1000 pseudoreplicates.

As this initial analysis suggested emergence of SaVs in the Netherlands, laboratories in the Food-borne Viruses in Europe (FBVE) network were contacted to investigate GE outbreaks in their respective countries (n=13). Sweden, Slovenia and Hungary (23) reported SaV outbreaks and sequences from outbreak strains and single or sporadic cases in those countries were included in the analyses, along with all available SaV sequences from GenBank (Table 2). For most outbreaks two sequences were available. The representative strains from the different outbreaks, reference strains from GenBank and strains from this study used in the phylogenetic analysis are presented in Table 2.

TABLE 1. Primers and probe with corresponding concentrations, and label, used for detection and typing of sapoviruses.

	Primer	Primer direction	Sequence 5' -3'	Primer position	Concentration used/ Probe label
SaV detection	SVLCF	FOR	GAYCWGGCYCTCGCCACCT	5074-5092[a]	10 pmol
	SVLCR	REV	GCCCTCCATYTCAAACACTA	5159-5178[a]	10 pmol
	SVLCP	-	TGYACCACCTATRAACCAVG	5101-5118[a]	5 pmol; Texas Red-BHQ2[b] (610nm)
SaV capsid typing (35)	SLV5749	FOR	CGGRCYTCAAAVSTACCBCCCCA	5494-5516[a]	10 pmol
	SLV5317	REV	CTCGCCACCTACRAWGCBTGGTT	5083-5105[a]	10 pmol

[a]AY 237422.3 Sapovirus Mc114
[b]BHQ: Black hole quencher

Analysis of evolutionary dynamics of new sapovirus strains.

Evolutionary dynamics of SaV strains were analysed using capsid nucleotide sequences and the time of sampling of these strains in the BEAST program package (2). Evolutionary dynamics were estimated using a Bayesian Markov chain Monte Carlo (MCMC) approach implemented in the BEAST program (BEAST version 1.4.7). BEAST MCMC analysis estimates posterior distributions of the timed evolutionary history distribution, based on the incorporation of sampling times in a molecular clock model, the substitution process and demographic history. The General Time Reversible (GTR) model of substitution, which allows different rates for all possible nucleotide changes and for different base frequencies was used for the analysis. Additionally we allowed for rate variation among all sites in the alignment, and for invariant sites to exist in the alignment. The uncorrelated lognormal relaxed clock model was used, meaning that different branches can evolve at different rates. The resulting samples of trees were summarized using Tree-Annotator (distributed in the BEAST program package), into Maximum Clade Credibility trees, which are scaled to a time-scale shown on the X-axis. The tips are aligned to detection dates of the samples, whereas the node heights represent genetic distances.

RESULTS

Etiology of outbreaks November 1st 2007 – January 1st 2009.

Of the 478 outbreaks sampled between November 2007 and January 2006, 74.6% were caused by noroviruses and 4.4% by rotaviruses, consistent with findings for previous seasons (Figure 1)(29). No adenovirus or astrovirus outbreaks were identified in outbreaks during this period. Surprisingly, however, SaVs were detected as the cause of 19 outbreaks, which corresponds to 4.0 % of all GE outbreaks reported in this study period (Figure 2). Additionally, 14 SaV positive samples were detected in individual patients from other outbreaks, including outbreaks attributed to other viruses. In total, 61 SaV positive samples were obtained in the Netherlands (Figure 2).

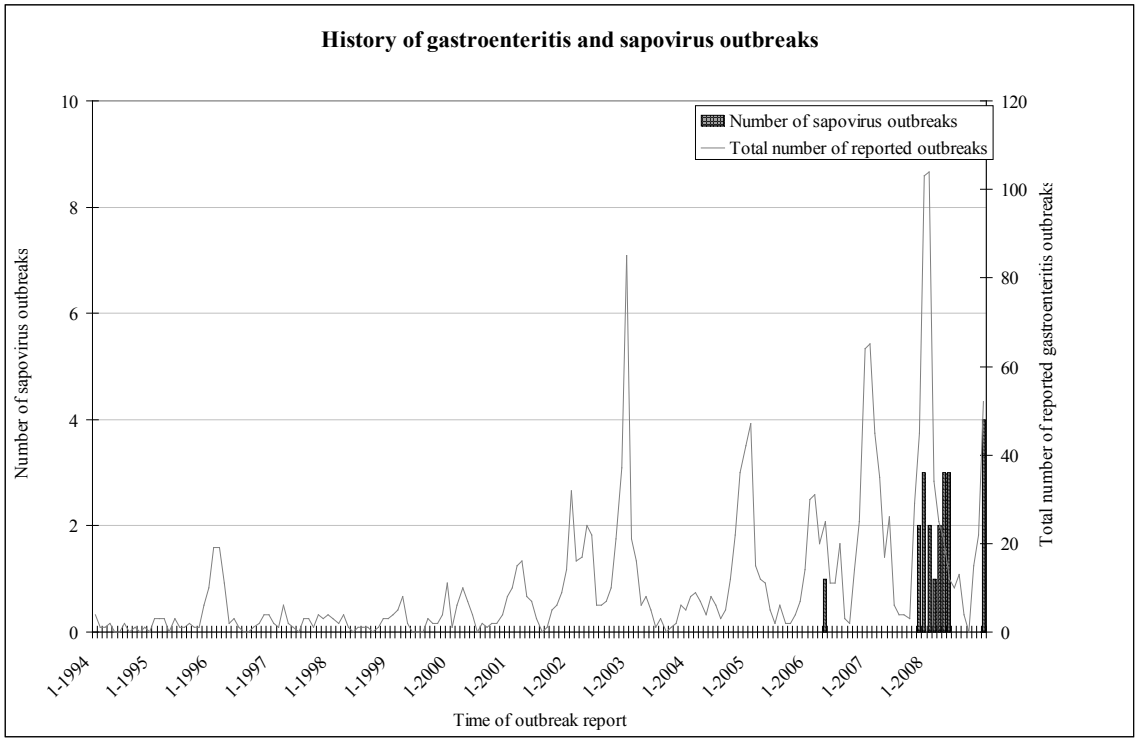


FIGURE 1. History of all reported gastroenteritis outbreaks and sapovirus outbreaks in the Netherlands.

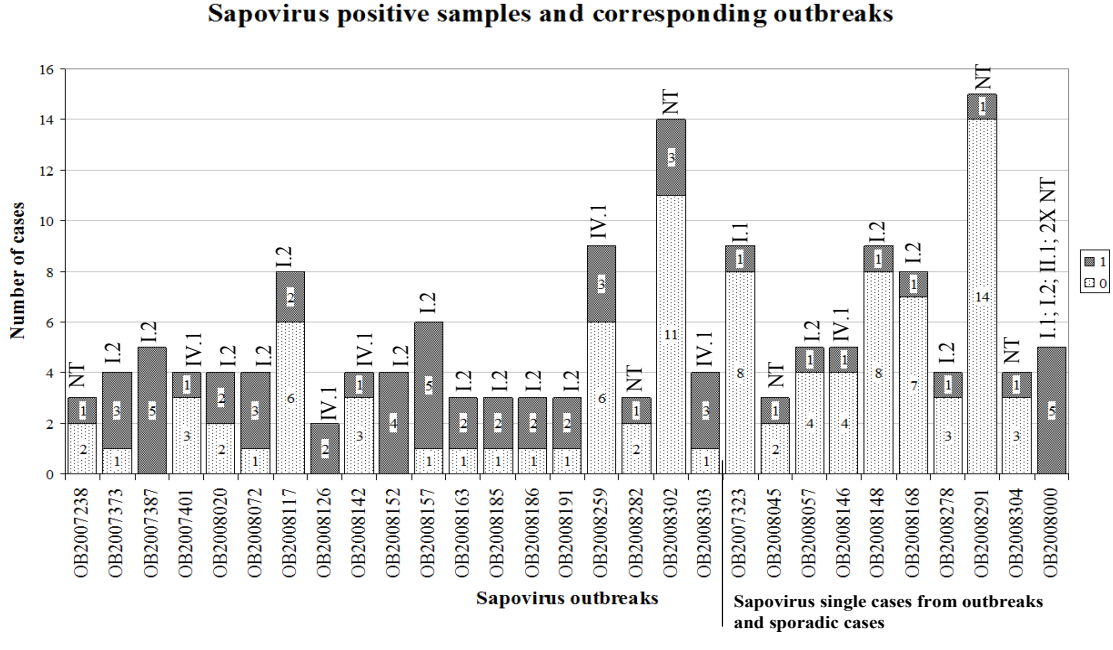


FIGURE 2. Number of sapovirus patient cases and corresponding outbreaks (OB numbers). Sapovirus genotypes which caused the infections are indicated above the bars. The bars from OB2007323 to OB2008168 indicate the single sapovirus infections in outbreaks, while bar OB2008000 indicates the sporadic cases.

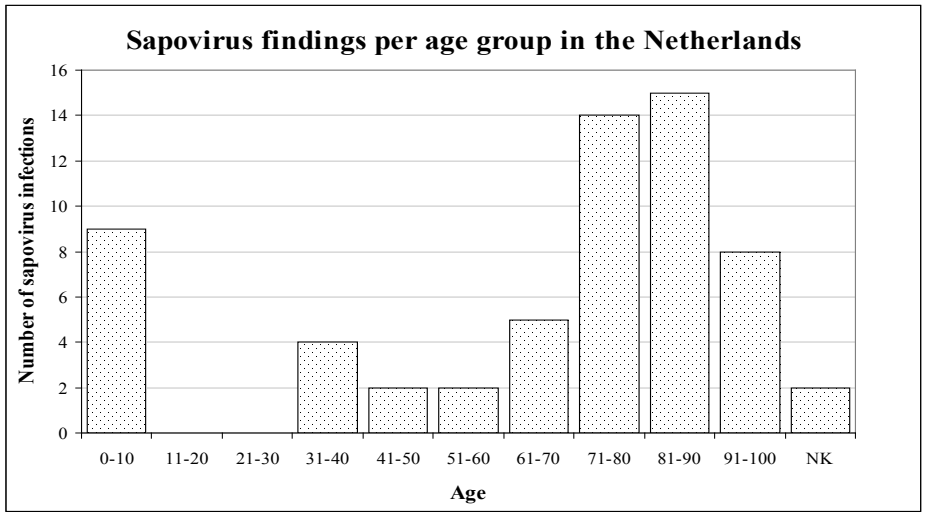


FIGURE 3. Age distribution of total number of sapovirus infections (cases involved in outbreaks and sporadic cases) in the Netherlands.
NK = Not known

TABLE 2. Summary of sequences used for comparison analysis

Sequence source	Capsid sequences used for Figure 4
The Netherlands	Old strains: SaV-1999-0601, SaV-1999-1795, SaV-1999-1841, SaV-1999-2360, SaV-1999-2686, SaV-1999-2672, SaV-1999-2786, SaV-1999-3466, SaV-1999-3632, SaV-2005-045 (sequences 228-235), SaV-2005-045-227, SaV-2005-009-035, SaV-2006-087-591 New strains: SaV-2008-000-439, SaV-2008-000-1406, SaV-2008-000-1438, SaV-2008-000-2015, SaV-2008-57-0507, SaV-2008-142-1441, SaV-2008-146-1471, SaV-2008-148-1543, SaV-2008-168-1624, SaV-2008-259-2025, SaV-2008-278-2129, SaV-2008-303-2314, SaV-2008-323-2388, SaV-2007-401-2478, SaV-2009-39-0344, SaV-2009-39-0590, SaV-2009-045-0385, SaV-2009-GEOPS SaV-2008-20 (consensus of two sequences 93, and 94), SaV-2008-72 (consensus of two sequences 723, and 724), SaV-2008-117 (consensus of two sequences 1222, and 1224), SaV-2008-126 (consensus of two sequences 1318, and 1319), SaV-2008-152 (consensus of four sequences 1503, 1504, 1505 and 1506), SaV-2008-157 (consensus of five sequences 1524, 1525, 1541, 1549, and 1550), SaV-2008-163 (consensus of two sequences 1583, and 1584), SaV-2008-185 (consensus of two sequences 1678, and 1679), SaV-2008-186 (consensus of two sequences 1672, and 1673), SaV-2008-191 (consensus of two sequences 1706, and 1707), SaV-2008-373 (consensus of three sequences 2238, 2343 and 2344), SaV-2008-387 (consensus of three sequences 2350, 2351, and 2474)
Sweden	Sweden1_08 (consensus of sequences 937_08, 939_08, 328_08, 329_08, 5896_08, 9175_08 from Sweden), Sweden2_08 (consensus of sequences 11881_08, 11640_08, 12986_08, 1529_08, 12031_08, 1530_08, 1529_08 from Sweden), Sweden3_08 (consensus of sequences 21414_08, 16216_08, 20468_08, 6499_08, 6673_08 from Sweden), Sweden4_08 (consensus of sequences 8211_08, 11535_08 from Sweden), Sweden5_08 (consensus of sequences 21413_08, 21717_08, 20656_08, 21091_08, 21476_08 from Sweden), Sweden6_08 (consensus of sequences 7580_08, 4878_08 from Sweden), Sweden19405_08, Sweden1645_08, Sweden12911_08, Sweden_1999-126, Sweden_1999-1422, Sweden_1999-1514
Slovenia	3951/07, 3791/07, 2615/07, 2124/07, 2276/07, 198/07, 2496/05, 1797/03, 2152/06
Hungary	
China	
Reference strains	U65427, U73124, AF194182, AF435814, AY289803, AY289804, AF435812, U95645, AF182760
GenBank	EU872293.1, EU872291, FJ214045, AB258427 (sequences EU86692, EU86691, EU86694, EU86693, EU86696, EU86695, EU124657, and EU022024 are identical to this sequence), FJ214046.1, FJ214043.1 (sequences FJ214042.1, FJ214056.1, FJ214055.1, FJ214054.1, FJ214053.1, and FJ214050.1 are identical to this sequence), EF600794.1, U73124, AJ606696.1, AF435813.1, AF194182U65427, AY646854.1, AF435814, AB1262498.1, DQ104357.1, AY894688.1 (sequences DQ125333.1, and DQ125334.1 are identical to this sequence), AJ786349.2 (sequences AB236378.1, AB046353.1, AJ412805.1, and AB236379.1 are identical to this sequence), DQ058829.1, AJ606697.1, DQ366346.1, AM049952.1, EF600796.1, AB305049.1, DQ155647, AB433785.1, AB327280.1, AB327282.1 (sequences AB327281, and AB327279.1 are identical to this sequence), AY289803, AJ606699.1, DQ915091.1, DQ845784.1, AJ606690.1, AJ412816.1, AM049951.1, AY289804, AF435812, U95645, AJ786351.1, AB181130.1, AY425671.1, AF182760

Outbreaks of sapoviruses in the Netherlands; epidemiology.
The most common setting of the 19 sapovirus-associated outbreaks was nursing homes (63%, 12), followed by hospitals (26%, 5) and child day-care centres (11%, 2), which is not significantly different from the distribution of settings for all GE outbreaks of suspected viral etiology during this period. The age of patients infected with SaVs ranged from under one year of age up to a 100 years old (Figure 3). Most patients were older than 60 years (69%, 42 of 61 cases) but this probably reflects the bias in reporting and sampling since 69% of all cases reported in this period were adults older than 60 years (Figure 3). Furthermore reporting of GE illness in nursing homes and healthcare settings is mandatory.
Geographically, most outbreaks were reported from the middle and south of the Netherlands (n =16, 84%). Sapovirus-associated outbreaks occurred in 7 of 12 provinces (17 different cities), with 5 (26.3%) of 19 outbreaks occurring in province Noord-Brabant, followed by Utrecht with 4 outbreaks (21.1%). Sapovirus-associated outbreaks occurred in different cities (and provinces) around the same time. SaV cases were identified between November 2007 and May 2008 (Figure 1), and between November 2008 and April 2009.

Genetic characterization of sapovirus strains from outbreaks and sporadic cases.
All positive samples were characterized by sequence analysis. Genotype I.2 SaVs were detected in 11 (57.9%) of 19 outbreaks (one was in a hospital, two in child day care centers, and eight in nursing homes). Five outbreaks (26.3%) caused by SaV genotype IV.1 strains involved adults only, two outbreaks occurred in hospitals and three in nursing homes. Three outbreaks could not be typed. Sporadic SaV infections were caused by four different genotypes, I.1, I.2, II.1, and IV.1 where genotype I.2 was responsible for 36% of sporadic cases.

Genetic analysis.
Comparison of partial capsid sequences, performed for genotypes I.2 and IV.1 sequences, showed that the genotype I.2 strains were highly similar (>99% nucleotide similarity over 312 nucleotides), whereas some diversity was observed for genotype IV.1 sequences (Figure 4 and Table 2). A full comparison between capsid sequences was performed using sequences obtained from other countries that also noticed SaV outbreaks, including Sweden, Slovenia, and Hungary, and all SaV sequences available from GenBank. This analysis showed high homology between genotype I.2 strains detected in different geographical regions (Sweden, Slovenia, Taiwan, Japan, and Russia) since 2007. Older strains detected in the Netherlands and Sweden were distinct, with the exception of one strain identified in 2004 in Thailand. For genotype IV.1 recent strain sequences from the Netherlands and Sweden, Japan and Hong Kong clustered together but more divergence was observed (97 % nucleotide similarity over 312 nucleotides) (Figure 4 and Table 2). Sequence analysis of polymerase gene segments from Hungary, Slovenia, China and the Netherlands for genotype I.2 strains confirmed the tight clustering.

Analysis of evolutionary dynamics of new sapovirus strains.
The evolutionary dynamics of partial capsid sequences of these two SaV genotypes were estimated and showed distinct phylogenies. The genotype IV.1 viruses diverged from a common node dating back to 1998 (Figure 5b), whereas for genotype I.2 the pattern suggested evolution of the dominant strain from an ancestor identified in 2002 (Figure 5a).

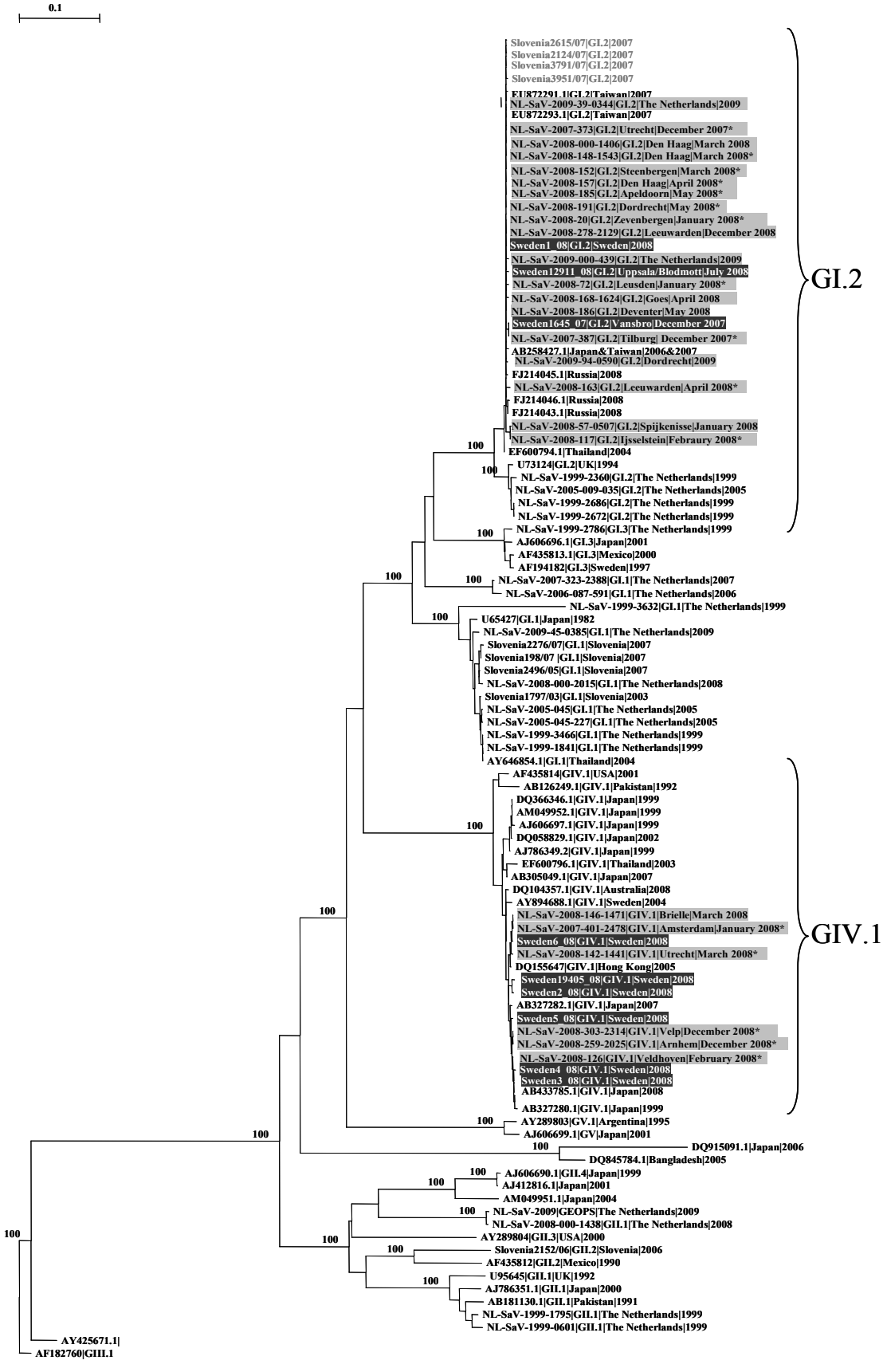
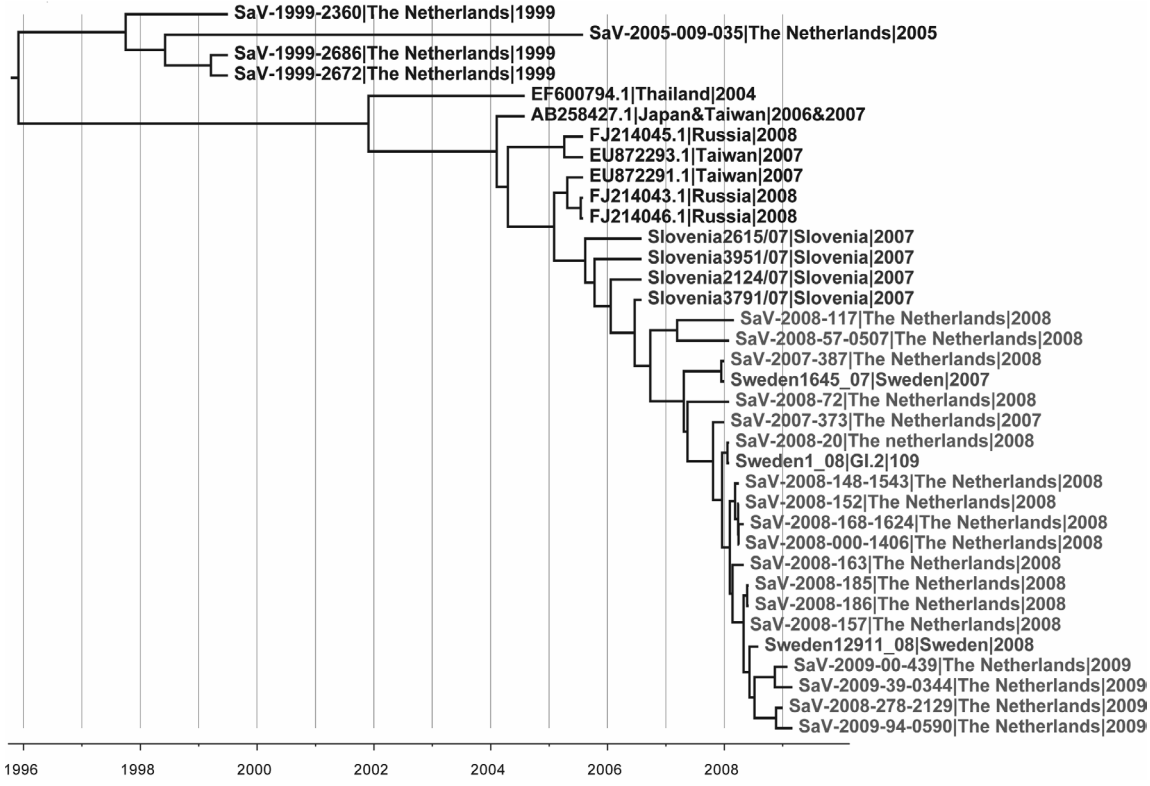
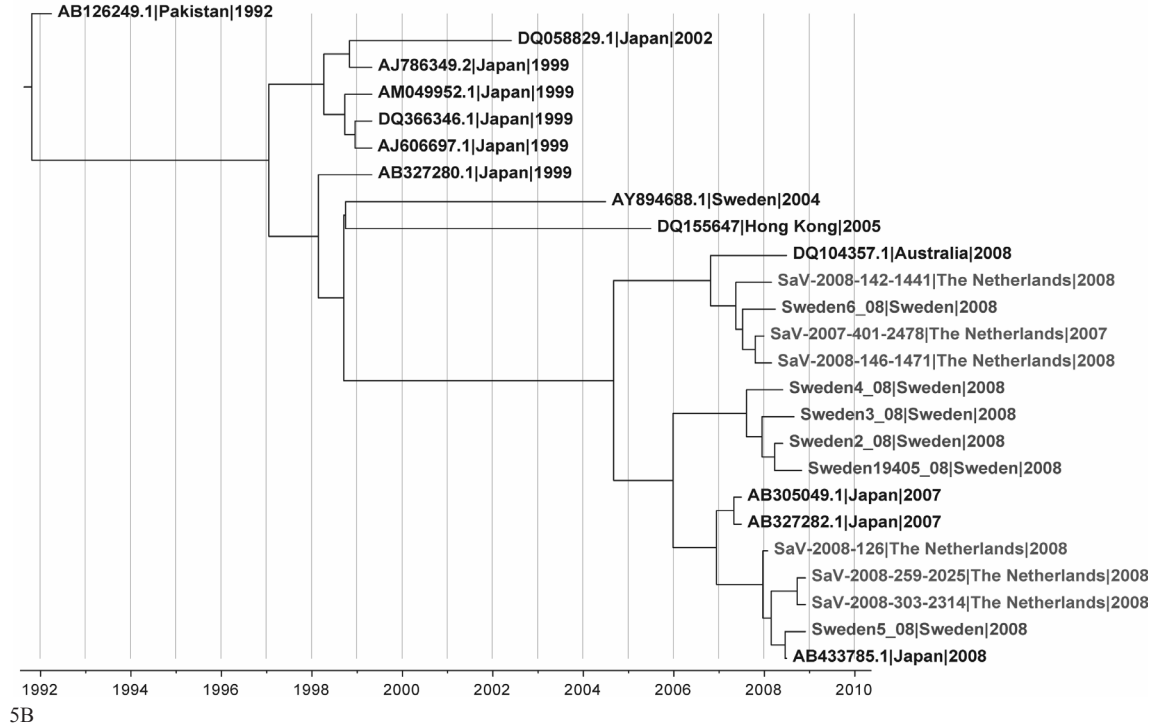

0.1
Slovenia2615/07|GI.2|2007
Slovenia2124/07|GI.2|2007
Slovenia3791/07|GI.2|2007
Slovenia3951/07|GI.2|2007
EU872291.1|GI.2|Taiwan|2007
NL-SaV-2009-39-0344|GI.2|The Netherlands|2009
EU872293.1|GI.2|Taiwan|2007
NL-SaV-2007-373|GI.2|Utrecht|December 2007*
NL-SaV-2008-000-1406|GI.2|Den Haag|March 2008
NL-SaV-2008-148-1543|GI.2|Den Haag|March 2008*
NL-SaV-2008-152|GI.2|Steenbergen|March 2008*
NL-SaV-2008-157|GI.2|Den Haag|April 2008*
NL-SaV-2008-185|GI.2|Apeldoorn|May 2008*
NL-SaV-2008-191|GI.2|Dordrecht|May 2008*
NL-SaV-2008-20|GI.2|Zevenbergen|January 2008*
NL-SaV-2008-278-2129|GI.2|Leeuwarden|December 2008
Sweden1_08|GI.2|Sweden|2008
NL-SaV-2009-000-439|GI.2|The Netherlands|2009
Sweden12911_08|GI.2|Uppsala/Blodmott|July 2008
NL-SaV-2008-72|GI.2|Leusden|January 2008*
NL-SaV-2008-168-1624|GI.2|Goes|April 2008
NL-SaV-2008-186|GI.2|Deventer|May 2008
Sweden1645_07|GI.2|Vansbro|December 2007
NL-SaV-2007-387|GI.2|Tilburg| December 2007*
AB258427.1|Japan&Taiwan|2006&2007
NL-SaV-2009-94-0590|GI.2|Dordrecht|2009
FJ214045.1|Russia|2008
NL-SaV-2008-163|GI.2|Leeuwarden|April 2008*
FJ214046.1|Russia|2008
FJ214043.1|Russia|2008
NL-SaV-2008-57-0507|GI.2|Spijkenisse|January 2008
NL-SaV-2008-117|GI.2|Ijsselstein|Febraury 2008*
EF600794.1|Thailand|2004
U73124|GI.2|UK|1994
NL-SaV-1999-2360|GI.2|The Netherlands|1999
NL-SaV-2005-009-035|GI.2|The Netherlands|2005
NL-SaV-1999-2686|GI.2|The Netherlands|1999
NL-SaV-1999-2672|GI.2|The Netherlands|1999
NL-SaV-1999-2786|GI.3|The Netherlands|1999
AJ606696.1|GI.3|Japan|2001
AF435813.1|GI.3|Mexico|2000
AF194182|GI.3|Sweden|1997
NL-SaV-2007-323-2388|GI.1|The Netherlands|2007
NL-SaV-2006-087-591|GI.1|The Netherlands|2006
NL-SaV-1999-3632|GI.1|The Netherlands|1999
U65427|GI.1|Japan|1982
NL-SaV-2009-45-0385|GI.1|The Netherlands|2009
Slovenia2276/07|GI.1|Slovenia|2007
Slovenia198/07 |GI.1|Slovenia|2007
Slovenia2496/05|GI.1|Slovenia|2007
NL-SaV-2008-000-2015|GI.1|The Netherlands|2008
Slovenia1797/03|GI.1|Slovenia|2003
NL-SaV-2005-045|GI.1|The Netherlands|2005
NL-SaV-2005-045-227|GI.1|The Netherlands|2005
NL-SaV-1999-3466|GI.1|The Netherlands|1999
NL-SaV-1999-1841|GI.1|The Netherlands|1999
AY646854.1|GI.1|Thailand|2004
AF435814|GIV.1|USA|2001
AB126249.1|GIV.1|Pakistan|1992
DQ366346.1|GIV.1|Japan|1999
AM049952.1|GIV.1|Japan|1999
AJ606697.1|GIV.1|Japan|1999
DQ058829.1|GIV.1|Japan|2002
AJ786349.2|GIV.1|Japan|1999
EF600796.1|GIV.1|Thailand|2003
AB305049.1|GIV.1|Japan|2007
DQ104357.1|GIV.1|Australia|2008
AY894688.1|GIV.1|Sweden|2004
NL-SaV-2008-146-1471|GIV.1|Brielle|March 2008
NL-SaV-2007-401-2478|GIV.1|Amsterdam|January 2008*
Sweden6_08|GIV.1|Sweden|2008
NL-SaV-2008-142-1441|GIV.1|Utrecht|March 2008*
DQ155647|GIV.1|Hong Kong|2005
Sweden19405_08|GIV.1|Sweden|2008
Sweden2_08|GIV.1|Sweden|2008
AB327282.1|GIV.1|Japan|2007
Sweden5_08|GIV.1|Sweden|2008
NL-SaV-2008-303-2314|GIV.1|Velp|December 2008*
NL-SaV-2008-259-2025|GIV.1|Arnhem|December 2008*
NL-SaV-2008-126|GIV.1|Veldhoven|February 2008*
Sweden4_08|GIV.1|Sweden|2008
Sweden3_08|GIV.1|Sweden|2008
AB433785.1|GIV.1|Japan|2008
AB327280.1|GIV.1|Japan|1999
AY289803|GV.1|Argentina|1995
AJ606699.1|GV|Japan|2001
DQ915091.1|Japan|2006
DQ845784.1|Bangladesh|2005
AJ606690.1|GII.4|Japan|1999
AJ412816.1|Japan|2001
AM049951.1|Japan|2004
NL-SaV-2009|GEOPS|The Netherlands|2009
NL-SaV-2008-000-1438|GII.1|The Netherlands|2008
AY289804|GII.3|USA|2000
Slovenia2152/06|GII.2|Slovenia|2006
AF435812|GII.2|Mexico|1990
U95645|GII.1|UK|1992
AJ786351.1|GII.1|Japan|2000
AB181130.1|GII.1|Pakistan|1991
NL-SaV-1999-1795|GII.1|The Netherlands|1999
NL-SaV-1999-0601|GII.1|The Netherlands|1999
AY425671.1|
AF182760|GIII.1
GI.2
GIV.1
100

FIGURE 4. Comparison analysis of SaV partial capsid nucleotide sequences using neighbor joining tree and showing different genogroups and clusters. The numbers of each branch indicate bootstrap values for the genotypes and isolates. Strain names of new sapovirus strains isolated in the Netherlands are in a light grey squre, black letters, and sapovirus outbreaks from the Netherlands are marked with an asterisk, new strains from Slovenia are in gray letters, and Sweden in are in a dark grey squre, white letters. GenBank accession numbers (in black) for the reference strains are as follows: U65427 (Hu/SaV/GI.1/Sapporo/1982/JP), U73124 (Hu/SaV/GI.2/Parkville/1994/US), AF194182 (Hu/SaV/GI.3/Stockholm318/1997/SE), AF435814 (Hu/SaV/GIV.1/Hou7-1181/1990/US), AY289803 (Hu/SaV/GV.1/Argentina39/AR), AY289804 (Hu/SaV/GII.3/Cruise ship/2000/US), AF435812 (Hu/SaV/GII.2/Mex340/1990/MX), U95645 (Hu/SaV/GII.1/London/1992/UK), AF182760 (Sw/SaV/GIII.1/PEC-Cowden/1980/US).



5A



5B

FIGURE 5A and B. Evolutionary dynamics of sapovirus, based on partial capsid nucleotide sequences of GI.2 (A) and GIV.1 (B). The time scale is shown on X-axis, the tips are aligned to detection dates of samples, and node heights represent distances between the isolates.

DISCUSSION

Since the start of the acute viral GE surveillance in the Netherlands in 1994 (29) through 2006 no GE outbreaks were assigned to SaV. However SaV was detected in outbreaks after November 2007. As this observed increase in SaV detection coincided with the introduction of a revised molecular detection method, we initially suspected that it reflected increased sensitivity of the PCR compared to previously used protocols. Therefore, samples from unexplained outbreaks from 2006 through 2007 were retested using the revised new method. The retrospective testing confirmed the absence of SaV in outbreaks that happened prior to November 2007 and, thus, the emergence of SaV infections in the Netherlands is not an artifact of changes in the protocols used. A possible alternative explanation has to do with changes in the referral bias of outbreak samples. Specifically, as norovirus testing is becoming more and more routine in the Netherlands, the selection of patient samples that are received and used for surveillance has been changing. Changes in patient demography would affect our conclusions if the increased incidence of SaV outbreaks was observed in children. However, the data indicated a higher incidence of SaV outbreaks in adults and nursing homes, which always have been included in our surveillance (1, 10, 16). SaV has been reported as a cause of occasional outbreaks in adults, but their prevalence seems to be increasing. Similar findings were 221 reported recently from Sweden (15), Canada (22), Japan (8, 10-13, 24), and Hungary (23). In addition, foodborne outbreaks and several novel recombinant SaV strains have been identified (8, 12, 17, 21, 31).

The increased reporting of SaV outbreaks was concomitant with increased prevalence of SaV genotype I.2 (58% of SaV-associated outbreaks in The Netherlands, 27% in Sweden, 40 % in Slovenia, and 100% in Hungary). SaV strains belonging to genotype I.2 have been causing infections worldwide recently (5, 11, 18,34). Again, this could reflect changes in the sensitivity of the methods used; nevertheless, previously used PCR methods are based on relatively conserved targets within the polymerase region. Therefore, the observed increase of genotype I.2 SaV infections is most likely not a consequence of new detection assays. Furthermore, recent genotype I.2 sequences from all over the world show high similarity, suggesting that there is a common source for the virus, such as contaminated food, as it has been found in other SaV outbreaks (21, 31). The near clonal nature of the outbreak strains belonging to genotype I.2 suggests that this specific lineage of this genotype has expanded more recently from a common ancestor. This was confirmed using the phylodynamic analysis, suggesting that recent strains emerged as drift variants, similar to what has been observed for norovirus genotype II.4 viruses where expansion of a successful variant has been observed for some norovirus genotypes (27). In addition, calicivirus infections are increasing globally (27) and sapovirus genotype I.2 may have the same mechanisms of persistence in humans as norovirus genotype II.4, such as low infectious dose, high infectivity, high level of shedding, high environmental persistence, multiple transmission routes and prolonged shedding after clinical recovery (20). Combined with data from Sweden, Hungary and Slovenia we conclude that SaV infections are increasing in Europe and, based on recent reports, are also emerging worldwide (5, 10, 11, 15, 18, 22, 34). Therefore we recommend inclusion of sapovirus diagnostic assay in the analysis of the outbreaks suspected of viral GE in other laboratories.

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Chapter 6

Clostridium difficile is not associated with outbreaks of viral gastroenteritis in the elderly in the Netherlands

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ABSTRACT

The coincidental increase in norovirus outbreaks and *Clostridium difficile* infection (CDI) raised the question of whether these events could be related, e.g. by enhancing spread by diarrhoeal disease outbreaks. Therefore, we studied the prevalence of *C. difficile* in outbreaks of viral gastroenteritis in nursing homes for the elderly and characterised enzyme immunoassay (EIA)-positive stool samples. Stool samples from nursing home residents ($n = 752$) in 137 outbreaks of viral aetiology were investigated by EIA for the presence of *C. difficile* toxins. Positive samples were further tested by a cell neutralisation cytotoxicity test, a second EIA and culture. Cultured isolates were tested for the presence of toxin genes, the production of toxins and characterised by 16S rRNA polymerase chain reaction (PCR) and sequencing. Twenty-four samples (3.2%) tested positive in the EIA. Of these 24 positive samples, only two were positive by cytotoxicity and three by a second EIA. Bacterial culture of 21 available stool samples yielded a toxinogenic *C. difficile* PCR ribotype 001 in one patient sample only. In conclusion, we found no evidence in this retrospective study for an association between viral gastroenteritis outbreaks and *C. difficile*. The high rate of false-positive EIA samples emphasises the need for second confirmation tests to diagnose CDI.

INTRODUCTION

Outbreaks of acute gastroenteritis (AGE) of suspected viral aetiology are reported to the National Institute for Public Health and the Environment (RIVM) as part of the ongoing viral gastroenteritis outbreak surveillance system. Although these outbreaks are selected based on criteria thought to be highly specific for viral gastroenteritis outbreaks, and despite extensive testing, the cause of approximately 12% of outbreaks of AGE remains unknown [1]. Of the outbreaks in which a viral pathogen was detected, the vast majority is associated with noroviruses (NoV).

Since 2002, the epidemiology of NoV appears to have changed, with the introduction of a novel lineage of the dominant genogroup (G) II.4 viruses. This introduction coincided with increased levels of reporting of NoV outbreaks across Europe and a noticeable peak in mortality in the elderly, which coincides with the NoV seasonal peak [2]. Simultaneously, Canada, the USA and, later, the United Kingdom (UK), the Netherlands, Belgium, France and Austria reported the emergence of *Clostridium difficile* ribotype 027, which appeared to have increased virulence compared to other *C. difficile* strains [3, 4]. In the Netherlands, this ribotype was detected in 26 hospitals and ten nursing homes by May 2008 [5, 6]. Antibiotic use has been associated with an increased risk of infection with *C. difficile* and is responsible for 15 to 25% of all cases of antibiotic-associated diarrhoea in hospitals [7]. Outbreaks and cases outside hospitals are less frequently recognised but have been described, sometimes without the typical risk factors [8, 9]. *C. difficile* outbreaks and cases in nursing homes have been studied less systematically and, although *C. difficile* type 027 has been found in at least ten nursing homes in the Netherlands, detailed information about these infections is absent [6].

The coincidental increase in norovirus GII.4 outbreaks and the spread of *C. difficile* ribotype 027 raised the question of whether these events could be related [2, 10]. In theory, mixed infections of NoV and *C. difficile* ribotype 027 could increase the severity of the disease. Furthermore, norovirus-induced episodes of vomiting and diarrhoea might trigger or exacerbate *C. difficile* diarrhoea, resulting in increased spread of *C. difficile* due to multiple episodes of vomiting and diarrhoea, as has been suggested in some recent publications [11–13]. Therefore, we decided to investigate a possible association of norovirus and rotavirus outbreaks with *C. difficile* infections (CDIs), particularly with respect to *C. difficile* polymerase chain reaction (PCR) ribotype 027.

MATERIAL AND METHODS

Sample collection.

In total, we tested 752 faecal samples from 137 outbreaks of gastroenteritis (number of stool samples varying from 2 to 25 per outbreak). All outbreaks occurred in 2006 in nursing homes and were selected because the *C. difficile* ribotype 027 problem emerged in that period in the Netherlands and these samples provided a snapshot of what is present within the elderly population. Viral detection has been performed as described previously by Svraka et al. [1]. The outbreaks were first tested using the assays for NoV. Then, all outbreaks that remained negative were further tested using the assays for rotaviruses, adenoviruses, astroviruses and sapoviruses [1]. Of these, 109 were confirmed norovirus outbreaks (644 stool samples), 14 outbreaks were confirmed rotavirus outbreaks (70 samples) and 14 outbreaks (38 samples) were of unexplained aetiology, reflecting the typical aetiology of such outbreaks. The outbreaks were reported throughout the year, but with a peak in winter, as part of our ongoing surveillance for viral gastroenteritis outbreaks, which explains the high number of NoV outbreaks in this study [1]. Undiluted stool samples had been stored at 4°C, as recommended for viral gastroenteritis diarrhoea, before testing for the presence of *C. difficile*.

Diagnostic assays used for the screening of samples.

The screening of 752 outbreak specimens for the presence of *C. difficile* was done using the PTAB enzyme immunoassay (EIA, Premier *C. difficile* Toxin A&B, Meridian Diagnostics, Inc., Cincinnati, OH, USA) on all stool samples. Assay was performed as described by the manufacturer. The stool samples had been stored at 4°C for periods longer than 3–4 days, which is in accordance with recommendations for the

diagnostics of viral gastroenteritis, but not in accordance to the manufacturer's recommendations, before testing for the presence of *C. difficile*. Consequently, we validated the PTAB EIA assay for use in samples that were stored longer at 4°C by the parallel testing of 96 stool samples that were aliquoted directly after receipt and stored for five months at −20 and 4°C.

The PTAB EIA was interpreted visually (within 15 min) and by spectrophotometer (within 30 min), as described by the manufacturer; 376 samples were read both visually and on a Labsystems Multiskan RC spectrophotometer plate reader (Thermo Fisher Scientific, Breda, the Netherlands) using dual wavelengths of 450 and 450/630 nm. The samples were described as positive if optical density (OD) values of 0.100 or more were measured at a wavelength of 450/630 nm and/or 0.150 or more at a wavelength of 450 nm, as described by the manufacturer. There was 100% concordance between reading visually and by using the spectrophotometer plate reader. The remaining 376 samples were read visually only.

Confirmatory testing on PTAB-positive samples

Stool samples found positive using the PTAB EIA assay were cultured and tested using a neutralisation cytotoxicity assay and the automated immunoanalyzer VIDAS® *C. difficile* A & B (bioMérieux) [14].

For culture, stool samples found positive in the PTAB EIA assay were treated with absolute ethanol (96%) prior to inoculation onto Columbia agar containing colistin and nalidixic acid, *C. difficile*-selective agar with cefoxitin, amphotericin B and cycloserine (CLO; bioMérieux) and blood agar. CLO medium was also used to inoculate faecal samples without pre-treatment with ethanol. Inoculated faecal samples were incubated in an anaerobic environment at 37°C for 2 days.

Clostridium-suspected colonies were tested further using PCR assays for *C. difficile*-specific GluD targeting the GDH gene [15], *C. difficile* ribotyping [16, 17], enterotoxin A (tcdA) [5], cytotoxin B (tcdB) [18] and 16S rRNA PCR [19]. For this, DNA was isolated from suspected colonies by a QIAamp DNA isolation column (Qiagen, Holden, Germany) according to the manufacturer's recommendations, including 10 min incubation at 55°C with proteinase K. The final volume of the DNA extracts was 200 µL.

The neutralisation cytotoxicity assay was performed using Vero cells in a 24-well format. Faecal samples were diluted in 1:4 Eagle's minimum essential medium (EMEM) containing 5% foetal bovine serum and centrifuged for 10 min at 3,000 rpm. The supernatant was filtered through a 0.45-µm-pore filter, 150 µL supernatant was mixed with 150 µl EMEM and neutralisation of the cytotoxic effect was performed using 150 µL faecal supernatant and 150 µl of 1:25 dilution of specific *C. difficile* antitoxin (Techlab, Blacksburg, VA, USA). These two mixtures were incubated for 30 min at room temperature; subsequently, 200 µl of each mix was inoculated on Vero cells. The cytotoxic effect was evaluated daily for 3 days microscopically.

Stool samples positive in the PTAB EIA were also tested using the VIDAS assay. Stool samples were centrifuged for 10 min at maximum speed (14,000 rpm) and 300 µl of supernatant was used, according to the manufacturer's instructions. Samples with OD value lower than 0.13 were described as negative, OD values between 0.13 and 0.37 were equivocal and OD values higher than 0.37 were described as positive.

Bacterial isolates cultured from EIA-positive faeces samples were also investigated for the production of tcdA and tcdB by VIDAS and EIA. Bacteria were cultured in liquid brain heart infusion (BHI) bouillon medium for 4 days. Subsequently, the BHI cultures were centrifuged at 3,000 rpm for 10 min and the supernatant was further investigated. The same cut-off values of EIA and VIDAS were used for in vitro bacterial toxin production as for toxin detection in faeces samples.

Evaluation of the approach of *Clostridium* detection.

Optimal sample storage conditions differ for viral testing and bacterial testing, and this raised the question whether *Clostridium* EIA test results could be explained by suboptimal use of the assay. Therefore, we validated this approach by testing original total nucleic acids isolated from 154 (20% of all samples tested in the EIA) faecal samples within 3 days of arrival from the stool samples which were included in our study. These extracts had been stored at −80°C. We used a real-time PCR for the tcdB gene, as described by van den Berg et al. [14, 20].

RESULTS

Testing of stool samples using the PTAB EIA.
For the validation of the use of the PTAB EIA on our sample set, we used 96 stool samples that were stored for 5 months at −20 and at 4°C. Of the samples stored at −20°C, two were positive, and of the samples stored at 4°C, three were positive, including the ones that were positive after storage at −20°C.
In total, 24 (3.2%) of 752 stool samples from outbreaks of acute gastroenteritis in nursing homes in 2006 tested positive in the PTAB EIA assay (Table 1). Of the 24 PTAB EIA-positive stool samples, 22 were from 17 norovirus outbreaks, one was from a rotavirus outbreak and the other one was from an outbreak of unexplained aetiology.

Confirmatory testing of PTAB-positive stool samples.
Specialised stool culture for *Clostridium* spp. (Table 1) was performed on 24 stool samples. For three stool samples, culture was negative, and in 21 stool samples, *Clostridium*-suspected colonies were found. Subsequently, neutralisation cytotoxicity assay and VIDAS assay were performed on 21 stool specimens of which sufficient amounts of material were available. This yielded two positive samples both by neutralisation cytotoxicity assay and VIDAS, and an additional weakly positive result using the VIDAS assay (Table 1).

Analysis of bacterial isolates from the stool samples.
From the 21 *Clostridium*-suspected colonies, which were grown on the CLO medium plates, DNA was extracted and analysed by 16S DNA sequencing and further using a range of assays for the characterisation of toxin-producing *C. difficile* (Table 1). 16S DNA sequencing revealed *Lactobacilli* in six stool samples (37.5%) and different *Clostridium* species in 15 samples (62.5%): *C. difficile*, *C. disporicum*, *C. perfringens*, *C. sordellii*, *C. boltei*, *C. butyricum*, *C. baratii*, *C. subterminale* and *C. bifermentans* (Table 1). Using the GluD, ribotyping PCR, tcdA and tcdB assays, one sample was positive and identified as *C. difficile*. One isolate of *C. disporicum* was weakly positive for tcdA and one isolate of *C. subterminale* for tcdB.
One *Clostridium* species from a faecal sample (13/8, Table 1) tested positive by EIA, cytotoxicity and VIDAS was identified as *C. sordellii*. This isolate was negative for tcdA and tcdB and did not produce a cytotoxin.
The results of the in vitro bacterial toxin production measured by cytotoxicity, PTAB and VIDAS revealed only strongly positive results for one sample that was identified as *C. difficile*. Using cut-off values of PTAB and VIDAS as applied on stool samples, (weakly) positive reactions were obtained for 14 samples using PTAB and six samples using the VIDAS assay. However, none of these isolates produced cytotoxins.

Epidemiological data on *C. difficile*- and *C. sordellii*-positive samples.
Only one stool sample and the corresponding bacterial isolate were positive in all assays and proven to be *C. difficile* type 001 (23/18, Table 1). This sample was from an outbreak of unexplained aetiology that occurred in November 2006, with eight cases being reported. All eight samples were tested using the PTAB EIA; however, only one was positive and tested further in other assays.
The *C. sordellii*-positive stool sample (13/8, Table 1) was from a norovirus outbreak that affected four persons from a residential institution in an endemic region for *C. difficile* in November 2006. The faeces samples of the three other patients were tested for CDI, but were negative.

Evaluation of the approach of *Clostridium* detection.
Of the 154 total nucleic acids that had been isolated from stool samples within 3 days of their arrival, the two samples that were cytotoxic and VIDAS-positive were positive by tcdB real-time PCR assay (Table 1 samples 13/8 and 23/18). All other samples were negative.

TABLE 1. Results on stool samples and characterisation of the isolates from the stool samples. These results are obtained for stool samples positive in the PTAB EIA and tested further using neutralisation cytotoxicity, VIDAS and culture assays. OD values of the VIDAS assay are given in the brackets. Results on the isolates cultured from the stool samples were by polymerase chain reaction (PCR) assays for GluD, ribotyping, tcdA, tcdB, and 16S rRNA and sequencing. Additionally, grown cultures were tested using neutralisation cytotoxicity assay, PTAB EIA and VIDAS. OD values of VIDAS and PTAB EIA (measured at 450 nm) are given in the brackets.

PTAB-positive samples/ outbreak number	Viral diagnosis	Cytotoxicity assay	VIDAS	Culture	16S rRNA PCR and sequencing	GluD and ribotyping PCR	tcdA PCR	tcdB PCR	Assays on cultured bacteria		
									Cytotoxicity assay	PTAB EIA	VIDAS
1/1	Norovirus (II.4 2006b)	−	− [0.02]	+	Lactobacillus casei	−	−	−	−	− [0.121]	− [0.03]
2/2	Norovirus (II.4 2004)	ND	ND	+	Clostridium disporicum	−	−	−	−	+ [0.324]	+ [0.44]
3/2	Norovirus (II.4 2004)	−	− [0.02]	+	Clostridium perfringens / Clostridium disporicum	−	−	−	−	+ [0.470]	+ [1.12]
4/2	Norovirus (II.4 2004)	−	− [0.03]	+	Clostridium disporicum	−	+	−	−	+ [0.427]	+ [0.62]
5/2	Norovirus (II.4 2004)	−	− [0.10]	+	Clostridium disporicum	−	−	−	−	+ [0.342]	+ [0.61]
6/2	Norovirus (II.4 2004)	−	− [0.03]	+	Clostridium subterminale	−	−	+	−	+ [0.287]	− [0.02]
7/2	Norovirus (II.4 2004)	−	− [0.01]	−	ND	ND	ND	ND	ND	ND	ND
8/3	Norovirus (II.4 2004)	ND	ND	+	Clostridium boltei	−	−	−	−	+ [0.440]	+ [0.48]
9/4	Norovirus (II.4 2004)	−	− [0.03]	+	Clostridium butyricum	−	−	−	−	+ [0.179]	− [0.02]
10/5	Norovirus (II.4 2006b)	−	− [0.13]	+	Clostridium bifermentans	−	−	−	−	+ [0.384]	+/− [0.29]
11/6	Norovirus (II.4 2006b)	−	− [0.01]	−	ND	ND	ND	ND	ND	ND	ND
12/7	Norovirus (II.4 2006b)	−	− [0.08]	+	Clostridium disporicum	−	−	−	−	+ [0.208]	− [0.02]
13/8	Norovirus (II.4 2006a)	+	+ [2.32]	+	Clostridium sordellii	−	−	−	−	+ [0.178]	− [0.02]
14/9	Norovirus (II.4 2006a)	−	− [0.04]	+	Clostridium baratii	−	−	−	−	+ [0.190]	− [0.09]
15/10	Norovirus (II.4 2006b)	−	− [0.03]	+	Lactobacillus	−	−	−	−	− [0.069]	− [0.04]
16/11	Norovirus (II.4 2006a)	−	− [0.01]	+	Lactobacillus	−	−	−	−	− [0.100]	− [0.05]
17/12	Norovirus (II.4 2004)	−	− [0.07]	+	Clostridium perfringensfringes	−	−	−	−	+ [0.165]	− [0.04]
18/13	Norovirus (I.2)	−	− [0.01]	+	Lactobacillus paracasei	−	−	−	−	− [0.120]	− [0.02]
19/14	Norovirus (II.4 2006a)	−	− [0.01]	+	Clostridium disporicum	−	−	−	−	+ [0.169]	− [0.05]
20/15	Norovirus (II.4 2004)	−	+ [0.55]	+	Lactobacillus paracasei	−	−	−	−	− [0.125]	− [0.04]
21/16	Norovirus (II.4 2006a)	−	− [0.01]	−	ND	ND	ND	ND	ND	ND	ND
22/17	Norovirus (II.4 2006a)	−	− [0.04]	+	Lactobacillus casei	−	−	−	−	− [0.069]	− [0.03]
23/18	Unexplained aetiology	+	+ [6.49]	+	Clostridium difficile type 001	+	+	+	+	+ [3.137]	+ [4.24]
24/19	Rotavirus	ND	ND	+	Clostridium disporicum	−	−	−	−	+ [0.196]	− [0.07]

−: negative; +: positive; ND: not done; OD: optical density; GluD: glutamate dehydrogenase gene; tcdA: enterotoxin A gene; tcdB: cytotoxin B gene
VIDAS cut-off OD values: negative <0.13, equivocal ≥ 0.13 to <0.37, positive ≥ 0.37
PTAB EIA: Premier *C. difficile* Toxin A&B enzyme immunoassay ; PTAB cut-off OD values: negative < 0.150, positive >0.150

DISCUSSION

In this study, we investigated whether the emergence of a successful norovirus strain [10] could coincide with the spread of *C. difficile* and whether the emergence of *C. difficile* could explain some unresolved outbreaks of acute gastroenteritis in nursing homes, as has been postulated. This was initiated by publications suggesting that such an association may exist [11–13, 21]. We did not find any evidence for such an association in our retrospective study. *C. difficile* PCR ribotype 001 was found in a single patient in one gastroenteritis outbreak of unexplained aetiology in a nursing home. This type is one of the most frequently circulating and detected types in the Netherlands.

However, our study was performed on outbreaks in nursing homes, while other studies describe hospital outbreaks, where CDIs are more common [3–5]. Wilcox and Fawley have shown that CDI rates are higher in closed hospital units which are affected by viral gastroenteritis than in open units where no viral gastroenteritis was detected [11]. Their results were not confirmed using *C. difficile*-specific assays, and our data suggest that false-positivity may explain previously noted associations between norovirus and *Clostridium*. We were unable to confirm the presence of *C. difficile* in all but one PTAB EIA-positive stool. This is not explained by their inability to culture, because other *Clostridium* species were isolated from 63.3% of all reactive samples.

Our study does have some limitations: first, stool samples had been sent by regular mail and stored at 4°C with a maximum of 16 months. This is in accordance with recommendations for the diagnostics of viral gastroenteritis, but is unusual for CDI tests. We did, however, find that the sensitivity of the PTAB EIA was not affected by storage of 5 months at 4°C relative to storage at −20°C. Furthermore, the testing of original total nucleic acids isolated from faecal samples within 3 days of arrival yielded the same positives as the combination of cytotoxicity test and VIDAS. Therefore, we conclude that our findings are not due to the degradation of *C. difficile* toxins [22, 23].

In addition, this was a retrospective study and stool samples were selected from patients with a suspected viral gastroenteritis [24]. Criteria for viral gastroenteritis differ from those for *Clostridium* gastroenteritis, since symptoms for CDIs are highly variable, ranging from mild diarrhoea to life-threatening colitis, and including watery diarrhoea [25].

Bignardi et al. noted that, if a large number of stool specimens are submitted for the testing of the prevalence of *C. difficile* during norovirus outbreaks, it is likely that the number of false-positive results will increase [12]. The *Clostridium* species found in this study, such as *C. disporicum*, *C. perfringens*, *C. sordellii*, *C. boltei*, *C. butyricum*, *C. baratii*, *C. subterminale* and *C. bifermentans*, are bacteria that are commonly found in the gut. These bacterial isolates did react weakly positive, using the cut-off values for the faecal material, by *C. difficile* PTAB EIA, confirming the specificity problem on stool samples stored for a long period at 4°C [26–31]. The PTAB EIA has a positive predictive value (PPV) of 87.4% according to the manufacturer's assessment when tested according to the manufacturer's recommendations. A prospective multicentre study using the cytotoxicity assay as a gold standard revealed a PPV value of 50.9% and a specificity of 94.3%, indicating that this assay is not highly specific when used for broader testing [14]. A recently performed meta-analysis by the European Study Group on *Clostridium difficile* (ESGCD) confirmed the PTAB to have an unacceptably low PPV of 50% at a prevalence rate of 5% [32].

A possible explanation for the high rate of false-positive PTAB results could be that suboptimal sample storage influenced the tests with weakly positive results just near to the cut-off values. Unfortunately, we were unable to test this hypothesis by a comparison of OD values of the false-positive samples with OD values of CDI-confirmed samples. Nonetheless, we recommend additional confirmatory testing, preferably a cell neutralisation cytotoxicity assay, which is the reference testing method for detection, or a molecular detection test including typing, specifically when samples are not sent in and stored according to *C. difficile* diagnostic criteria.

In summary, we found no evidence for an association between the spread of norovirus and *C. difficile*. Previous reports that suggested this association may be explained by false-positive PTAB tests.

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Chapter 7

Metagenomic sequencing for virus identification in a public health setting

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ABSTRACT

The use of metagenomics for virus discovery in clinical samples has opened new opportunities for understanding the etiology of unexplained illness. This study explores the potential of this sequence-independent approach in a public health setting, by systematic analysis of samples cultured from patients with unexplained illness through a combination of PCR-based assays and viral metagenomics. In total, 1834 cell culture isolates were collected between 1994 and 2007 through the Enterovirus Surveillance program in the Netherlands. During the 13-year period, a total of seven samples that exhibited reproducible cytopathogenic effects in cell culture tested negative in standard PCR assays for a range of viruses. In order to fill the diagnostic gap, viral metagenomics was applied to these culture supernatants. Viral metagenomics resulted in the rapid identification of viruses in all the samples. The unexplained samples contained BK polyomavirus, Herpes simplex virus, Newcastle disease virus, and the recently discovered Saffold viruses (which dominated the unexplained samples; n = 4). The full genomic sequences of four SAFV genotype 3 (SAFV-3) viruses, which share 88 to 93% nucleotide identity to known SAFV-3 viruses, are reported. Further screening for SAFV in additional cultured unidentified clinical isolates from 2008 and 2009 resulted in identification of another SAFV-positive sample. Although the pathogenicity of the identified viruses has not been established, this study demonstrated that viral metagenomics is a powerful tool that can be integrated into public health monitoring efforts to investigate unidentified viruses in cell cultures from clinical isolates where standard PCR assays fail to detect viruses.

INTRODUCTION

Diagnostic assays for pathogen detection are a critical component of public health monitoring efforts. The detection and identification of viruses in clinical samples relies on a range of traditional and modern techniques (26). Traditionally, electron microscopy and cell culture are used to identify the presence of viral agents. These traditional techniques have now been combined or replaced by molecular or serological assays designed to screen for specific "known" viruses. Subsequently, if no viral pathogens are found, more investigative methods like generic PCR assays that target viral groups are used, rather than species-specific assays (37-39). The combination of these molecular techniques is often successful but fails to produce conclusive results when novel viruses or divergent variants of a known viral family are involved. Moreover, viruses do not have ubiquitously conserved genetic elements such as ribosomal DNA that can be used to amplify and identify all viruses and, thus, there is no universal PCR assay that can target all the viruses in a sample (36). Since new viruses are discovered frequently, there is a need to constantly update PCR assays (1, 2, 43). In view of the limitations of methods that target specific viruses, new approaches are required for the identification of novel or "unsuspected" viruses in public health monitoring schemes. One promising approach for rapid viral identification is the use of viral purification and metagenomic sequencing (viral metagenomics).

In contrast to specific assays that are designed to recover a single virus or a group of closely related viruses, viral metagenomics can identify all the viruses in a sample without *a priori* knowledge of the viral types present (16, 21). It is important to distinguish viral metagenomics, where viruses are purified before sequencing and yield of viral sequences is high (42), from a direct metagenomics approach, where total homogenates are sequenced and viruses only account for a small proportion of the sequences (15). Viral metagenomics has been used to characterize viral communities present in the environment (4, 7, 17, 18), and to describe viruses in mammalian feces (6, 8, 9, 11, 27, 47, 51), cell culture (23, 25), respiratory tract aspirates (2, 48), blood (10, 24), and animal tissues (30, 31).

The present study describes the use of a combination of molecular approaches, including specific and generic PCRs for various viruses as well as metagenomic sequencing to identify viruses from clinical specimens in a public health context. Virological data from 1834 cultured clinical specimens collected between 1994 and 2007 through the Enterovirus Surveillance (EVS) program in the Netherlands are summarized. Metagenomic sequencing led to the identification of viruses in all the samples that exhibited consistent cytopathogenic effects (CPE) and remained unexplained after PCR testing.

MATERIAL AND METHODS

Clinical Specimens.

All the clinical specimens reported in this study were collected through the Enterovirus Surveillance (EVS) program in the Netherlands. The goal of this program is to monitor poliovirus circulation and ensure its eradication from the Netherlands. Although 90% of enterovirus infections are asymptomatic, these infections can result in a broad spectrum of clinical symptoms (50). Therefore, the EVS collects samples from patients with a variety of clinical manifestations such as meningitis, hepatitis, respiratory illness and gastrointestinal disorders. Since virus shedding of both symptomatic and asymptomatic patients occurs through feces, most of the samples collected through the EVS program are stool specimens, which provides information about the general circulation of enteroviruses. The surveillance in its current form was started after a poliovirus type 3 outbreak in 1992-1993 (14, 34). In order to detect polioviruses, all samples were initially tested for a broad range of enteroviruses in routine virological laboratories (32, 33), which are linked to academic hospitals or to regional public health centers throughout the entire country. For enterovirus surveillance, clinical samples were cultured on combinations of cell lines routinely used in the different laboratories including human rhabdomyosarcoma (RD), tertiary monkey kidney (tMK), rhesus monkey kidney cells (LLC-MK2), Vero, HEp-2, human epithelial carcinoma *cell line (HeLa),* human embryonic lung fibroblast *cell line* (Gabi), and various human fibroblast cell lines, and confirmed as enteroviruses by local laboratories. Cell culture samples that exhibited cytopathogenic effects (CPE) but tested negative for enteroviruses or were not genetically typed were submitted to the National Institute for

Public Health and Environment, Bilthoven, The Netherlands (RIVM) for virus identification and typing by PCR. Between 1994 and 2007, a total of 1834 cell culture isolates collected throughout the Netherlands were submitted to RIVM for analysis.

Systematic analysis of cell culture isolates from EVS samples at RIVM.
Cell culture isolates submitted to RIVM were passaged once, and subsequently tested using PCR assays for enteroviruses (32, 33), followed by parechoviruses (44). Samples that remained negative for enteroviruses and parechoviruses were screened using a broad range of PCR assays for noroviruses, rotaviruses A, B and C, adenoviruses, astroviruses, sapoviruses, vesiviruses, reoviruses, generic PCR that detects both enteroviruses and rhinoviruses, hepatitis A and E viruses, Influenza A and B viruses, Aichi Virus, Coronaviruses 229E, NL63, and OC43, human respiratory syncytial viruses A and B, and human metapneumovirus (hMPV) as described previously (37-39, 44, 46). Samples that tested negative in all assays but showed consistent CPE were processed for metagenomic sequencing (Table 1 and 2). Information regarding sample description for these unexplained cell cultures and associated patient clinical symptoms are given in Table 2.

TABLE 1. Total number of isolates obtained through the EVS program from 1994 through 2007 and results from the PCR-based virological analysis (44, 45).

PCR assays	Total number of samples 1994-2007 (%)
Enterovirus	1513 (82.5)
Parechovirus	189 (10.3)
Rhinovirus	9 (0.5)
Adenovirus	14 (0.8)
Reovirus	9 (0.5)
Astrovirus	1 (0.1)
No CPE	92 (5.0)
No virus identified (consistent CPE)	7 (0.4)
Total number of samples	1834 (100)

Virus particle purification and metagenomic sequencing.
Virus particles were purified from selected cell cultures prior to metagenomic sequencing. For this purpose cell culture samples were vortexed vigorously for two minutes. A one milliliter aliquot was collected and mixed with 1 ml of SM Buffer (0.1 M NaCl, 50 mM Tris-HCl [pH 7.4], 10 mM $MgSO_4$), vortexed once again, and filtered through a 0.22 µm Sterivex filter (Millipore, Billerica, MA, USA). The filtrate (i.e. viral fraction) was treated with 20% (v/v) chloroform followed by treatment with DNase I (2.5 U/µL) and RNase (0.25 U/µL) to remove free nucleic acids. If no viruses were detected after this purification procedure, the purification was repeated without chloroform and treated with only DNase I (2.5 U/µL) to recover enveloped viruses. The methods used for virus purification for each of the samples are summarized in Table 2. Prior to extracting total viral nucleic acids, EDTA (20 mM final concentration) was added to a 200 µL aliquot of purified virus particles to chemically inactivate nucleases. For analysis of RNA viruses, total RNA was extracted from virus particles using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA) followed by randomly primed RNA amplification with the Transplex Whole Transcriptome Amplification (WTA) kit (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's protocol using 50 and 65 PCR cycles. For analysis of DNA viruses, total viral nucleic acids were extracted using the QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, CA, USA), followed by random amplification using the GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. GenomiPhi products were further amplified using the Whole Genome Amplification (WGA) Kit (Sigma-Aldrich, Saint Louis, MO, USA).
Since it was unknown if the selected cell culture samples contained DNA or RNA viruses, WTA and WGA products were examined on a 2% agarose gel and positive reactions (defined as a smear between 200

- 400 bp) were purified using UltraClean™ PCR Clean-Up™ kit (MO BIO Laboratories Inc., Carlsbad, California, USA). The purified products were cloned into competent Escherichia coli cells using the TOPO TA® system (Invitrogen, Carlsbad, California, USA) and sequenced. To identify the different viruses in the cell culture samples approximately 48 clones were selected from each sample for sequencing. The sequenced fragments were compared against the GenBank non-redundant (nr) nucleotide database through tBLASTx (E-value < 0.001) (3).

Genome completion of Saffold viruses.

WTA fragments sequenced from four samples had significant similarities to Saffold viruses (SAFV). In order to complete the genome of the SAFV-like viruses, WTA fragments were assembled against the SAFV genome with the highest similarities to the sequenced WTA fragments (i.e. SAFV-3 NL2007; accession no. FM207487). The assembly was performed using the Sequencher software (version 4.1; Gene Codes) using the assembly algorithm for dirty data with optimization for gap alignment, 70% minimum match percentage, and a minimum overlap of 10 bp. Since WTA fragments did not overlap into a complete genome, the assembly was used to design primers for genome walking based on the consensus sequence between WTA fragments and SAFV-3 NL2007. Complementary DNA was produced from RNA extracted from purified viral particles using the Synthesis Superscript III reverse transcription kit (Invitrogen, Carlsbad, CA) with random primers. Each PCR was performed in a 50 µL reaction containing 4 µL of cDNA, 1X REDTaq PCR Reaction Buffer (Sigma-Aldrich), 1 µM of each primer (Table 3), 0.2 mM dNTPs, and 1 U REDTaq DNA Polymerase (Sigma-Aldrich). Each PCR reaction proceeded at 94°C for 5 min, followed by 50 cycles of [94°C for 1 min, annealing temperature (Table 3) for 45 sec, and 72°C for 2 min], and a final extension at 72°C for 10 min. The 5'UTR region was verified with the 5' RACE System (Invitrogen, Carlsbad, California, USA) using gene-specific primers (Table 3) according to the manufacturer's suggested protocol for high GC transcripts. SAFV genome sequences have been submitted to GenBank (accession no. HM181996 – HM181999).

Phylogenetic analysis of identified SAFV.

The SAFV genome sequences were used for phylogenetic analysis (Figure 1). All alignments were performed using the CLUSTALW algorithm (41) and BLOSUM62 as the similarity matrix in BioEdit version 7.0.9.0 (22). A neighbor-joining phylogenetic tree was constructed in MEGA4 (40) using the Maximum Composite Likelihood method to calculate evolutionary distances. All gaps and missing data were eliminated from the dataset and one-thousand bootstrap resamplings were performed to assess statistical support.

A similarity plot analysis showing the relationship among the detected SAFV and previously reported genomes was performed using SimPlot software, version 3.5.1 (29). The following parameters were used to calculate the similarity plot: 400 bp window size, 20 bp step size, and an empirical transition to transversion ratio. Sequences that originated from the same region and were 98-99% identical at the nucleotide level were grouped for the SimPlot analysis.

SAFV screening of additional unexplained samples from EVS.

The original cell culture isolates examined for this study were collected through the EVS between 1994 and 2007. To further assess the prevalence of SAFV in unexplained cell culture samples, eight additional unidentified cell culture isolates collected between 2008 and 2009 were tested using a PCR assay targeting the VP1 region of SAFV (5). The VP1 region is the most diverse protein of picornaviruses and it has been used to classify different SAFV genotypes (5). For the PCR assay, cDNA was obtained from randomly-primed reverse transcription (RT) of total RNA from cell cultures using the Superscript III RT kit (Invitrogen, Carlsbad, California, USA).

The RT-PCR reaction was prepared by adding 2.5 µL of the RT-mixture with random primers to 22.5 µL of PCR-mixture containing 12.5 µL of the HotStarTaq Mastermix (Qiagen, Valencia, CA, USA), 1.25 µL of forward and reverse primers [10 pmol/µL], and 7.5 µL H_2O. For the VP1 region VP1F1 (5'-ACWCTTGGTTTCDGGHGG-3') and VP1R1 (5'- TCGCCCATRCASACRAGRA-3') primers were used in the first round of nested PCR, while VP1F2 (5'- GACTTYACYCTBAGAATGCC-3') and VP1R2 (5'-ACTGTTCTAYCRTGAACTTTGTA-3') were used in the second round (5). The PCR reaction proceeded

as follows: denaturation at 95°C for 15 min, 4 cycles of amplification at 95°C for 1 min, 55°C for 1 min, and 68°C for 1 min 30 s, followed by 35 cycles of amplification at 95°C for 30 s, 52°C for 30 s, and 68°C for 1 min 30 s, then a final extension at 68°C for 10 min. A 2.5 µL sample from the first round PCR was used as template for the nested PCR reaction. The same PCR reaction mixture and PCR conditions were used for both rounds of PCR (5).

Sequences from the SAFV VP1 PCR products, along with publicly available sequences that were previously classified into SAFV genotypes (3), were used for phylogenetic analysis. A neighbor joining tree was constructed in MEGA4 using the same parameters that were used to construct the phylogenetic tree of full-length SAFV genomes (Figure 2).

RESULTS

Characterization of isolates from enterovirus surveillance at RIVM.
From 1994 through 2007 a total of 1834 cell culture isolates were submitted to the RIVM to identify viruses in cell cultures with CPE. Upon passaging at RIVM, 92 cell culture isolates did not show reproducible CPEs on cell lines and, thus, were not further investigated (Table 1). Of the 1742 samples with reproducible CPEs, 1513 were confirmed as enterovirus positive, and 189 as parechovirus positive. Extended PCR testing resulted in the identification of other viral agents, such as adenoviruses, reoviruses, rhinoviruses, and astroviruses, in 35 cell culture samples (Table 1). The seven remaining cell culture samples exhibiting consistent CPE tested negative for all viral PCR assays and were processed for viral metagenomics (Table 2).

Viral metagenomics of cell culture samples with unidentified viruses.
Viral metagenomics was performed on seven cell culture samples where PCR assays failed to identify potential viral pathogens (Table 2). This approach allowed the identification of viral agents in all the samples with minimal sequencing (< 50 clones per sample). Viral sequences from four of the cell cultures were related to the recently discovered SAFVs. The other unexplained cell culture samples contained a paramyxovirus related to Newcastle disease virus (1 isolate, will be described elsewhere), BK polyomavirus (1 isolate) and Herpes simplex virus (1 isolate) (Table 2). Sequences related to BK polyomavirus and Herpes simplex virus were 100% identical at the nucleotide level to known viruses and were not further examined. Since SAFV-like sequences were identified in four isolates, the complete genomes of these viruses were sequenced. The genomes were identified as SAFV NL1999-590, NL2005-1035, NL2007-2686, and NL2007-2690 (accession no. HM181996-HM181999). These isolates represent samples collected between 1999 and 2005 (Table 2).

Phylogenetic analysis of identified SAFV.
Available SAFV complete or near complete genome sequences were used for phylogenetic analysis (Figure 1). All the SAFV isolated from EVS samples clustered with SAFV genotype 3 (SAFV-3) genomes from the Netherlands and Germany. A similarity plot analysis was performed to evaluate sequence similarities among the different SAFV genotypes (Figure 3). SAFV-3 NL2007 (accession no. FM207487) was selected as the query sequence to evaluate how the new genomes compared to this genome, which was discovered in the Netherlands from a stool sample collected in 2007 (52). The SAFV-NL2007-2686, NL2007-2690 and

NL2005-1035 isolates were almost identical to each other, sharing 98-99% nucleotide identity. The SAFV-NL1999-590 isolate showed the least similarity with SAFV-3 NL2007 and the other SAFV-like genomes detected in this study (88% overall nt identity to all SAFV from the Netherlands). The region encoding the structural protein of the SAFV-NL1999-590 genome showed the most divergence compared to other SAFV-3 including the SAFV-3 sequences from Germany (Figure 3). NL1999-590 shared between 80 and 87% nt identity to the query sequence, while the other SAFV-3 genomes including those from Germany ranged between 90 and 95% nt identity. The SimPlot analysis did not reveal any potential recombination among SAFV-3 and other genotypes (data not shown).

TABLE 2. Description of isolates used for viral metagenomics.

ID	Year Collected	Age	Gender	Place	Clinical symptoms	Sample type	Growth on cell line1	Cell line used for viral metagenomics1	Virus purification	Virus identified2
NL1994-1104	1994	Unknown	Unknown	Netherlands	Unknown	Feces	tMK	tMK	Chloroform, DNase, RNase	BK polyomavirus
NL1999-590	1998	11 months	Male	Uithoorn	Malaise, fever	Feces	tMK	tMK	Chloroform, DNase, RNase	SAFV
NL2003-116	2003	7 months	Female	Ter Apel	Diarrhea, vomiting, nauseous	Feces	HeLa, Rd, Gabi	Rd	DNase	Herpes simplex
NL2003-579	2003	54 years	Female	Leiden	Fever	Infiltrate	HeLa, Rd, Gabi	Rd	DNase	NCDV
NL2005-1035	2005	5 years	Male	Amersfoort	Unknown	Feces	tMK	tMK	Chloroform, DNase, RNase	SAFV
NL2007-2686	2004	4 years	Unknown	Nijmegen	Unknown	Feces	HeLa, tMK	tMK	Chloroform, DNase, RNase	SAFV
NL2007-2690	2004	8 months	Unknown	Nijmegen	Unknown	Feces	HeLa	HeLa	Chloroform, DNase, RNase	SAFV

[1]tMK: tertiary monkey kidney; HeLA: human epithelial carcinoma cell line; Rd: human rhabdomyosarcoma; Gabi: human embryonic lung fibroblast cell line
[2] SAFV: Saffold virus; NCDV: Newcastle disease virus

TABLE 3. Primers used for genome walking of SAFV genomes

Genome	Primer ID[a]	Sequence	Annealing (ºC)[b]
All	200R	TGACTTTCCTCTGATTAGCC	RACE 5'c
All	400R	GGCATGWTAGCACTTTCGTC	RACE 5'
HM181996	640R	AAAGACCGCCGTGTGAAT	RACE 5'
HM181997; HM181998; HM181999	650R	CACTTATTGAGAGACGGA	RACE 5'
HM181996	1F	TTTCAAAGGGGGCCCTG	49ºC
	640R	AAAGACCGCCGTGTGAAT	
	640F	ATTCACACGGCGGTCTTT	49ºC
	1480R	GTGTTTGTGGCCGTGTTTC	
	1418F	TGAACATTCTTGGTAGTGCTG	49ºC
	2480R	TTCTCTTTTTGTTAGAGTCC	
	2400F	CACCACCGTTCCTGTTTACG	49ºC
	3860R	CCAAAGCCAGGGATTTCATA	
	5157F	GCCAAATGGTCTCAAGTACA	49ºC
	6450R	ATACCGCCACCACCAGC	
	6600F	CGCAGAACAGTAGCACACC	49ºC
	7480R	GGTTAAGTGTAATGCTGCTC	
	7124F	ACTGTTAGGGCGTTTTGCATC	49ºC
	8125R	TTTTTTTTTTTTTTGTTCTCATTTCCAA	
HM181997; HM181998; HM181999	1F	TTTCAAAGGGGGCCCTG	49 ºC
	650R	CACTTATTGAGAGACGGA	
	528F	GGTCTAAGCCGCTYGGAAT	60ºC
	1092R	CAAYRGCWGTGCAAAKAGGGCACAC	
	1087F	CRKWTKTGTGCCCTMTTTGCACWGCYRTTG	60 ºC
	1585R	ASAAACGACCAACWGTKGATT	
	1418F	TGAACATTCTTGGTAGTGCTG	49 ºC
	2480R	TTCTCTTTTTGTTAGAGTCC	
	2300F	GTCAGGGAACATGCTGGAAC	49 ºC
	2790R	TCCCAAATGGCGTAAGT	
	2690F	TGGAGCAGGAGAACCCAAA	49 ºC
	3750R	TTTGGTGTCTTGTGGGTTTG	
	3670F	TCAGTACATTACAGATACAA	44 ºC
	4100R	AAGTAGAAAATCCATGATCG	
	3750F	CAAACCCACAAGACACCAAA	49 ºC
	5050R	GCGGCCAAATGAGAGTTTAG	
	5175F	TTGTCAAATGGTTTCAAGTAC	44 ºC
	6470R	GCATTGCGTACACAGC	
	6470F	GCTGTGTACGCAATGC	49 ºC
	7190R	CTGAGCCAAGATCCAAACCA	
	6720F	CGTAGACCAGGTCGCTTTCT	44 ºC
	7570R	AGTCATCACCATAAGATAAA	
	7570F	TTTATCTTATGGTGATGACT	44 ºC
	8125R	TTTTTTTTTTTTTTGTTCTCATTTCCAA	

[a] Primer number indicates approximate nucleotide position in reference genome SAFV-3 (FM207487).
[b] All PCR reactions were performed with a touchdown program to incrementally decrease the annealing temperature by 0.2°C each cycle.
[c] Indicates gene specific primers used for the 5' RACE System (Invitrogen) and nested reactions according to manufacturer's instructions.

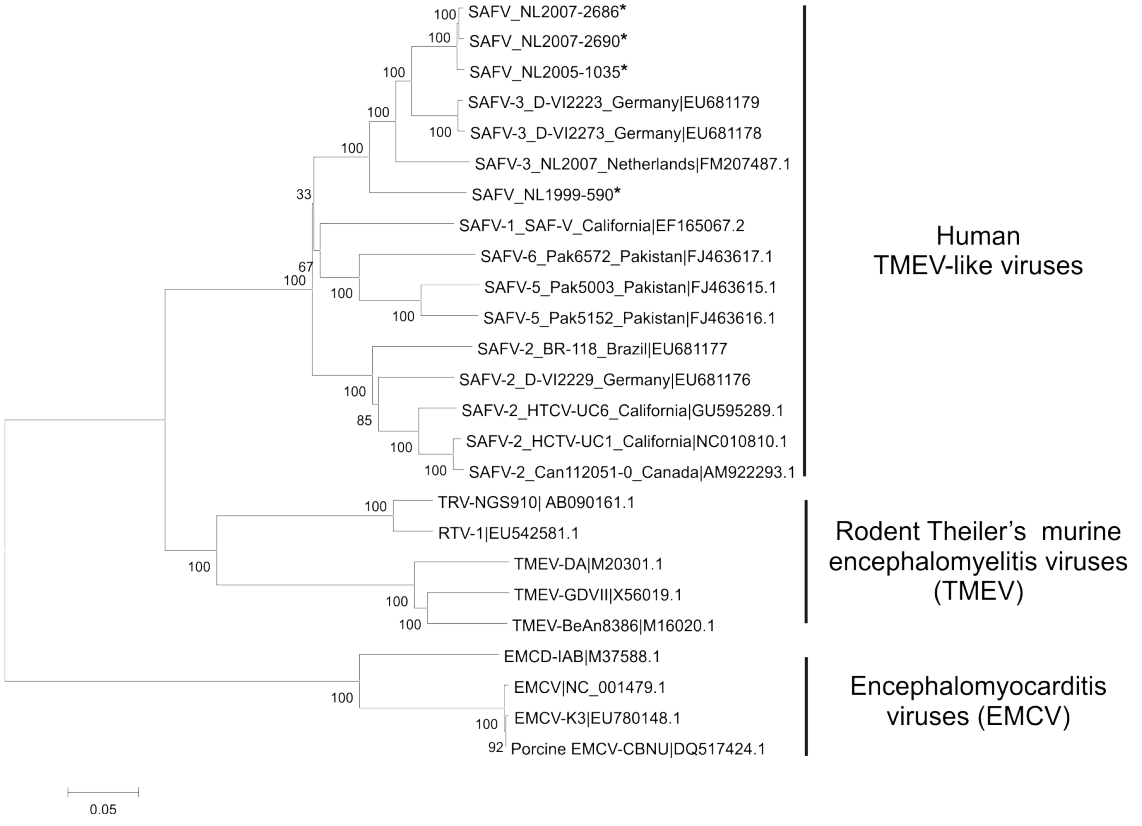

FIGURE 1. Neighbor-joining phylogenetic tree of complete or near complete SAFVgenomes and their relationship to other cardioviruses. All the SAFV genomes used for phylogenetic analysis are identified with their genotype (SAFV- #), the name used in their original publication, and the country where the genomes were identified. Genomes reported in this study are marked with an asterisk. The tree was constructed in MEGA4 using the Maximum Composite Likelihood method to calculate evolutionary distances (number of base substitutions per site). One-thousand bootstrap resamplings were performed to assess statistical support.

Frequency of SAFV infections in cell culture samples with unidentified viruses from EVS and phylogenetic analysis of identified SAFV.

A PCR assay targeting the VP1 region of SAFV was used to screen eight additional EVS samples with CPE of unidentified origin collected between 2008 and 2009. SAFV was detected in one of the samples collected in 2008, bringing the total number of SAFV positive cell culture isolates to five in the period from 1994 through 2009. Overall, SAFV accounted for 33% of the unidentified cell culture isolates, but <0.3% of the total EVS samples.

To further describe the five SAFV-like viruses identified in samples from the EVS program, complete VP1 sequences were acquired and used for classification. Phylogenetic analysis using a neighbor joining tree was performed on approximately 825 nucleotides of VP1 protein (Figure 2). All the detected SAFV clustered with SAFV-3 sequences from the Netherlands, California, and Germany (Figure 2) (13, 20, 52).

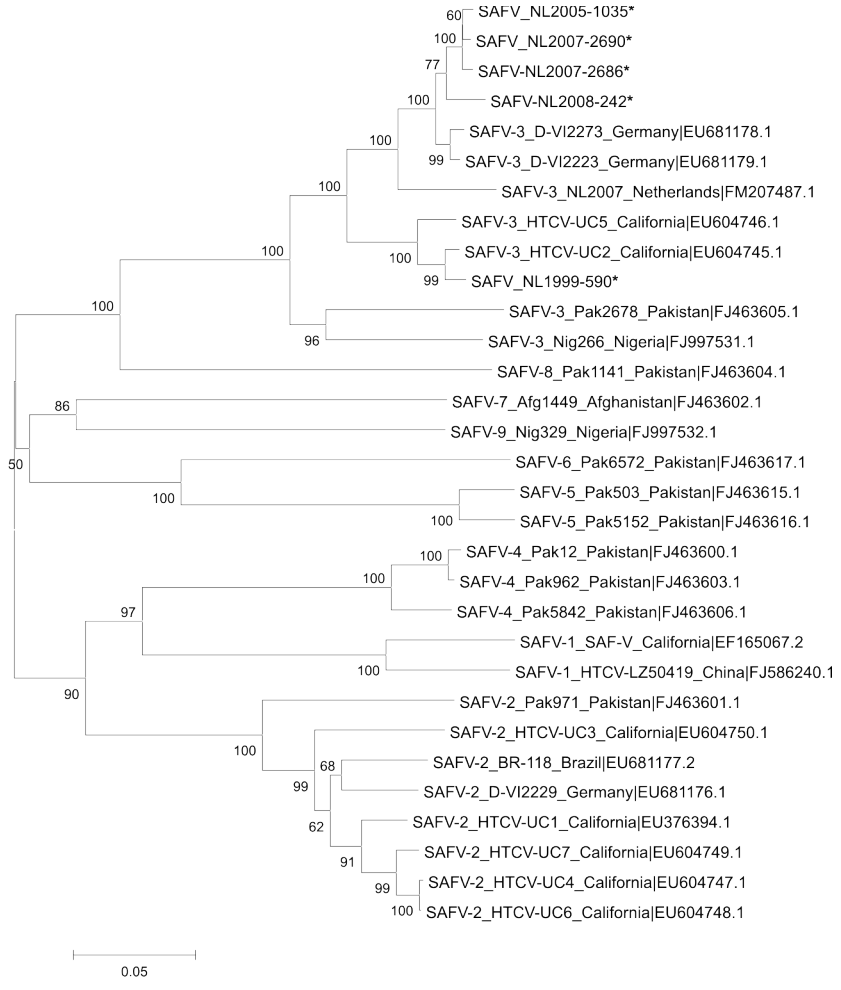

FIGURE 2. Neighbor-joining phylogenetic tree of SAFV VP1 sequences. All the SAFV sequences used for phylogenetic analysis are identified with their genotype (SAFV- #), the name used in their original publication, and the country where the sequences were identified. Sequences reported in this study are marked with an asterisk. The tree was constructed in MEGA4 using the Maximum Composite Likelihood method to calculate evolutionary distances (number of base substitutions per site). One-thousand bootstrap resamplings were performed to assess statistical support.

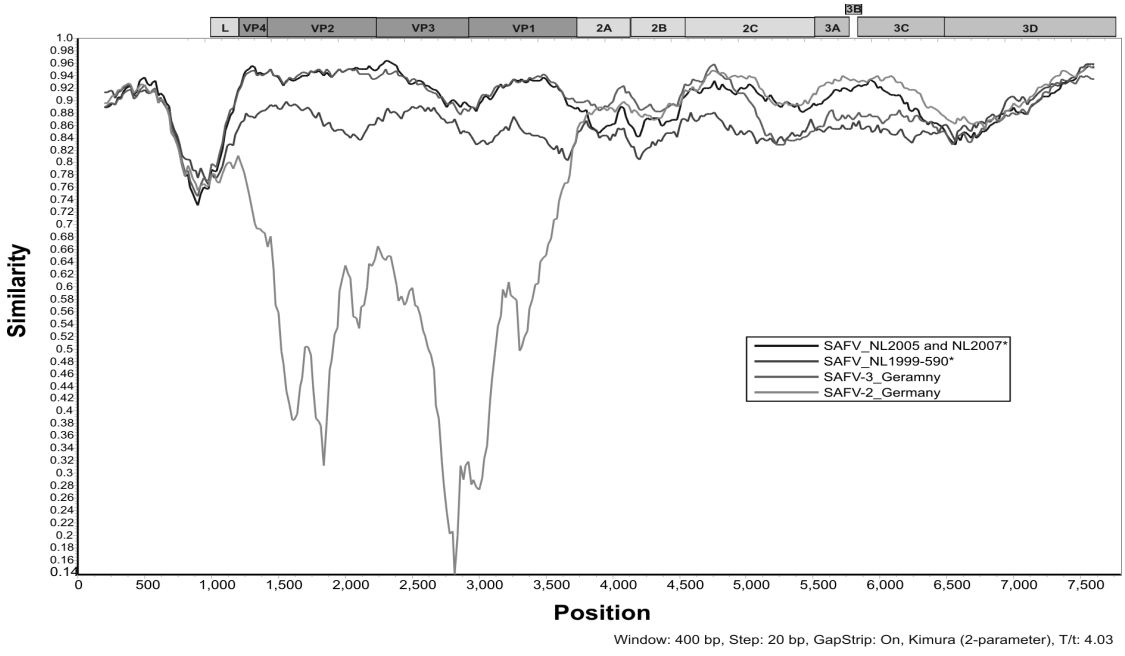

FIGURE 3. Similarity plot analysis of SAFV genomes identified in the Netherlands and Germany using SAFV-3 NL2007 (accession no. FM207487) as the query sequence. Analysis was performed in SimPlot version 3.5.1 using a window size of 400 bp, a step size of 20 bp, and an empirical transition (Ts) to transversion (Tv) ratio. Sequences or groups marked with an asterisk include sequences reported in the present study (accession no. HM181996 – HM181999). The sequences from Germany include the SAFV genotype 2 (SAFV-2) strain D/2229 (accession no. EU681176) and SAFV-3 strains D/2223 and D/2273 (accession no. EU681178-79).

SAFV-3 VP1 CD Loops I and II

```
                                  I                  II
SAFV3_UC2_CA        LTPLPSDIVNNSA LPEQERWISF ASPTTQAPPY KTKQDWNFVM FSPFT
SAFV3_UC5_CA        LTPLPSDIVNNSA LPEQERWISF ASPTTQAPPY KTKQDWNFVM FSPFT
SAFV-3_D2223        LTPLPSDIVNNSV LPEQERWISF ASPTTQAPPY KTKQDWNFVM FSPFT
SAFV-3_D2273        LTPLPSDIVNNSV LPEQERWISF ASPTTQAPPY KTKQDWNFVM FSPFT
SAFV_NL1999-590     LTPLPSDIVNNSA LPEQERWISF ASPTTQAPPY KTKQDWNFVM FSPFT
SAFV_NL2007-2686    LTPLPSDIVNNSV LPEQERWISF ASPTTQAPPY KTKQDWNFVM FSPFT
SAFV_NL2007-2690    LTPLPSDIVNNSV LPEQERWISF ASPKTQAPPY KTKQDWNFVM FSPFT
SAFV_NL2005-1035    LTPLPSDIVNNSV LPEQERWISF ASPTTQAPPY KTKQDWNFVM FSPFT
SAFV-3_NL2007       LTPLPSDIVNNSV LPEQERWISF ASPTTQKPPY KTKQDWNFIM FSPFT
```

SAFV-3 VP2 EF Loops I and II

```
                                        I                              II
SAFV_NL1999-590     ASQFHAGSLL VFMAPEFDTS NHSTEVEPRA DTAFKVDVNW QKHTQILTGH AYVNTTTKIN VPLALNHQNF
SAFV_NL2007-2686    ASQFHAGSLL VFMAPEFDTS NHSTEVEPRA DTAFKVDANW QKHTQILTGH AYVNTTTKVN VPLALNHQNF
SAFV_NL2007-2690    ASQFHAGSLL VFMAPEFDTS NHSTEVEPRA DTAFKVDTNW QKHTQILTGH AYVNTTTKVN VPLALNHQNF
SAFV_NL2005-1035    ASQFHAGSLL VFMAPEFDTS NHSTEVEPRA DTAFKVDANW QKHTQILTGH AYVNTTTKVN VPLALNHQNF
SAFV-3_DVI2223      ASQFHAGSLL VFMAPEFDTS NHSTEVEPRA DTAFKVDANW QKHTQILTGH AYVNTTTKVN VPLALNHQNF
SAFV-3_DVI2273      ASQFHAGSLL VFMAPEFDTS NHSTEVEPRA DTAFKVDANW QKHTQILTGH AYVNTTTKVN VPLALNHQNF
SAFV-3_NL2007       ASQFHAGSLL VFMAPEFDTS NHSTEVEPRA DTAFKVDANW QKHAQILTGH AYVNTTTKVN VPLALNHQNF
```

FIGURE 4. Alignment of putative SAFV genotype 3 (SAFV-3) VP1 CD (top) and VP2 EF (bottom) loop amino acid sequences. Amino acids 716 – 765 and 261 -330 are shown for VP1 CD and VP2 EF loops, respectively. Alignment shows conservation of amino acids in these structural loop sequences, except for one amino acid in the VP1 CD loop II sequence (highlighted in yellow) in SAFV-3 NL2007.

DISCUSSION

RIVM is a poliovirus reference laboratory, and laboratories from across the Netherlands submit cell culture isolates for confirmation and typing of enteroviruses through PCR assays and serological testing. This program started in 1994, after a poliovirus type 3 outbreak in 1992-1993, to monitor the eradication of poliovirus from the Netherlands (14, 34). For samples collected between 1994 and 2007, RIVM confirmed the presence of a range of known viruses in all but seven samples. Enteroviruses (including human rhinoviruses) accounted for 82.5% of the samples, and parechoviruses were the second most common group of viruses detected, accounting for 10.3% of the samples collected through EVS (44, 45). Extended PCR testing allowed the identification of other viruses in a low proportion of samples (<1% for each virus). Finally, viral metagenomics enabled the identification of additional DNA and RNA viruses in all of the remaining unexplained samples. Of note, none of these were enteroviruses, indicating that the existing enterovirus PCR assays effectively capture the diversity of enteroviruses circulating in the Netherlands.

As expected, two genera from the family *Picornaviridae*, *Enterovirus* and *Parechovirus*, comprised the bulk of viruses detected during the 13-year EVS period investigated in this study. The newly described SAFV dominated the unexplained cases (~33% of the cell cultures with unidentified viruses from 1994-2009), and also belong to the family *Picornaviridae*, but to a third genus, named *Cardiovirus*.

Since its initial discovery in 2007 from a stool sample collected in 1981 from California, USA (23), SAFV has been detected in stool and respiratory samples from patients in Europe, North and South America, and Asia (1, 5, 13, 20, 28, 35, 49, 52). Currently there are eight SAFV genotypes based on variants of the VP1 protein (SAFV-1 - SAFV-8), which is a determinant of viral tropism and antibody neutralization sensitivity (5). To date most of the SAFV diversity has been identified from samples collected in Pakistan where genotypes two through six and eight have been identified (5). In contrast, SAFV-3 is the only genotype that has been detected in samples collected in the Netherlands previously and in our study (52).

One of the SAFV-3 genomes, SAFV-NL1999-590, is more divergent in the structural protein-encoding region of the genome when compared to other SAFV-3 genomes, but conservation of amino acids in this region suggests that SAFV-NL1999-590 belongs to the same genotype (Figure 4). It is possible that the divergence observed in SAFV-NL1999-590 is due to mutational changes over time, indicating prolonged circulation of these viruses as has been described for other enteroviruses (44, 45). Since there is limited data regarding SAFV-3 viruses, it is difficult to calculate their substitution rates. Alternatively, SAFV-NL1999-590 may be recombinant relative to genotype 3 strains from the USA as the VP1 phylogenetic analysis indicated that SAFV-NL1999-590 clusters with SAFV-3 strains from California instead of strains from the Netherlands and Germany. Within-genotype recombination has been reported for SAFV-2 (19), but BootScan analysis failed to show evidence for recombination between SAFV-NL1999-590, SAFV-3 sequences from the Netherlands and Germany, and SAFV- 1 and -2 sequences from California, Canada, and Brazil (data not shown). For definitive conclusions, more complete genome sequences of SAFV strains identified in the US are needed.

The identification of different SAFV-3 in 0.25% of the tested samples is in the same range as other viruses from the *Picornaviridae* family such as Coxsackie A viruses (van der Sanden, manuscript in preparation), indicating endemic circulation in the Netherlands. Based on a virus neutralization assay, it has been shown that SAFV-3 infection indeed is highly widespread in humans in at least three continents (i.e. Europe, Africa, and Asia (52). Consistent with the hypothesis of endemic circulation, infection seems to occur in early life as 92% to 98% of a group of 150 individuals between 4- and 40- years old from the Netherlands had neutralizing antibodies against SAFV-3 (52). Seroprevalence data also indicated that SAFV-3 NL2007 or a related virus has been present in the Netherlands for at least 10 years (52), again confirmed by the identification of SAFV-NL1999-590 (Figure 3). In the United States, it has been shown that 91% of adults carry antibodies against SAFV-2 with 80% of studied individuals generating neutralizing antibodies against this genotype (12).

Although SAFV infection is highly prevalent, the pathogenicity of this virus remains unknown. SAFV has been detected in stool and respiratory secretion samples from children with gastroenteritis (13, 20, 35, 49), respiratory illness (1, 13), and non-polio acute flaccid paralysis (5), as well as in asymptomatic individuals (5, 13, 49, 52). In several cases, SAFV has been detected in addition to other viruses known

to cause gastroenteritis, thus, hindering interpretation regarding SAFV infection and observed pathology (13, 20, 35). SAFV-2 have been recently linked to infection in a child with diarrhea and vomiting based on an acute seroconversion event supporting an association between this virus and diarrheal disease (12, 49). Although SAFV-3 were the only viral agents identified in five out of fifteen of the unexplained cell culture samples in this study, it cannot be concluded that these viruses were causing illness or clinical symptoms. It is well known that enteroviruses may manifest illness in a minority of infected persons, but this does not mean that infections are trivial. Polio, for instance, develops in less than 1% of infected persons, but is a debilitating disease that results in life-long disabilities. Therefore, further research is needed to unravel the possible role of SAFV in human illness.

Sequence-independent methods are becoming more important to identify emerging viruses in public health monitoring efforts (1). Viral metagenomics is a relatively new technique that has been used increasingly to identify viruses in clinical specimens (2, 8-10, 23-25, 47, 48, 51). Purifying viral particles before sequencing minimizes the amount of sequencing that needs to be performed for viral identification. If sufficient budget exists, combining the viral purification procedure presented here with next generation sequencing technologies will enable rapid sequencing of the complete genomes of viruses of interest. In this study, viruses were identified in a relatively short amount of time (less than a week) with limited sequencing (< 50 clones per sample), demonstrating that metagenomic analysis can be added as a routine tool to investigate unidentified viruses in cell cultures from clinical samples in a public health setting. This method proved to be a more efficient and cost-effective alternative compared to individual PCR assays for different viruses. Therefore we recommend the use of viral metagenomics on cell culture samples that test negative for established PCR assays in virus surveillance programs. However, we emphasize that embedding these techniques with follow-up studies is essential for establishing relevance of the findings for public health.

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Chapter 8

General Discussion and Concluding Remarks

INTRODUCTION

Viral gastroenteritis is one of the most common diseases in developing and developed countries. In the Netherlands, surveillance of viral gastroenteritis outbreaks started in 1994, and is still ongoing. For this period fecal samples were tested for noroviruses, rotaviruses, sapoviruses, adenoviruses, astroviruses, vesiviruses, coronaviruses and Aichi viruses. Despite this extensive testing, however, a certain proportion of gastroenteritis outbreaks remains without etiological diagnosis (Figure 1). The studies described in this thesis aimed to elucidate the cause of these unexplained gastroenteritis outbreaks in order to set up intervention measures. To achieve this goal an overview was made of all gastroenteritis outbreaks in the period from 1994 through 2005, and molecular assays were used for detection of a range of enteric viruses that could be involved according to outbreak reports or other specific studies (e.g. coronaviruses, porcine sapoviruses, enteroviruses, parechoviruses). Following the emergence of a virulent clone of *Clostridium difficile* in hospitals, we assayed our outbreak specimens to rule out missed Clostridium outbreaks. Multiplex PCR assays were developed, to allow faster and more sensitive screening. Subsequently, we addressed the possible role of less well or only recently recognized pathogens in viral gastroenteritis outbreaks and developed molecular methods to detect some newly described viruses. Finally, since no unusual viruses were detected and a proportion of outbreaks remained without diagnosis, a viral discovery technique was applied on the remaining stool samples from unresolved outbreaks. In this chapter the results described in this thesis and their public health implications are discussed. Furthermore, suggestions for future research and surveillance of viral gastroenteritis are done.

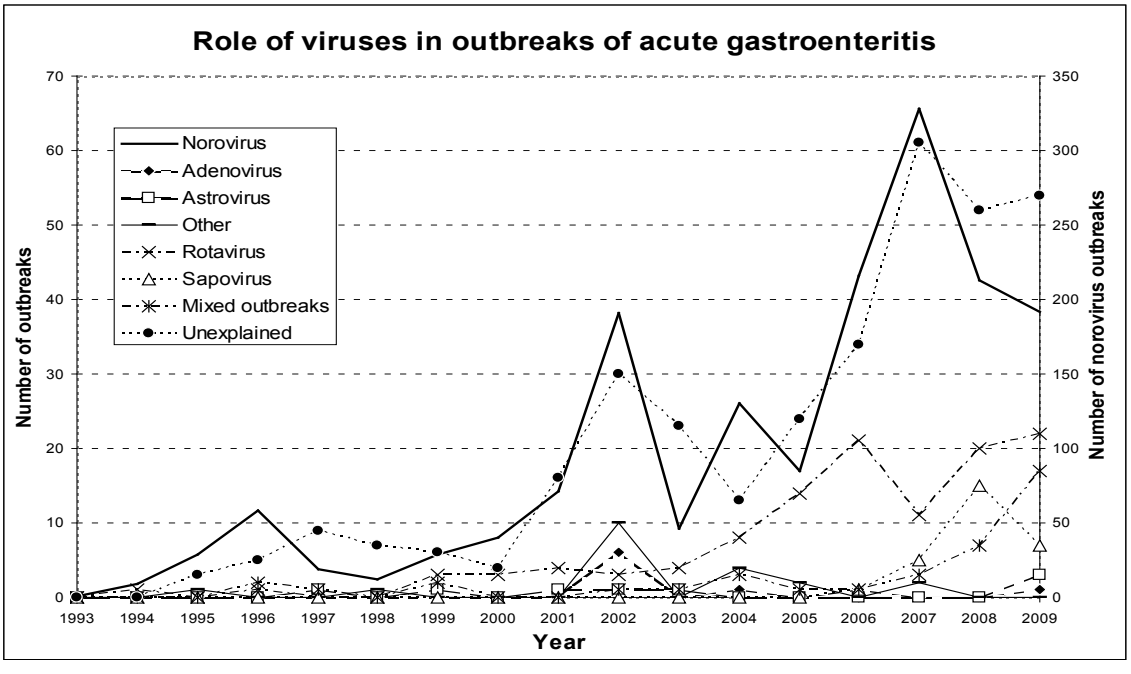

FIGURE 1. Role of viruses in outbreaks of acute gastroenteritis from 1993 through 2009. Y-as for noroviruses has been indicated on the right side of the figure, the y-as for other viruses and unexplained outbreaks is on the left.

VIRAL PATHOGENS OF GASTROENTERITIS AND THEIR DETECTION

Major causes of viral gastroenteritis outbreaks in the Netherlands.
The main aim of this thesis was to elucidate unexplained outbreaks of viral gastroenteritis. In order to do this, a systematic evaluation of common etiologies is needed as a first step. As indicated in this thesis (chapter 2 and 3) and other studies performed worldwide noroviruses were found to be the major cause of outbreaks. This is followed by outbreaks caused by rotaviruses, and more recently, sapoviruses (Figure 1). We validated our findings by consulting the population studies from the Netherlands and different countries worldwide. These studies revealed that noroviruses and rotaviruses are leading causes of infectious intestinal diseases, especially in children. Based on a population study in 1999, the incidence rate of acute gastroenteritis in the Netherlands was measured to be 283 per 1,000 person-years (14), with an estimated 4.5 million cases per year. A high proportion, 33.5% (2.0% adenoviruses – 16.1% noroviruses), of this was explained by viral etiologies, compared to 2.4% caused by bacterial and 6.8% by parasitic pathogens. In all, about 54% of cases remained unexplained (14). Similarly, studies worldwide showed that noroviruses and rotaviruses predominate as causes of acute gastroenteritis (3, 14, 24, 27, 41). However, there are clear differences in their representation in outbreak surveillance, partly depending on the target population. We have shown that norovirus outbreaks are very common in all age groups, whereas rotavirus - which has a similar incidence in the community -, is far less frequently detected in outbreaks in the adult population. The low number of rotavirus outbreaks notified in The Netherlands might reflect a bias in reporting: the current surveillance is based on the mandatory notification of outbreaks in healthcare settings including elderly homes, thus preferentially capturing norovirus outbreaks. Notification of outbreaks in child day-care centres is not mandatory and thus mostly not reported. Rotaviruses are known to cause infections primarily in children, have a similar incidence in the population as noroviruses, and are shed to high viral loads. Therefore, we assume that more rotavirus outbreaks would be detected if more outbreaks that involve children were reported through our surveillance system. In addition to noroviruses and rotaviruses, we screened for established diarrheal viruses (sapoviruses, adenoviruses, and astroviruses), but these were less common, explaining in total 2 % of outbreaks overall.

The routine diagnostic screening for viral gastroenteritis outbreaks at the RIVM is done by a combination of multiplex assays wherein noroviruses genotype II and rotaviruses are screened in one multiplex PCR (chapter 3), and norovirus genotype I, sapoviruses, adenoviruses, and astrovirues in other multiplex PCR assays. Since noroviruses and rotaviruses are major causes of viral gastroenteritis outbreaks, the order of screening for viruses causing outbreaks as performed at RIVM is justified according to algorithm for testing of outbreaks (28) and will explain about 80% of the outbreaks of viral gastroenteritis as was shown in this thesis (Figure 1).

Extraction and detection methods.
The correct identification of the possible cause of an outbreak relies on high performance of laboratory methods. Thorough validation of each of the steps in detection of enteric viruses (or other pathogens, for that matter) is needed, in order to be able to draw the conclusions described in this thesis. Some essential steps will be discussed below.

Extraction of the nucleic acids
Multiplex PCR detection assays are developed and used for detection of microbial DNA/RNA, but preparation of the stool samples and extraction of the nucleic acids is an essential step in detection of viruses. Routine diagnostic testing of pathogens in stool samples involves a large number of samples that need to be processed. Therefore, the hands-on-time for sample preparation and analysis can become a major bottleneck and human errors may occur more easily. These difficulties can be eliminated by use of robotic workstations for DNA/RNA isolation and real-time PCR. Next to the reduction in hands-on time, the use of robotic workstations would improve consistency of the process and reproducibility of DNA/RNA extraction and analysis.

Several platforms are available for automated nucleic acids extraction, such as MagNA Pure (Roche, Almere, The Netherlands), EasyMAG (bioMérieux, Boxtel, The Netherlands), Xtractor (Qiagen, Venlo, The Netherlands), Qiasymphony (Qiagen, Venlo, The Netherlands). They are being used for extraction of viral, bacterial and parasitic pathogens. However, comparative studies designed to specifically evaluate the performance of the extraction method are scarce. A comparison study was performed by Schuurman et al (2007) who compared MiniMAG (bioMérieux, Boxtel, The Netherlands) and MagNA Pure Pure (Roche, Almere, The Netherlands) systems for detection of *Salmonella* species from stool samples and showed a difference of 1.26 cycles was observed in the Ct values after real-time PCR of RNA extracted using the MagNA Pure compared with the miniMAG machine. Also, differences were observed in the ability to remove PCR inhibitors (48).

Another study comparing 8 extraction methods for detection of SARS CoV from stool samples, of which 4 extractions were automated and involved the MagNA Pure (Roche, Almere, The Netherlands), EasyMAG and MiniMAG (bioMérieux, Boxtel, The Netherlands), and QIAamp MDx (Qiagen, Venlo, The Netherlands). The sensitivities of PCR detection of a panel of defined samples following the different extraction techniques ranged from 75% (9 of 12 samples detected) to 100% (12 of 12 samples detected). When the Roche MAgNA Pure system was used higher numbers of viruses were detected for diluted samples than for undiluted samples, which suggests the presence of inhibitors after the extraction (37).

Comparative studies were also performed on other clinical samples. For example, a comparative study was done for extraction of respiratory tract pathogens *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* using easyMAG/EasyQ (bioMérieux, Boxtel, The Netherlands) and MagNA Pure (Roche, Almere, The Netherlands) extractions on respiratory tract specimens. In this study the analytical specificities of PCR assays following both extraction methods were similar, but a higher analytical sensitivity was found for *C. pneumoniae* PCR using the easyMAG/EasyQ combination, since the easyMAG/EasyQ system detected nucleic acid extracts about 10 times more diluted than the MagNA Pure with in-house PCR. Both combinations were equivalent when detecting *M. pneumoniae* in positive respiratory tract samples (5).

Another study compared five commercial methods, of which three automated extraction systems, for toxoplasma DNA extraction from amniotic fluid. In this study the MagNA Pure Compact (Roche, Almere, The Netherlands) provided the best results at low concentrations (lower than 5 tachyzoites/mL of amniotic fluid), for example at a concentration of 2.5 tachyzoites/mL 83.3 % (Taqman PCR) and 95.8 % (FRET PCR) of positive Toxoplasma DNA was detected by use of MagNAPure Compact, and 63.6% and 70.8 % by use of NucliSens easyMAG method. However using MagNA Pure Compact method inhibition in 3.2% of the samples tested was seen (62).

The optimal method for the extraction of RNA and DNA for PCR detection would provide pure nucleic acid free of amplification inhibitors and at the same time recover RNA/DNA quantitatively across a range of concentrations (37), but - as seen in the comparisons above – there is no single best method. The optimal results found in one study for a certain sample type and a pathogen can not be generalized to other sample types and pathogens. Therefore an evaluation of extraction methods should be done for each pathogen, sample type, and PCR examined.

In the studies described in this thesis, we used MagNA Pure (Roche, Almere, The Netherlands) for extraction of nucleic acids, and therefore, inhibitors of PCR may have influenced the outcomes. However, for outbreak diagnosis, where one expects to find pathogens in multiple samples collected from acutely ill patients, this is less of a problem than when using PCR methods for evaluation of sporadic cases. From this evaluation it is clear that optimal results, including assay sensitivity and quantitation, require an optimal extraction method. In addition, variations in the sample matrixes, differences in the virus copy numbers (pathogen copy numbers) present, and the properties of the virus (e.g. enveloped or non-enveloped) should be considered. Ideally, an evaluation of extraction methods should be done for each virus/pathogen and sample type being examined.

As indicated above, another essential factor that may effect the detection of viruses are various materials, inhibitors, that are present in faecal specimens. They can cause inhibition of the DNA amplification or reverse-transcription (used for amplification of RNA viruses), and therefore it is essential to control the efficiency of the extraction and amplification process by use of an internal control (21, 52).A fixed amount

of an internal control is added to the lysis buffer that is used for extraction of nucleic acids. Less efficient amplification of this control template may reveal possible false negative results for the specific targets. In addition to this, adding positive control specimens to the series is necessary in order to follow the extraction and amplification of the specific target. During the studies described in this thesis, we used these procedures, and therefore we believe that we did not fail to spot viruses because of bad performance of the nucleic acid extractions and inhibitors present in faecal samples.

Involvement of other viral pathogens in outb reaks of viral gastroenteritis.

Known viral pathogens

After ruling out the common gastroenteritis viruses, as well as possible technical issues, 15 % of outbreaks remain without diagnosis. In recent literature, several enteric viruses have been recognized that might play a role, but have not been systematically evaluated. Parechoviruses were detected as significant cause of sporadic cases of acute gastroenteritis, involving children that were hospitalized due to gastrointestinal complaints in China (64). A study in Japan also involving children detected enteroviruses, parechoviruses, and human bocaviruses in sporadic cases (38). In France, Aichi viruses were detected in oyster-related gastro-enteritis outbreaks (29). Lastly, the recently discovered Saffold viruses were suggested to play a possible role as a cause of gastroenteritis (8, 9, 15, 43).

Therefore, the second step in our approach was the systematic exclusion of enteroviruses, Aichi viruses, parechoviruses, and Saffold viruses, and additionally coronaviruses, and vesiviruses, as causes of illness in our patient population. This screening did not result in explaining any viral outbreaks and therefore it is not likely that these viruses play an important role as cause of outbreaks of viral gastroenteritis in the Netherlands. Typically, high loads of viruses are expected in stool samples from outbreaks, as seen in outbreaks caused by noroviruses (34), rotaviruses (32, 35, 66) and sapoviruses (63) if the stool samples are collected during the peak of the infection. The detection limits of the specific assays for Aichi viruses and parechoviruses were low. Also, generic assays for vesiviruses, enteroviruses and coronaviruses had previously been used on clinical samples (29). Therefore, although we did not measure detection limits for each of the assays, we assume that these viruses would have been detected if present in clinically relevant quantities.

The seeming contrast between finding for instance parechoviruses and Saffold viruses in hospitalized children and not in our studies could again be explained by the clear biases in our outbreak surveillance, in which outbreaks in children are underrepresented. Alternatively, this may reflect differences in viral properties: the success of spread of noroviruses lies in their extreme transmissibility, resulting in effective person to person spread (58). As Aichi viruses, parechoviruses, enteroviruses, coronaviruses are mostly found in sporadic cases, it is therefore possible that they do not spread as efficiently as noroviruses, resulting in a lower outbreak potential. This also could explain why they were not detected in outbreaks used for examination during this thesis.

A third option is that -although these viruses may be shed by humans- , their clinical relevance is limited. This points at a problem with the novel pathogen discovery techniques, that are based on random amplification of genomic sequences, for instance in the human gut. Studies have shown that the majority of the obtained sequences were similar to plant pathogenic RNA viruses, for example pepper mild mottle virus (PPMV) was found in two-thirds of individual from different countries, suggesting that PMMV is prevalent in human populations (65). Therefore, the mere finding of a virus in stool does not constitute evidence for its relevance. Evaluation of relevance of a virus in a disease can be done by use of Koch's postulates. Although this gold standard has limitations, not all viruses can be cultured of which noroviruses are a good example, the application of the four criteria may help identification of a causative agent. The Koch's postulates are:

1. The microorganism must be found in all organisms suffering from the disease.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be reisolated from the diseased experimental host and identified as being identical to the original specific causative agent.

As indicated above, Saffold virus was detected in different studies in cases of gastroenteritis in children and it was isolated and grown from stool samples of children with gastroenteritis as shown in chapter 7 covering the first two of Koch's postulates. We should keep in mind, however, that this might be an asymptomatic infection or a subclinical infection. In any case, we did show that none of the above pathogens are a significant cause of outbreaks of gastroenteritis. Therefore inclusion of the specific assays for newly discovered viruses is not recommended for routine surveillance or outbreak diagnosis.

Novel methods for evaluation of possible etiologies: the metagenomic analysis

After finalising testing for a broad panel as discussed above, another approach needs to be explored for remaining samples from outbreaks with unresolved etiology. Therefore we decided to use viral metagenomic on a selected set of outbreaks that did not yield a known virus. This selected set of outbreaks occurred around the same time, between January and March 2009, and took place in the same region (southern part of the country). Furthermore, all of these outbreaks contained a substantial number of infected patients. A 10 % faecal suspension was made, centrifuged and the supernatant was treated with chloroform in order to get rid off the bacteria, parasites and other faecal material that could disturb the reaction. Subsequently, the viral nucleic acids were extracted and used for viral metagenomics. However, no unusual viruses were detected in those stool samples and use of metagenomic analysis did not explain any of the outbreaks. This suggests that either the outbreaks were not viral, or they were caused by a virus for which the approach chosen is less suitable.

Again, one has to consider the technical specifics of the approach in order to interpret the negative results. The protocol was less optimal for identification of enveloped viruses, because of rigorous purification methods, including chloroform extraction. Therefore, we could conclude that the outbreaks that were tested by use of viral metagenomics were not caused by non enveloped viruses, but we were less certain about the involvement of enveloped viruses. We performed a small set of experiments without using chloroform treatment. Furthermore, a caesium chloride gradient purification step was done for purification of viral particles from the stool. No viruses, enveloped or non enveloped, were detected.

Viral metagenomics on stool specimens is hampered by the presence of numerous bacteria and bacteriophages (54). The number of clones that can be sequenced is a limiting step, and the predominance of competing nucleic acid may mask presence of viral sequences. This can be circumvented by use of ultra deep sequencing or so called "454" sequencing, that provides a great amount of DNA sequence data and allows large scale sequencing when compared to Sanger sequencing (44-47). Ultra deep sequencing has been applied on clinical samples and successfully identified viruses in stool (new adenoviruses) (17) and a new arenavirus transmitted through solid-organ transplantation (36). But, "454" sequencing is still costly and therefore not one of the most common methods to use for patient diagnostics, but can be applied with success in different research investigations.

Biases in the outbreak surveillance?

During the years spanned by the studies in this thesis, more sensitive methods for five common viral gastroenteritis pathogens and additional detection methods for known, but less common viral gastroenteritis pathogens have been implemented in routine diagnostics, but the number and proportion of unexplained outbreaks increased (Chapter 2 and Figure 1). In the period from 1994 through 2005, about 12% of all outbreaks remained unexplained. For the years 2007, 2008, and 2009 the proportion of unexplained outbreaks increased to 15.8%, 16.9%, and 18.2% respectively. This is remarkable since outbreaks are screened for more viruses and with more sensitive techniques than was done previously.

One of the reasons for the increasing proportion of unexplained outbreaks can be changes in the criteria for referral of specimens from outbreaks, which initially included testing for the absence of bacterial and parasitic pathogens in stool samples. Nowadays, the most common cause of viral gastroenteritis, norovirus, is a well known pathogen and has been a popular topic in different media with reports on outbreaks on cruise ships, college campuses, and closures of hospital wards (18-20, 22, 23, 30, 33, 55-57). Therefore, diagnostic testing prior to referral may have changed, raising the question if the bacterial and parasitic pathogens are excluded before these outbreak samples were sent for virological testing. This information is not usually provided by the peripheral laboratories that are testing and subsequently sending in the samples.

Lately, guidelines for evaluation and control of viral gastroenteritis outbreaks and food-borne infections have been distributed and these guidelines also involve an algorithm for gastroenteritis outbreaks (28). To prepare this algorithm an overview was made of the most likely causes of outbreaks in different settings and age groups. This background information could be used for more cost-effective diagnostic testing. For instance: most reported healthcare outbreaks of acute gastroenteritis were caused by viral pathogens, but outbreaks that occurred after visiting a restaurant had bacterial pathogens as commonly involved. In day care centres, parasitic infections need to be considered, in addition to viral pathogens (12-14, 28, 53, 61). However, this can only be done if requests to diagnostic laboratories provide background information, a practice that is uncommon.

Another explanation for the increased proportion of unexplained outbreaks may be some fatigue in obtaining the requested sample size for outbreak evaluation. For instance, in 2009 11.1% of all outbreaks submitted contained a single sample, which is suboptimal for establishing a diagnosis. The sensitivity of outbreak diagnosis increases with greater sample number (14, 16). In Figure 1 the number of outbreaks as reported during the surveillance has been given, and high number of norovirus outbreaks are followed by high number of unexplained outbreaks (peaks in 2002 and 2007). Although this is only speculation, it is possible that these peaks with unexplained outbreaks are actually norovirus outbreaks that could not be identified because of low sample number that were obtained for these outbreaks. Therefore, laboratories and physicians submitting samples for outbreak testing should be educated to increase the sampling of outbreak cases when possible and better communication between the reference laboratory (RIVM) and peripheral laboratories could improve this situation.

This can be assisted by the newly founded working group for diagnostics of enteric infections. Most of the clinical laboratories that perform diagnostics on gastrointestinal illness participate in this working group, and RIVM could take a more active part in this group. RIVM has an experience of 15 years with viral outbreaks, while other laboratories are now starting to test for viral pathogens causing gastroenteritis. On the other hand, clinical laboratories have more experience with sporadic gastrointestinal illness caused by bacteria and parasites. Combining these areas of expertise could lead to a more effective diagnosis of pathogens involved in gastrointestinal infections. For outbreaks analysis, RIVM could educate these laboratories on testing and sending in of stool samples (to RIVM) for viral gastroenteritis outbreaks. A diagnostic work flow can be set-up in which different diagnostic tasks could be divided according to the specialties of the laboratories. Laboratory analysis should be performed according to the outbreak algorithm (viral pathogens at RIVM or another specialized laboratory and bacterial and parasitic pathogens in other laboratories) in order to exclude most common pathogens causing gastroenteritis. Subsequently, RIVM could continue with diagnosing of unexplained outbreaks using for example, viral metagenomics.

Consequences of evolution of diagnostic methods.

Detection of viral causes of gastroenteritis has been done by PCR since 1994, but the approach of detection of viruses has been changing from single to multiple pathogen testing. Previously, a stool sample was tested first for noroviruses and only negative samples were tested further for rotaviruses, which was followed by testing of adenoviruses, astroviruses and sapoviruses when negative. Use of multiplex PCR assays allows simultaneous detection of all these viruses (Chapter 3). This automatically leads to increased detection of mixed infections. Mixed infections are also commonly found in studies that involve multiplex testing of respiratory samples, especially in samples collected from children (25, 26, 31).

Real-time PCR assays are sensitive methods and it should be kept in mind that by use of these assays viruses can be detected that are shed in low numbers and that may not be involved in the disease (40, 41). Asymptomatic shedding of viruses causing gastroenteritis, for example noroviruses, has also been documented, and the proportion of asymptomatic shedders increases with increasing sensitivity of the technique used for their detection (39, 42). Therefore, finding more mixed infections most likely reflects technical changes, rather than changes in epidemiology, as shown in England, where higher norovirus incidence was determined by use of new and more sensitive assays (41). In recent studies on etiology of acute gastroenteritis, mixed infections were detected in 40% and 22% of hospitalized children and adults (Friesema I, submitted for publication). Therefore it is difficult to assign one pathogen responsible for disease.

A question is whether the use of viral load estimates would help distinguish clinically relevant levels of shedding from that found typically in asymptomatic shedders. An attempt can be made by looking at cycle threshold (CT) of the detected pathogens. A lower CT value indicates a higher load, and possibility that this pathogen was involved in an infection or even caused the disease is therefore high. For example, norovirus viral loads are highest in the acute stages of illness, but shedding may be detected for up to 8 weeks after norovirus infection (median about 4 weeks) (2). Patients as well as experimentally infected healthy volunteers may shed $>10^{11}$ norovirus copies/g faeces at the peak of the infection, and with a median of 10^7 – 10^8 (4, 7). There is some evidence that shedding is longer in symptomatic patients, suggesting that viral load determination might aid clinical diagnosis (1). A recent study in England (40) investigated how faecal viral load can be used to determine when norovirus is the most likely cause of illness in an infectious intestinal disease case. Cycle threshold values from the real-time RT-PCR were used as an alternative measure of viral load. The optimal Ct value cut-off for norovirus was 31. However, clinical and epidemiological information should be considered in the diagnosis of disease with Ct values close to the cut-off, and the approach described here should be validated in other studies.

To assess relevance of mixed infections and viral loads in a patient with acute gastroenteritis infection new population studies are needed. This is even more so with the routine implementation of increasingly broad syndromic multiplex detection methods in clinical laboratories, not only for viruses. Detection of parasites by microscopic examination has been replaced by real-time PCR assays that detect *Entamoeba histolytica*, *Giardia lamblia, Cryptosporidium parvum*/*Cryptosporidium hominis, Dientamoeba fragilis,* and *Blastocystis hominis* (6, 51, 59, 60). Assays for detection of bacterial pathogens, *Salmonella*, *Campylobacter*, *Yersinia*, *Shigella*/EIEC species and *Clostridium difficile*, in stool samples by real-time PCR assays are also developed, and culture methods are being replaced (10, 11, 49, 50).

HOW TO PROCEED

As discussed, PCR assays for detection of common viral pathogens have been developed, validated, tested and used on the clinical samples from outbreaks of viral gastroenteritis. These assays are being adapted in other laboratories across the Netherlands to use for patient diagnostics. Extensive evaluation of unexplained outbreaks did not yield clinically relevant additional pathogens, suggesting that the current virological work-up is exhaustive. The recent emergence of sapoviruses as a cause of outbreaks of viral gastroenteritis indicates that this pathogen should also be added to the list, or be used in a second line of screening. It also shows that periodic evaluation of causes of illness in the community through systematic studies is needed. So, first step in viral gastroenteritis outbreak diagnosis would be to follow science based algorithm for outbreaks (28).

In order to notice an increase or emergence of a pathogen causing outbreaks, as was noticed for rotaviruses and sapoviruses during this thesis, after testing for viruses according to algorithm, unexplained outbreaks should be tested for other known viral gastroenteritis pathogens. The remaining unexplained outbreaks can be tested by use of new molecular approaches, as viral metagenomics or so called “454” sequencing. Although, “454” sequencing is still too costly for routine laboratories, and the bioinformatics analysis challenging, these techniques hold promise for the future, and should be accessible through collaborations with research institutions, such as universities and other academic centers, and in reference laboratories.

CONCLUDING REMARKS

In conclusion, with our systematic approach for evaluation of gastroenteritis outbreaks, we confirmed that noroviruses cause the majority of the gastroenteritis, followed by rotaviruses. Therefore, stool samples collected during outbreaks should be tested first for these pathogens. A wider spectrum of etiologies can be included for evaluation of outbreaks that test negative, including testing for adenoviruses and sapoviruses, bacterial (*Salmonella* and *Campylobacter*) and parasitic (*Giardia lamblia* and *Cryptosporidium* species) pathogens, and *Clostridium difficile* and STEC toxins. The remaining unexplained outbreaks can be tested

for new viruses by use of viral metagenomics by a reference laboratory.
Now, it is widely accepted that viral pathogens cause the majority of the gastroenteritis outbreaks, and RIVM has a long experience with viral diagnosis, therefore it is recommended for the laboratories that are starting viral diagnostics to use this. The routine laboratories in the Netherlands could profit from the knowledge that RIVM has, if RIVM would take an educative role and share their experience with other laboratories. Since more laboratories are performing the diagnosis of viral gastroenteritis it is of great interest to continue the reporting of outbreaks to RIVM in order to type the norovirus outbreaks and keep track of other pathogens involved in outbreaks. To get more information on outbreaks in for example child day-care centres it would be recommended to report outbreaks from different settings.
Furthermore, we would recommend new population based studies that use new detection assays, rather than testing the old samples with new assay as performed in England (41). This would be of great interest to assess prevalence of different pathogens in general healthy population, the relevance of mixed infections and the influence of viral loads in relation to clinical manifestations.

The use of the assays developed during this thesis will cover most probable viruses that are involved in gastroenteritis outbreaks. Investigation on samples from unexplained outbreaks is exciting and should be continued, but detection of a pathogen in almost 90% of all outbreaks is a great achievement.

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Summary

Samenvatting

Sažetak

SUMMARY

This thesis will provide an overview of my research done on viral gastroenteritis outbreaks in the Netherlands, specific and generic assays for detection of viral pathogens in fecal samples, and reference assays for detection of viruses in cases of gastroenteritis. It describes an approach that can serve as a basis for an efficient use of detection techniques on samples from gastroenteritis outbreaks suspected of viral etiology.
The knowledge obtained in this thesis may help to reduce the diagnostic gap in viral gastroenteritis outbreaks by use of a systemic approach, which includes specific and generic viral assays.

In the introduction of this thesis, **chapter 1**, I present a review of viral agents that cause viral gastroenteritis, and their detection methods. Also, surveillance of viral gastroenteritis outbreaks and used detection methods in the period from 1994 through 2009 is described.

To determine the role of viruses in outbreaks of acute gastroenteritis in the Netherlands all the data collected from 1994 through 2005 were analyzed. In a study, described in **chapter 2**, a total of 6707 stool samples from 942 outbreaks were investigated. Stool samples were tested for the presence of noroviruses, sapoviruses, rotaviruses, adenoviruses, astroviruses and Aichi viruses by electron microscopy, enzyme-linked immune assays, and/or reverse transcription PCR. Since the start of the gastroenteritis surveillance in 1994, the number of outbreaks reported had increased, mainly due to rising numbers of outbreaks caused by noroviruses, rotaviruses and outbreaks that remained unexplained. We showed that 78% of the outbreaks of gastroenteritis were caused by noroviruses, 4.9% by rotaviruses, 1% by adenoviruses, 0.5% by astroviruses and none by sapoviruses or Aichiviruses. Re-testing of the viral gastroenteritis outbreaks, which remained without diagnosis, with the new and at that time state-of-the-art detection assays, led to finding the causative agent in 17.9% of the previously unexplained outbreaks. Despite thorough testing, 12% of the outbreaks suspected of having viral etiologies remained unexplained.
To allow more sensitive, specific, and faster diagnostics for common viral gastroenteritis pathogens the method for detecting the four most important viruses in gastroenteritis outbreaks and sapovirus, that were detected in outbreaks since 2007, was updated again, in 2009. **Chapter 3** describes the novel approach for detection of these enteric viruses in which the virus specific reverse transcriptase primers were replaced by random priming, an internal control was included and the separate assays were adapted to allow for parallel testing in a multiplex format. This assay is a two-step method in which in the first step we screened for the most common causes of viral gastroenteritis, noroviruses of genogroup II and rotaviruses of group A, with equine arteritis virus used as the internal control. Subsequently, in the second step, two parallel PCR assays were developed for the detection of noroviruses of genogroup I and the internal control equine arteritis virus in one run and adenoviruses, sapoviruses, and astroviruses in the other run. This approach allows rapid and cost-effective elucidation of the common causes, noroviruses, rotaviruses, sapoviruses, adenoviruses and astroviruses, of acute gastroenteritis outbreaks. The results obtained in this chapter showed that this new method is highly sensitive and specific for the differentiation of viruses. If this approach is used 80 to 90% of gastroenteritis outbreaks can be diagnosed. The detection of all these viruses is rapid, with a turnaround time of 1 day, whereas at least 3 days were required when the previous methods were used. Hands-on-time was reduced from 8 to 2 hours. This approach leads to reductions in labor and reagent costs, which allows high-throughput testing for pathogens that cause viral gastroenteritis. Furthermore, due to the parallel testing, the detection of mixed infections is much more likely and this will increase the insight in the real causes of acute gastroenteritis. The inclusion of an internal control enables screening for sporadic cases of acute gastroenteritis.

In order to discover if other viruses were related to gastroenteritis outbreaks, novel detection methods were developed and applied on the stool samples from all viral gastroenteritis outbreaks that remained without diagnosis after testing for the pathogens as described in chapter 2 and 3. A new generic real-time assay for detection of vesiviruses was developed, as described in **chapter 4**. This genus of family *Caliciviridae* causes gastroenteritis, vesicular skin lesions, hemorrhagic disease, respiratory diseases and other conditions in animals. A report on *Vesivirus* viremia, as detected by PCR, in samples from patients with hepatitis of unknown etiology in the USA suggested a zoonotic potential for vesiviruses, but this

finding was not confirmed by others. In order to investigate its role in viral gastroenteritis outbreaks, or more specifically, to see if it has been causing any of the unexplained outbreaks, we applied this assay on all samples from outbreaks of unexplained etiologies. No vesivirus was detected in any of the outbreaks of gastroenteritis. Subsequently, this generic assay was used on serum samples from patients with hepatitis, throat-swab specimens of patients with rash illnesses, throat-swab and nose-swabs of patients with acute respiratory diseases, cell cultures with cytopathologic effect from enterovirus surveillance. Again, in none of these samples vesiviruses were found. Therefore, it can be concluded that vesiviruses are an unlikely cause of acute hepatitis and gastroenteritis, rash illnesses, respiratory and intestinal diseases in humans in the Netherlands.

In 2007 sapoviruses were found to cause outbreaks of viral gastroenteritis for the first time in the Netherlands, as described in **chapter 5.** Sapoviruses belong to the *Caliciviridae* family and can cause gastroenteritis in humans and swine. From November 2007 to September 2009, 478 outbreaks of acute gastroenteritis were tested for sapoviruses, and the viruses were detected in 4% of all outbreaks. Sapoviruses are known to cause sporadic infections in children, and although outbreaks were tested that involve children and adults, sapovirus outbreaks were mostly found in adults and were therefore remarkable. The majority of outbreaks was caused by sapoviruses genotype I.2. Phylogenetic analysis showed high homology between genotype I.2 strains detected in different geographical regions (Sweden, Slovenia, Taiwan, Japan, and Russia). Combined with data from Sweden, Hungary and Slovenia we concluded that sapovirus infections were increasing in Europe. Therefore the inclusion of sapovirus diagnostic assay in the analysis of the outbreaks suspected of viral gastroenteritis was recommended for the reference laboratories

Chapter 6 describes a study on *Clostridium difficile* in outbreaks of viral gastroenteritis in elderly. These bacteria have been described to cause outbreaks simultaneously with noroviruses. The coincidental increase in norovirus outbreaks and *Clostridium difficile* infections raised the question if these events could be related. Therefore, we studied the prevalence of *C. difficile* in outbreaks of viral gastroenteritis in nursing homes for elderly and characterized enzyme-immunoassay positive stool samples. Stool samples from nursing homes residents in 137 outbreaks of viral etiology were investigated for the presence of *C. difficile* toxins. We found no association between *C. difficile* and viral gastroenteritis outbreaks in elderly in the Netherlands. We did find that *C. difficile* enzyme-immunoassay reacted positive with several other clostridia species in the stored stool samples we used. The high rate of false-positive EIA samples showed the low specificity of this assay when used in retrospective studies. We suggest the use of a confirmation test when this test is used for surveillance purposes.

The optimized and novel molecular detection assays that were developed during this thesis detecting the common viral gastro-enteritis pathogens (noroviruses, rotaviruses, adenoviruses, astroviruses and sapoviruses) led to elucidation of the previously unexplained outbreaks. Other viruses such as Aichi viruses, coronaviruses and vesiviruses, as well as *C. difficile* were not detected in these outbreaks. After thorough testing about 12 % of the outbreaks remained unexplained we decided to use viral discovery techniques in order to detect other viruses. Viral metagenomics is described in **chapter 7**. The first set of samples used for viral metagenomics were unexplained cell culture samples from enterovirus surveillance. These samples had a cytopathologic effect on cell lines, but tested negative for a range of different viruses, such as enteroviruses, parechoviruses, noroviruses, sapoviruses, rotaviruses, adenoviruses, astroviruses, reoviruses, rhinoviruses, hepatitis A en E viruses, Influenza A and B viruses, Aichi viruses, Coronaviruses 229E, NL63, and OC43, RSV A and B, and hMPV. In aall of the samples a virus was detected within a week. In the majority of these samples (four of seven) newly identified Saffold viruses were detected, and four complete genome sequences of Saffold viruses were obtained. The pathogenicity of the identified viruses in humans has not been established. This study demonstrated that viral metagenomics is a powerful tool that can be used to investigate samples with unexplained etiology and can be integrated into public health monitoring efforts to investigate for viruses in unidentified clinical samples where a standard pathogen specific approach such as PCR fails to detect viruses. Use of metagenomic analysis directly on stool samples was hampered by numerous bacterial sequences. Viral sequences were detected, however more in-depth sequencing, as done by 454 sequencing, is needed for identification of virus sequences in stool samples.

Finally, the general discussion and concluding remarks are described in **chapter 8.** Here we

conclude that detection of a pathogen in almost 90% of all outbreaks suspected of viral etiology is a great achievement. With our systematic approach for evaluation of gastroenteritis outbreaks, we confirmed that noroviruses cause the majority of the gastroenteritis, followed by rotaviruses. If outbreaks test negative, it is recommended to test for a wider spectrum of etiologies, such as sapoviruses, adenoviruses, bacterial pathogens *Salmonella* and *Campylobacter*, and pathogenic parasites *Giardia lamblia* and *Cryptosporidium*. The remaining unexplained outbreaks can be tested by reference centers or universities for new viruses or other pathogens by use of (viral) metagenomics in combination with 454 sequencing.

SAMENVATTING

Dit proefschrift geeft een overzicht van mijn onderzoek naar uitbraken van virale gastro-enteritis, beter bekend als buik griep, in Nederland. Ik beschrijf de ontwikkeling en resultaten van verschillende testen voor de detectie van virale ziekteverwekkers in fecale monsters van gastro-enteritis uitbraken. Sommige testen zijn specifiek, die detecteren één bepaald virus, andere zijn meer generiek, die kunnen meerdere aan elkaar verwante virussen detecteren. Ook worden in dit proefschrift de huidige referentie testen voor de detectie van virussen in gastro-enteritis uitbraken gepresenteerd. Dit proefschrift kan dienen als basis voor een efficiënt gebruik van detectie technieken op de patiënten monsters van gastro-enteritis uitbraken waarvoor een virale ziekteverwekker wordt verdacht. Indien de systematische aanpak, die in dit proefschrift beschreven is en die specifieke en generieke virale testen omvat, gevolgd wordt, kan een groot aantal uitbraken van gastro-enteritis verklaard worden.

In de introductie van dit proefschrift, **hoofdstuk 1**, beschrijf ik de ziekteverwekkers van de virale gastro-enteritis, hun moleculaire detectie methoden en het gebruik ervan. Verder is er ook een kort overzicht gemaakt van de detectie technieken die gebruikt zijn gedurende de surveillance van de virale gastro-enteritis uitbraken in Nederland in de periode van 1994 tot en met 2009.

Om de rol van de virale ziekteverwekkers in de uitbraken van acute gastro-enteritis in Nederland te kunnen bepalen zijn de data van 1994 tot en met 2005 verzameld en geanalyseerd. Tijdens deze studie, die beschreven is in **hoofdstuk 2**, zijn er in totaal 6707 fecale monsters uit 942 uitbraken verzameld en onderzocht. Deze fecale monsters zijn getest op de aanwezigheid van de meest bekende virussen die gastro-enteritis veroorzaken, namelijk norovirussen, rotavirussen en adenovirussen. Ook hebben we getest voor andere virussen waarvoor gerapporteerd was dat ze gastro-enteritis kunnen veroorzaken, zoals sapovirussen, astrovirussen en Aichi virussen. We hebben gebruik gemaakt van de elektronen microscopie, enzyme–linked immuno assays en reverse transcriptase PCRs. Sinds de start van de gastro-enteritis uitbraken surveillance in 1994 is er een toename gezien in de aantallen van de gerapporteerde uitbraken. Dit was voornamelijk het gevolg van stijgende aantallen uitbraken veroorzaakt door norovirussen, rotavirussen en onverklaarde uitbraken (uitbraken zonder diagnose). We toonden aan dat 78% van de uitbraken van gastro-enteritis veroorzaakt zijn door norovirussen, 4,9% door rotavirussen, 1% door adenovirussen, en 0,5% door astroviruses. Hertesten van de virale gastro-enteritis uitbraken, zonder diagnose, met de nieuwe en verbeterde detectie methoden heeft geleid tot het vinden van een virus in 17,9% van deze eerder onverklaarde uitbraken. Hoewel alle gerapporteerde gastro-enteritis uitbraken, met een verdenking voor een virale infectie grondig getest zijn voor virussen, bleven ongeveer 12% van de uitbraken onverklaard.
Om een meer gevoelige, specifieke en snellere diagnostiek voor de verwekkers van de virale gastro-enteritis te kunnen doen is een nieuwe detectie methode ontwikkeld in 2009. Deze methode detecteert de vier belangrijke virussen die gastro-enteritis uitbraken veroorzaken, namelijk norovirussen, rotavirussen, adenovirussen, astrovirussen en daarnaast ook sapovirussen, die vanaf 2007 gevonden worden in de gastro-enteritis uitbraken. In **hoofdstuk 3** wordt deze nieuwe benadering voor de detectie van deze enterale virussen beschreven. Tijdens deze studie zijn de specifieke primers, die aan één bepaalde virus kunnen binden en die voorheen gebruikt zijn in de specifieke PCRs, vervangen door de multiplex PCR testen, die dus meerdere virussen in één keer kunnen detecteren, en die gebruik maken van random primers die aan alle virussen kunnen binden tijdens de reverse transcriptie reactie. Deze test is een twee-staps methode waarbij in de eerste stap gezocht wordt naar de meest voorkomende veroorzakers van virale gastro-enteritis, norovirussen (van genogroep II) en rotavirussen en een interne controle (equine arteritis virus, EAV) waarmee de reactie gecontroleerd wordt. Vervolgens, in de tweede stap, worden er twee parallelle PCR-testen ingezet voor de detectie van norovirussen (genogroep I) en opnieuw de interne controle; in de andere run worden adenovirussen, sapoviruses en astroviruses gedetecteerd. Deze benadering geeft een snelle en kosteneffectieve detectie/diagnose van de meest voorkomende virale verwekkers van de acute gastro-enteritis uitbraken. Als de multiplex PCR gebruikt wordt, dan worden gemiddeld tussen de 80 tot 90% van de uitbraken gediagnosticeerd. De resultaten verkregen in dit hoofdstuk gaven aan dat deze nieuwe methode zeer gevoelig en specifiek is voor de detectie van virussen. De detectie van deze virussen is snel. Doorlooptijd (het vinden van deze virussen als ze aanwezig zijn in een fecaal monster) van deze test is 1,

terwijl we met de voorheen gebruikte methode ten minste 3 dagen nodig hadden om voor al deze virussen te kunnen testen. Ook is de hands-on-time (tijd dat je werkelijk bezig bent met het inzetten van deze test) aanzienlijk verminderd, van 8 uur naar ongeveer 2 uur. Deze aanpak leidt tevens tot vermindering van de arbeids- en reagens kosten, en maakt het high-throughput testen voor ziekteverwekkers van virale gastro-enteritis mogelijk. Bovendien, als gevolg van het parallel testen, is de detectie van gemengde infecties, waarin meer dan een virus betrokken is, ook veel waarschijnlijker. Deze nieuwe test zal het inzicht in de echte veroorzakers van acute gastro-enteritis vergroten. Deze nieuwe methode bevat ook een interne controle die extractie controleert op efficiëntie en maakt de controle op de remming in de PCR mogelijk, daarom is deze test ook beter geschikt voor diagnostiek op monsters van sporadische gevallen.
Om te ontdekken of andere virussen in verband met gastro-enteritis uitbraken gebracht kunnen worden, zijn nieuwe detectiemethoden ontwikkeld en toegepast op de monsters van alle virale gastro-enteritis uitbraken zonder diagnose. Een nieuwe generieke real-time test voor de detectie van vesiviruses werd ontwikkeld, dit is beschreven in **hoofdstuk 4**. Vesivirussen behoren net als de norovirussen en sapovirusen tot de familie *Caliciviridae,* deze virussen veroorzaken gastro-enteritis, vesiculaire huidletsels, hemorragische ziekte, aandoeningen aan de luchtwegen en andere aandoeningen bij dieren. Door een onderzoeksgroep in de VS is *Vesivirus* viremie in mensen beschreven. Deze bevindingen zijn gedaan door gebruik te maken van een PCR detectie test op monsters van patiënten met hepatitis van onbekende etiologie (oorzaak). Het werd gesuggereerd dat vesivirussen potentieel zoonotisch zijn, maar deze bevindingen zijn niet bevestigd door andere onderzoeksgroepen. Om de rol van de vesivirussen in virale gastro-enteritis uitbraken te onderzoeken, of meer specifiek, om te bepalen of deze virussen mogelijke veroorzakers zijn van de onverklaarde uitbraken van gastro-enteritis in Nederland, hebben we een generieke moleculaire test ontwikkeld en toegepast op de fecale monsters uit de onverklaarde uitbraken. Er zijn hierin geen vesivirussen gedetecteerd. Vervolgens werd deze generieke test gebruikt op serummonsters van patiënten met hepatitis, keeluitstrijkjes van patiënten met huiduitslag, keel- en neusuitstrijkjes van patiënten met acute aandoeningen van de luchtwegen en de celculturen met cytopathologisch effect uit de materialen verzameld in de enterovirus surveillance. Ook in deze monsters zijn geen vesivirusen gevonden. Daarom kan er worden geconcludeerd dat vesivirussen waarschijnlijk geen oorzaak zijn van acute hepatitis en gastro-enteritis, dermatologische aandoeningen, respiratoire en intestinale ziekten bij de mens in Nederland.

In 2007 werden de sapovirussen voor het eerst als veroorzakers van uitbraken van virale gastro-enteritis in Nederland gevonden en dit wordt beschreven in **hoofdstuk 5**. Sapovirussen behoren tot de *Caliciviridae* familie en kunnen gastro-enteritis bij de mens en varkens veroorzaken. In de periode van november 2007 tot september 2009, werd in 4% van de 478 uitbraken van acute gastro-enteritis sapovirus gedetecteerd. Het is bekend dat sapovirussen sporadische infecties bij kinderen kunnen veroorzaken, en in deze studie hebben we uitbraken getest die voorkwamen bij kinderen en volwassenen. Opmerkelijk was dat sapovirus uitbraken vooral bij volwassenen werden gevonden. De meerderheid van de uitbraken werd veroorzaakt door sapovirus genotype I.2. Door gebruik te maken van de fylogenetische analyse werd een hoge homologie (overeenkomst) gevonden tussen genotype I.2 stammen die in dezelfde periode circuleerden in Zweden, Slovenië, Taiwan, Japan en Rusland. Gecombineerd met gegevens uit Zweden, Hongarije en Slovenië hebben we geconcludeerd dat sapovirus infecties in Europa toenemen. De opname van een diagnostische test voor sapovirus is aanbevolen voor de analyse van de virale gastro-enteritis uitbraken.
In **hoofdstuk 6** wordt een onderzoek beschreven naar *Clostridium difficile* in de uitbraken van virale gastro-enteritis bij ouderen. Een stijging van de norovirus uitbraken en *Clostridium difficile* infecties leidde tot de vraag of deze gebeurtenissen aan elkaar gerelateerd zijn. Een studie naar de prevalentie (voorkomen) van *C. difficile* in de uitbraken van virale gastro-enteritis in verpleeghuizen werd gestart. Feces van 137 uitbraken van virale gastro-enteritis werden onderzocht op de aanwezigheid van *C. difficile* toxines met een enzyme-immunoassay. Wij vonden geen associatie tussen *C. difficile* en virale gastro-enteritis uitbraken bij ouderen in Nederland. We zagen wel dat de gebruikte enzyme-immunoassay, positief geregeerde met een aantal andere *Clostridia* soorten die aanwezig waren in de fecale monsters. Het hoge percentage van vals-positieve monsters benadrukt de ongeschiktheid van deze enzym-immunoassay test voor surveillance doeleinden wanneer wordt getest op bewaarde monsters. In dat soort gevallen is het raadzaam de positieve bevindingen te bevestigen met een andere test.

De geoptimaliseerde en nieuwe moleculaire detectie testen die ontwikkeld zijn gedurende dit

promotie traject en die de algemene gastro-enteritis pathogenen detecteren, namelijk norovirussen, rotavirussen, adenovirussen, astrovirussen en sapovirussen, hebben bijgedragen tot het verklaren van een gedeelte van de voorheen onverklaarde uitbraken. Andere virussen zoals Aichi virussen, coronavirussen en vesivirussen, evenals *C. difficile* zijn niet gevonden in deze uitbraken. Omdat we na uitgebreid testen voor norovirussen, sapoviruses, rotavirussen, adenovirussen, astroviruses, enterovirussen en parechovirussen nog 12% van de uitbraken niet konden verklaren hebben we gebruik gemaakt van, in ontwikkeling zijnde, virale ontdekkingsmethoden in een poging om andere virussen te detecteren. In **hoofdstuk 7** is de virale metagenomic analyse in detail beschreven. Hiervoor zijn in eerste instantie kweek suspensies uit fecale monsters van de enterovirus surveillance gebruikt . Deze monsters hadden een cytopatologisch effect op cellijnen getoond, maar waren negatief in een reeks van testen voor verschillende enterale virussen, zoals enterovirussen, parechoviruses, norovirussen, sapoviruses, rotavirussen, adenovirussen, astroviruses, reovirussen, rhinovirussen, hepatitis A en E virussen, Influenza A en B virussen, Aichi virussen, Coronavirussen 229E, NL63, en OC43, RSV A en B, en hMPV virussen. In elk van deze monsters werd binnen een week, een virus geïdentificeerd met behulp van virale metagenomics. In vier van de zeven geteste monsters zijn Saffold virussen gedetecteerd waaroor tevens de complete genoomsequenties zijn verkregen. De pathogeniciteit van de geïdentificeerde virussen is niet vastgesteld. Deze studie heeft aangetoond dat virale metagenomics een krachtig instrument is die gebruikt kan worden voor de identificatie van virussen in monsters die in de standaard pathogeen specifieke PCR testen negatief zijn gebleven. Deze techniek zou toepast kunnen worden in onderzoeks- en referentiecentra. Het gebruikt van deze methode rechtstreeks op de fecale materialen was gehinderd door de talrijke bacteriële sequenties die aanwezig zijn in deze monsters. Het is mogelijk dat virale ontdekkingsmethode in combinatie met bijvoorbeeld 454 sequencing wel geschikt is voor identificatie van virus sequenties in dergelijke monsters.

In de laatste hoofdstuk van dit proefschrift, **hoofdstuk 8**, zijn de algemene discussie en concluderende opmerkingen beschreven. We concluderen dat de detectie van een pathogeen in bijna 90% van alle gastro-enteritis uitbraken een grote prestatie is. Met onze systematische aanpak hebben we bevestigd dat norovirussen de meerderheid van de gastro-enteritis uitbraken veroorzaken, gevolgd door rotavirussen. Indien de uitbraken negatief zijn voor norovirussen en rotavirussen, is het aangeraden om deze uitbraken verder te testen voor een breder spectrum van pathogenen, zoals sapovirussen, adenovirussen, bacteriële pathogenen *Salmonella* en *Campylobacter* en parasitaire pathogenen *Giardia lamblia* en *Cryptosporidium*. De resterende, onverklaarde uitbraken kunnen door gebruik te maken van virale metagenomics in combinatie met 454 sequencing getest worden voor nieuwe virussen of andere pathogenen. Dit zou gedaan kunnen worden door de universiteiten en referentie centra die deze methode tot hun beschikking hebben.

SAŽETAK

Ova teza će ukratko pružiti pregled mojih istraživanja koji su urađeni na polju epidemija virusnog gastroenteritisa, to jest epidemija stomačne gripe prouzrokovane virusima, u Nizozemskoj (Holandiji). U ovoj tezi su prezentirani resultati, opisani specifični testovi , koji detektiraju samo jedan virus, i generični (opšti) testovi, koji detektiraju nekoliko srodnih ili vezanih virusa odjednom. Ovi testovi se mogu koristiti za otkrivanje virusnih patogena u uzorcima stolica gastroenteritisa. Također, u ovom radu su prezentirani referentni testovi ili bolje rečeno sistematski pristup za otkrivanje virusa u uzorcima stolica gastroenteritisa. Ovaj sistematski pristup može poslužiti kao osnova za efikasnu upotrebu tehnike i tehničkih sredstava za detekciju virusa u uzorcima stolica od pacijenata koji imaju stomačnu gripu i gdje postoji sumnja da su virusi prouzrokovali ovu infekciju. Korištenje ovog sistematskog pristupa omogućuje pronalazak virusa u velikom dijelu od gastro-enteritis episoda.

U uvodu ove teze, **poglavlju 1**, predstavljam pregled uzročnika (patogena) virusnog gastroenteritisa, te molekularne metode za detekciju ovih virusa i korištenje opisanih metoda. Tu je opisan i kratak pregled tehinke koji se koristio tokom nadzora (praćenja) nad epidemijama virusnog gastroenteritisa u Nizozemskoj od 1994 do 2009 godine.

Da bi se uloga virusa u epidemijama akutnog gastroenteritisa mogla odrediti svi podatci o epidemijama koje su se desile u Nizozemskoj od 1994 do 2005 godine su prikupljeni i analizirani. Tokom ovog studija, koji je opisan u **poglavlju 2**, ukupno 6707 uzoraka stolica, prikupljenih iz 942 epidemija gastroenteritisa su ispitani (jedan epidemija se sastoji od najmanje dva uzorka stolice). Stolični uzorci su ponovo testirani na prisustvo virusa koji su poznati kao najčešći uzroci stomačnu gripe, naime, norovirusa, rotavirusa i adenovirusa. Također, ovi stolični uzorci su testirani na prisustvo drugih virusa koji prouzrokuju stomačnu gripu, kao sto su sapovirusi, astrovirusi i Aichi virusi, koristeći elektronsku mikroskopiju, enzim-linked imunološke testove, te reverznu transkriptazu polimeraznu lančanu reakciju, to je način da se jednolančana DNK pretvori u dvolančanu DNA, te da se pristuni DNK umnoži). Od početka nadzora gastroenteritis epidemija, s početkom u 1994 godini,vidio se porast u broju prijavljenih slučajeva epidemija. Uzrok povećanja prijavljenih broja epidemija je direktno povezano sa povećanjem norovirus i rotavirus epidemija, ali i brojem epidemija u kojima se nije mogao pronaci virus. Mi smo pokazali da je 78% slučajeva epidemija gastroenteritisa bilo uzrokovano od norovirusa, 4,9% epidemija od rotavirusa, 1% od adenovirusa i 0,5% od astrovirusa. Ponovno testiranje uzoraka stolica iz neobjašenjenih epidemija stomacne gripre (virusnog gastroenteritisa) sa novim i poboljšanim metodama je dovelo do pronalaženja virusa u 17,9% od prethodno neobjašnjenih epidemija. Iako su sve prijavljene epidemije gastroenteritisa, za koje je sumnjano da su prouzrokovane virusima, temeljito testirane za sve gore navedene viruse oko 12% slučajeva je ostalo neobjašnjeno.

Osjetljivija, specifičnija i brža dijagnostika virusa u uzorcina stolica gastroenteritisa je omogućena sa novom metodom za detekciju koja je razvijena u 2009 godini. Ova metoda detektira četiri glavna virusa koji uzrokuju gastroenteritis epidemije, naime, noroviruse, rotaviruse, adenoviruse, astroviruse, ali i sapoviruse, koji su početkom 2007 godine pronađeni kao uzročnici epidemija gastroenteritisa u Nizozemskoj. U **poglavlju 3**, ovaj novi način za detekciju gore navedenih virusa je opisan. Tokom ovog istraživanja, specifični prajmeri, a to su početnici reakcije da se određeni virus može vezati i umnožiti, koji su se koristili u PCR testovima su zamjenjeni sa multipleks PCR testovima u kojima više virusa odjednom mogu biti detektirani pomoću prajmera koji se za sve viruse se mogu vezati u toku reakcije. Ovaj dio reakcije je poznat kao reverzna transkripcija. Ova metoda se sastoji iz dva stepena, u prvom stepenu se traže virus koji najčešće prouzrokuju gastroenteritis, norovirusi (genetska grupa II) i rotavirusi. Tu se također koristi unutarnja kontrola (EAV) sa kojom se čitava reakcija, to jest detekcija ovih virusa, kontroliše. Zatim, u drugom stepenu detekcije, dva paralelna PCR testa se koriste za detekciju norovirusa (genetske grupe I) i unutarnje kontrole u jednoj reakciji i u drugoj reakciji se vrši detekcija adenovirusa, astrovirusa i sapovirusa. Ovaj pristup omogućuje brzu i ekonomičnu detekciju virusa koji najčešće prouzrokuju akutne gastroenteritis epidemije, naime norovirusa, sapovirusa, rotavirusa, adenovirusa i astrovirusa. Korišćenje multipleks PCR testa ce omogućiti dijagnozu u prosječno 80 do 90% gastroenteritis epidemija. Rezultati prezentirani u ovom poglavlju pokazuju da je ova nova metoda vrlo osjetljiva i specifična za razlikovanje virusa, ako su virusi prisutni u uzorcima

stolica. Otkrivanje navedenih virusa je brza, uz vrijeme obrta od jednog dana, dok su najmanje tri dana bila potrebna kada su prethodne metode bile korištene. Radno vrijeme koje je potrebno da bi se resultati dobili je skraćeno sa 8 na 2 sata. Ovakav sistematski pristup dovodi do smanjenja troškova rada i reagensa, te omogućuje visoki obrt za testiranje virusa. S obzirom na paralelno ispitivanje, više virusa se moze detektirati istovermeno, omogućujući otkrivanje miješanih infekcija, to jest infekcija u kojima je više virusa prisutno. Korištenje ove metode će povećati uvid u prave uzroke akutnog viralnog gastroenteritisa. Uključivanje unutarnje kontrole, omogućuje kontrolu reakcije, te dozvoljava da se pojedinačni slučajevi stomačne gripe, znači pojedinačne osobe koje imaju stomačnu gripu, mogu testirati ovom metodom.

Da bismo otkrili dali su drugi virusi povezani sa gastroenteritisom, nove metode za detekciju su razvijene i primjenjene na uzorke iz gastroenteritis epidemija bez dijagnoze (takozvane neobjašnjenje epidemije). Novi generični test za otkrivanje vesivirusa je razvijen i opisan u **poglavlju 4**. Vesivirusi, dio od familije *Caliciviridae,* prouzrokuju gastroenteritis, vezikularne kožne lezije, hemoragijske bolesti, bolesti dišnih organa i druge bolesti kod životinja. Od strane istraživačke grupe u SAD-u, Vesivirus viremija kod ljudi je opisana. Ovi nalazi su otkriveni pomoću PCR testa u uzorcima od pacijenata sa hepatitisom, u kojima nijedan drugi virus osim vesivirusa nije bio pronađen. Tu je predloženo da vesivirusi potencijalno prouzrokuju zo-onoze, bolesti koji prelaze za životinja na ljude, ali ti rezultati nisu potvrđeni od drugih istraživačkih grupa. Zbog toga smo odlučili da se uloga vesivirusa u gastroenteritis epidemijama treba ispitati, ili preciznije treba utvrditi dali su vesivirusi mogući uzroci neobjašnjenih gastroenteritis epidemija u Nizozemskoj. Za ovu istragu je generični molekularni test razvijen i primjenjen na stoličnim uzorcima iz neobjašnjenih epidemija gastroenterisa. Vesivirusi nisu otkriveni u ovim uzorcima. Nakon toga, ovaj generički test korišten je na uzorcima seruma od bolesnika s hepatitisom, na brisevima iz grla od bolesnika s osip bolestima, grlo-nos-obriscima od bolesnika s akutnim respiratornim bolestima, i na kulturama stanica u kojima je primjećeno da je neki virus prisutan. Ove kulturne stanice su prikupljene tokom nadzora infekcija od enterovirusa. Opet, ni u jednom od tih uzoraka vesivirusi nisu pronađeni. Zbog toga se može zaključiti da vesivirusi vjerojatno nisu uzrok akutnog hepatitisa i gastroenteritisa, osip i dermatoloskih bolesti, respiratornih i crijevnih bolesti kod ljudi u Nizozemskoj.

U 2007 godini je po prvi put utvrđeno da su sapovirusi uzroci pojava virusnog gastroenteritisa u Nizozemskoj, kao što je opisano u **poglavlju 5**. Sapovirusi pripadaju *Caliciviridae* familiji i mogu prouzrokovati gastroenteritis kod ljudi i svinja. U razdoblju od novembera 2007 do septembra 2009, 478 epidemija akutnog gastroenteritisa je testirano na prisustvo sapovirusa u ovim uzorcima. Otkriveno je da su sapovirus prisutni u 4% od 478 epidemija gastroenteritisa. Poznato je da sapovirusi uzrokuju sporadične (pojedinačne) infekcije gastroenterisa kod djece, međutim ovdje su testirani uzorci iz epidemija koje uključuju djecu. ali i odrasle. Nadalje, sapovirusi su većinom pronađeni u epidemijama koje uključuju odrasle, nešto što se vidi često i zbog toga su ovi rezultati izuzetni. Većina sapovirus epidemija je bila prouzrokovana od sapovirusa genotipa I.2. Filogenetske analize su pokazale visoku homologiju (sličnost) između sapovirusa genotipa I.2 koje su otkrivene u različitim geografskim regijama (Švedska, Slovenija, Tajvan, Japan i Rusija). U kombinaciji s podacima iz Švedske, Mađarske i Slovenije zaključili smo da su sapovirus gastroenteritis infekcije u porastu u Europi. Uključenje sapovirus dijagnostičkih metoda za analizu gastroenteritis epidemija, gdje postoji sumnja da su virusi mogući uzroci infekcije, je zbog toga preporučeno za referentne laboratorije.

Poglavlje 6 opisuje studij o *Clostridium difficile* bakteriji u epidemijama virusnog gastroenteritisa kod starijih osoba. Te bakterije su opisane da prouzrokuju epidemije gastroenteritsa istovremeno s norovirusima. Porast norovirus epidemija i *Clostridium difficile* infekcija je dovelo do pitanja da li su ovi događaji povezani. Zbog toga je pokrenut studij koji istražuje učešće *C. difficile* u epidemijama virusnog gastroenteritisa koje su se desile u staračkim domovima. Fekalni uzorci od 137 epidemija virusnog gastroenteritisa su ispitani na prisutnost *C. difficile* toksina. Nismo pronašli povezanost između *C. difficile* i epidemija virusnog gastroenteritisa kod starijih osoba u Nizozemskoj. Vidjeli smo da je enzim-imuno test, koji je korišten, reagovao pozitivno s nekoliko drugih vrsta klostridija u uzorcima stolica koje su čuvane na 4°C i korištene za diagnostiku *C. difficile*. Visoki stupanj lažno-pozitivnih uzoraka ukazuje na nesposobnost ovog testa za nadzore svrhe. Prisutnost drugih vrsta klostridija može reagirati pozitivno ako se ovaj test koristi na fekalnim uzorcima koji su duže čuvani na 4°C, zbog toga je potrebna još jedna potvrda *za Clostridium difficile* infekcije (CDI) ako se ovaj test koristi.

Optimalizacija molekularnih metoda i razvijanje novih metoda za detekciju virusa tokom ovog doktorskog studija, koji detektiraju noroviruse, rotaviruse, adenoviruse, astroviruse i sapoviruse su pridonijele pronalaženju virusa u uzorcima od neobjašnjenih gastroenteritis epidemija. Drugi virusi kao što su Aichi virusi, coronavirusi, vesivirusi i *C. difficile* bakterija nisu otkriveni u stoličnim uzorcima iz epidemija. Pošto je poslije testiranja za sve ove viruse ostalo oko 12 % neobjašnjenih epidemija, odlučili smo da koristimo metode za otkrivanje novih virusa da bi smo pronašli neki drugi virus u ovim uzorcima. U **poglavlju 7** je metoda za otkrivanje novih virusa, virusna metagenomic analiza, opisana u detalj. Prvi set uzoraka korištenih za virusni metagenomics su bile neobjašnjene kulture iz uzoraka koje su skupljene u toku nadzora nad enterovirus infekcijama. Ovi uzorci su ostavili utisak na stanične linije, primjećeno je da je neki virus ili toksin prisutan u tim uzorcima, ali su bili negativni u nizu testova za različite viruse, kao što su naprimjer enterovirusi, parechovirusi, norovirusi, sapovirusi, rotavirusi, adenovirusi, astrovirusi, reovirusi, rhinovirusi, hepatitis A i E virusi, Influenza A i B virusi, Aichi virusi, Coronavirusi 229E, NL63, en OC43, RSV A en B, en hMPV virusi. Korištenjem ove tehnike za otkrivanje virusa je u svakom od tih uzoraka, u roku od sedam dana rada, virus identificiran. U većini uzoraka (četiri od sedam) Saffold virusi su otkriveni. Za ova četiri uzorka su potpune genom-sekvencije otkrivene. Patogenost, mogućnost da ovi virusi prouzrokuju infekciju, od ovih virusa nije utvrđen. Ovaj studij je pokazao da je virusni metagenomics snažna metoda koja može biti integrirana za prepoznavanje virusa kada standardni patogen-specifični PCR testovi nisu u stanju da detektiraju virus. Ova tehnika nudi mogućnost da se uzroci od neobjašnjenih infekcija mogu razumjeti i mogla bi se primijeniti u istraživanju u referalnim centrima. Korištenje metagenomic analize direktno na fekalnom materijalu, stolicama, je spriječena brojnim bakterijskim sekvencijama koje su prisutne u uzorcima. Virusne sekvencije su bile pronađene u ovim uzorcima, ali mnogo detaljnije sekvencije, kao sto se mogu dobiti sa 454 sekvenciranjem, su potrebne za identifikaciju patogena u uzorcima stolica.

U posljednjem poglavlju ove teze, **poglavlju 8**, opšta diskusija i zaključna razmatranja su opisana. Ovdje možemo zaključiti da je otkrivanje patogena u gotovo 90% od svih gastroenteritis epidemija, gdje postoji sumnja da je virus uključen u infekciju, veliki uspjeh. Uz naš sistematski pristup, potvrdili smo da norovirusi uzrokuju najveći dio gastroenteritis epidemija, zatim slijede rotavirusi. Ako epidemije ostanu negativne nakon testiranja za noroviruse i rotaviruse, onda savjetujemo da se ove epidemije testiraju za širi spektar drugih patogena, kao što su sapovirusi, adenovirusi, bakterije *Salmonella* i *Campylobacter*, te parasiti *Giardia lamblia* i *Cryptosporidium*. Preostale, neobjašnjene epidemije se mogu testirati u referalnim centrima ili univerzitetima sa metodama koje se koriste za otkrivanje novih virusa ili drugih patogena koje se treba kombinirati sa 454 sekvenciranjem.

Dankwoord

DANKWOORD

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En hier zet ik een .

Curriculum vitae

Publication list

PhD portfolio

CURRICULUM VITAE

Sanela Svraka-Latifovic werd geboren op 26 december 1978 in Prijedor, Bosnië en Herzegovina. Het HAVO diploma haalde ze in 1997 en vijf VWO certificaten in 1998 aan het Alexander Hegius Lyceum te Deventer. In 1998 begon ze een HLO studie Biologie en Medisch Laboratorium Onderzoek en doet afstudeeroprdracht in het Dijkzigt ziekenhuis te Rotterdam, en een stage in het Rijksinstituut voor de volksgezondheid en milieu (RIVM) te Bilthoven. In 2002 haalt ze haar HLO diploma en begint te werken bij het RIVM als onderzoeksmedewerker.
In 2003 begon ze aan HAN University te Nijmegen de (part-time) studie Master of Molecular Life Sciences. Tijdens deze studie deed ze twee afstudeeropdrachten waarvan één aan de Universiteit van Marseille, Frankrijk en één bij het RIVM in samenwerking met Slovak Academy of Sciences, Bratislava, Slovakije. Deze twee afstudeeropdrachten resulteerden in twee publicaties. In 2005 haalde ze het diploma Master of applied sciences en begon ze te werken als onderzoeker in opleiding bij het RIVM onder begeleiding van Prof.dr. Marion Koopmans en Dr. Erwin Duizer. Tijdens haar promotie traject deed ze onderzoek naar onbegrepen gastro-enteritis binnen afdeling virologie, Laboratorium voor Infectieziekten en Screening. Gedurende haar onderzoeksproject werkte ze aan het verbeteren en ontwikkelen van de diagnostische technieken die gebruikt worden om pathogenen aan te tonen in patiëntmonsters uit gastro-enteritis uitbraken. Ze deed drie maanden onderzoek in Verenigde Staten aan de University of South Florida en Lawrence Livermoore National Laboratory, waar ze werkte met de discovery methoden, metagenomic sequencing en discovery microarrays. In maart 2010 begon ze te werken in Tergooiziekenhuizen te Hilversum als moleculair bioloog.

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1. **Rondy, M., M. Koopmans, C. Rotsaert, V. A. N. L. T, B. Beljaars, V. A. N. D. G, J. Siebenga, S. Svraka, J. W. Rossen, P. Teunis, V. A. N. P. W, and L. Verhoef.** Norovirus disease associated with excess mortality and use of statins: a retrospective cohort study of an outbreak following a pilgrimage to Lourdes. Epidemiol Infect**:**1-11.
2. **Svraka, S., E. Duizer, H. Egberink, J. Dekkers, H. Vennema, and M. Koopmans.** 2009. A new generic real-time reverse transcription polymerase chain reaction assay for vesiviruses; vesiviruses were not detected in human samples. J Virol Methods **157:**1-7.
3. **Svraka, S., E. Duizer, H. Vennema, E. de Bruin, B. van der Veer, B. Dorresteijn, and M. Koopmans.** 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. J Clin Microbiol **45:**1389-94.
4. **Svraka, S., E. Kuijper, E. Duizer, D. Bakker, and M. Koopmans.** Clostridium difficile is not associated with outbreaks of viral gastroenteritis in the elderly in the Netherlands. Eur J Clin Microbiol Infect Dis **29:**677-82.
5. **Svraka, S., J. M. Rolain, Y. Bechah, J. Gatabazi, and D. Raoult.** 2006. Rickettsia prowazekii and real-time polymerase chain reaction. Emerg Infect Dis **12:**428-32.
6. **Svraka, S., K. Rosario, E. Duizer, H. van der Avoort, M. Breitbart, and M. Koopmans.** Metagenomic sequencing for virus identification in a public-health setting. J Gen Virol **91:**2846-56.
7. **Svraka, S., R. Toman, L. Skultety, K. Slaba, and W. L. Homan.** 2006. Establishment of a genotyping scheme for Coxiella burnetii. FEMS Microbiol Lett **254:**268-74.
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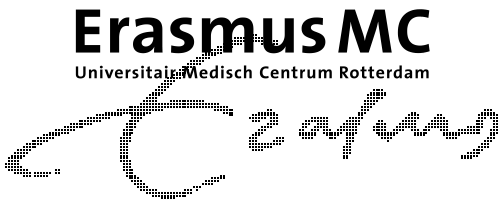
9. **Svraka, S., H. Vennema, B. van der Veer, K. O. Hedlund, M. Thorhagen, J. Siebenga, E. Duizer, and M. Koopmans.** Epidemiology and genotype analysis of emerging sapovirus-associated infections across Europe. J Clin Microbiol **48:**2191-8.
10. **Mattison, K., N. Corneau, I. Berg, A. Bosch, E. Duizer, I. Gutierrez-Aguirre, Y. L'Homme, Y. Lucero, Z. Luo, A. Martyres, M. Myrmel, M. O'Ryan, F. Pagotto, D. Sano, S. Svraka, U. Urzua, and S. Bidawid.** Development and validation of a microarray for the confirmation and typing of norovirus RT-PCR products. J Virol Methods.



PhD Portfolio
Summary of PhD training and teaching

Name PhD student: Ing. Sanela Svraka, Msc
Erasmus MC Department: Virology
Research School: Postgradute School Molecular Medicine

PhD period: Mei 2005- Maart 2010
Promotor(s): Prof. Dr. Marion Koopmans
Supervisor: Dr. Erwin Duizer

1. PhD Training	Year	Workload (Hours/ECTS)
General courses		
- Several courses including Time Management, Mindmapping, Speed reading.	2006-2009	8 hours
- Course "Learn to think differently".	2008	8 hours
- Projectmatig werken.	2007	24 hours
- Biomedical English Writing and Communication.	2007	3 ECTS
- Endnote course.	2007	8 hours
Specific courses (e.g. Research school, Medical Training)		
- Workshop Bioinformatic Analysis, Tools and Services.	2008	8 hours
- Virology course.	2006	40 hours
Seminars and workshops		
- Dutch Annual Virology Symposium 2009.	2009	8 hours
- Mini Symposium Norovirus infections.	2008	4 hours
- Workshop "How to improve your PhD?", Herman Lelieveldt (13-12-2007).	2007	4 hours
- Symposium "Post-infectious Diseases: Molecular Mimicry and beyond". provided by Postgraduate School Molecular Medicine and the department of Virology, Erasmus MC.	2007	8 hours
- BioNumerics database workshop.	2005	8 hours
Presentations		
Oral presentations		
- Samples of unexplained etiologies at RIVM and role of viruses in outbreaks of acute gastroenteritis in the Netherlands (invited speaker). Lawrence Livermore National Laboratory. Livermore, California, US.	2009	20 hours
- Role of viruses in outbreaks of acute gastroenteritis in the Netherlands (invited speaker). University of South Florida, St. Petersburg, Florida, US.	2009	20 hours
- Diagnosis of viral gastroenteritis (invited speaker). Werkgroep Moleculaire Diagnostiek van Infectieziekten. Utrecht, the Netherlands.	2009	40 hours
- Emergence of sapovirus infections in adults. 5th and Final Annual Meeting on the Foodborne Viruses in Europe Network. Uppsala, Sweden.	2009	20 hours
- Emergence of sapovirus infections in adults. European society of clinical virology congress. Winter meeting, Amsterdam, the Netherlands.	2009	40 hours
- Vesiviruses are an unlikely cause of acute hepatitis or gastroenteritis in humans in the Netherlands. European society of clinical virology congress. Saariselka, Finland.	2008	40 hours
- Zoonotic potential of vesiviruses: Do they cause outbreaks of viral gastroenteritis and/or acute hepatitis? Leids Universitair Medisch Centrum. Leiden, the Netherlands.	2008	20 hours
- Outbreaks of viral gastroenteritis in the Netherlands since 1994. (invited speaker). 2nd meeting of Bosnia-Herzegovian researchers from Bosnia and Herzegovina and abroad. Sarajevo, Bosnia-Herzegovina.	2008	40 hours
- Clostridium species found in Norovirus outbreaks in the Netherlands. 4th Annual Meeting on the Foodborne Viruses in Europe Network. Pecs, Hungary.	2007	30 hours
- Outbreaks of viral gastroenteritis in the Netherlands since 1994. American Society for Virology meeting. Madison, Wisconsin, US	2006	40 hours
- Proportion of unexplained outbreaks of gastroenteritis -10 year Dutch experience. 2nd Annual Meeting on the Foodborne Viruses in Europe Network. Ljubljana, Slovenia.	2005	40 hours

Poster presentations		
- Relevance of viral discovery in public health. European Society of Virology meeting. Como, Italy.	2010	40 hours
- Multiplex and parallel detection of multiple pathogens in fecal samples of outbreaks of gastroenteritis. World congress of molecular diagnostics, Microarrays, and Lab-on-a-chip. San Francisco, California, US.	2009	1 hour
- Multiplex and parallel detection of multiple pathogens in fecal samples of outbreaks of gastroenteritis. 5th and Final Annual Meeting on the Foodborne Viruses in Europe Network. Uppsala, Sweden.	2009	1 hour
- Multiplex and parallel detection of multiple pathogens in fecal samples of outbreaks of gastroenteritis. European society of clinical virology congress. Saariselka, Finland.	2008	40 hours
- Vesiviruses are an unlikely cause of acute hepatitis or gastroenteritis in humans in the Netherlands. European society of clinical virology congress. Saariselka, Finland.	2008	1 hour
- Clostridium species found in Norovirus outbreaks in residential institutions in the Netherlands. European Congress of Clinical Microbiology and Infectious Diseases. Barcelona, Spain.	2008	40 hours
- Vesiviruses are an unlikely cause of acute hepatitis or gastroenteritis in humans in the Netherlands. 4th Annual Meeting on the Foodborne Viruses in Europe Network. Pecs, Hungary.	2007	40 hours
- Outbreaks of viral gastroenteritis in the Netherlands since 1994. 3rd Annual Meeting on the Foodborne Viruses in Europe Network. Rome, Italy.	2006	40 hours
Inter(national) conferences		
- European Society of Virology meeting. Como, Italy.	2010	24 hours
- World congress of molecular diagnostics, Microarrays, and Lab-on-a-chip. San Francisco, California, US.	2009	16 hours
- European society of clinical virology congress. Winter meeting, Amsterdam, The Netherlands.	2009	20 hours
- 2nd meeting of Bosnia-Herzegovian researchers from Bosnia and Herzegovina and abroad. Sarajevo, Bosnia-Herzegovina.	2008	16 hours
- 5th and Final Annual Meeting on the Foodborne Viruses in Europe Network. Uppsala, Sweden.	2008	16 hours
- European society of clinical virology congress. Saariselka, Finland.	2008	3.5 ECTS
- 4th Annual Meeting on the Foodborne Viruses in Europe Network. Pecs, Hungary.	2007	16 hours
- 3rd Annual Meeting on the Foodborne Viruses in Europe Network. Rome, Italy.	2006	16 hours
- American Society for Virology meeting. Madison, Wisconsin, US.	2006	32 hours
- 2nd Annual Meeting on the Foodborne Viruses in Europe Network. Ljubljana, Slovenia.	2005	16 hours
Other		
- Monthly meeting of LIS Department.	2005-2010	
- Meetings of Virology department bi-weekly.	2005-2010	
2. Teaching	Year	Workload (Hours/ECTS)
Supervising practicals and excursions, Tutoring - Supervision of senior secondary education student.	2009	100 hours
Supervising Master's theses - Supervision of Master Thesis. - Supervision of Bachelor Thesis.	 2006-2007 2008-2009	 100 hours 100 hours
Other - Recipient of travel award of European Society of Virology at the European Society of Virology Meeting (ESV 2010). Lake Como, Italy. - Recipient of travel award of European Society of Clinical Virology at the Clinical Virology Annual Meeting (ESCV 2008). Saariselka, Finland. - Reviewer for Journal of Clinical Virology, and Journal of Medical Virology.	 2010 2008 2005-2010	 40 hours
		1343 hours (~48 ECTS) and 6.5 ECTS