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Etiological Role of Viruses in Outbreaks of Acute Gastroenteritis in The Netherlands from 1994 through 2005[∇]

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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.

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

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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	5279–5299 ^a	98
	Capsid	NV1LCR	–	CCTTAGACGCCATCATCATTTAC	5354–5376 ^a	
Norovirus GGI probe	Capsid	NV1LCpr		VIC-TGGACAGGAGAYCGCRATCT-TAMRA	5321–5340 ^a	
Norovirus GGII	Capsid	NV2LCF	+	GARYCIATGTTYAGRTGGATG	5000–5020 ^b	95
	Capsid	NV2LCR	–	TCGACGCCATCTTCATTCAC	5075–5094 ^b	
Norovirus GGII probe	Capsid	NV2LCpr		FAM-TGGGAGGGSGATCGCRATCT-TAMRA	5042–5061 ^b	
Rotavirus	VP1, core protein	ABC F	+	TAYACIGAYGTTTCICARTGGGA	1578–1600 ^c	385
	VP1, core protein	ABC R	–	GTAGTTGTCTGTCICCRCTCIAC	1896–1916 ^c	
Astrovirus	Capsid	AC'1	+	ATGGCTAGCAAGTCTGACAAG	4326–4346 ^d	231
	Capsid	AC230	–	GGTTTTGGTCCCTGTGACACC	4542–4561 ^d	
Adenovirus	Hexon protein	HEXAA1885	+	GCCGCAGTGGTCTTACATGCACAT	17663–17686 ^e	308
	Hexon protein	HEXAA1913	–	CAGCACGCCGCGGATGTCAAAGT	17941–17963 ^e	

^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.

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 MgCl₂, 1 mM deoxyribonucleoside triphosphates (dNTPs), and 5 U of avian myeloblastosis virus reverse transcriptase (Promega, Leiden, The Netherlands).

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 MgCl₂, 0.16 µl of TaqStart antibody (end concentration, 8.8 × 10⁻³ µg/µl; Clontech Laboratories, Inc.), and 11.84 µl of H₂O. 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

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.

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 MgCl₂ (RV mix) or 2 mM MgCl₂ 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 MgCl₂, 5 U of avian myeloblastosis virus, 10 pmol of both AdV primers (32) (Table 1), and 35.6 µl of H₂O. 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.

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)	
Unknown ^a	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)

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 centers.

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 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	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 centers, 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 centers 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 centers, 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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