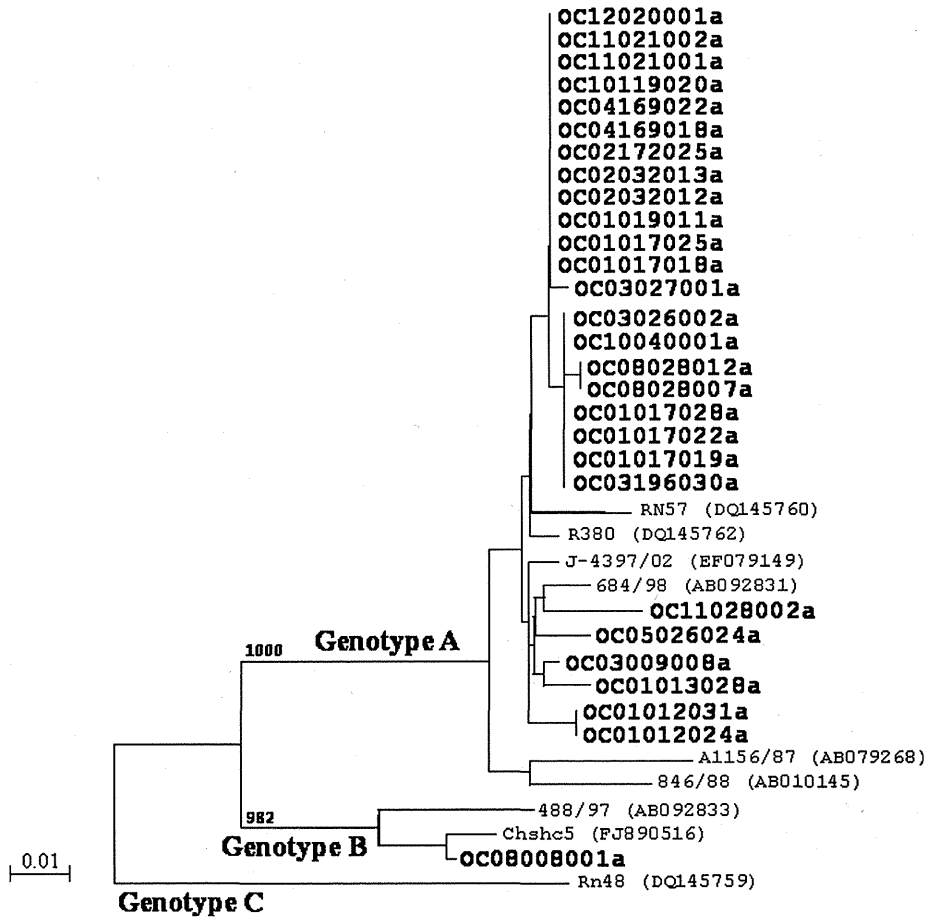

Fig. 1. Phylogenetic analysis of the partial 3CD-encoding gene (367 nucleotide sequence) of aichiviruses using the neighbor-joining method. The aichivirus strains in this study are presented in boldface. The bootstrap values for the genotype are indicated on the branches. The scale shows the number of substitutions per site.

analyzed. Oberste et al. [2005] classified EVs into the same serotype when they had at least 75% nt identity in the VP1 sequence. Phylogenetic analysis of complete VP1 nt sequences among HEV-C viruses also showed that strain OC01017025e was classified into the CVA19 cluster (Fig. 3). Therefore, it was concluded that these two HEV-C strains were type CVA19. Isolation of these two strains using five cell lines (A549, BEAS-2B, LLC-MK2, RD-18S, and Vero E6) by three blind passages with more than 7 days' incubation was unsuccessful (data not shown). Of the five EV strains, strain EV76 was detected in a single isolate. This EV-positive outbreak occurred in January 2001. RVC, EAdVs, HPeVs, and HBoVs were not detected in this study.

The number of virus-positive patients in five outbreaks (OC01012, OC01017, OC08005, OC08028, and OC11021) increased by investigation of other enteric viruses excluding NoVs (Table III). Outbreak OC08005 was NoV-negative and SaV-positive (data not shown). In outbreaks OC01012 and OC01017 with few NoV-positive patients, investigation of other enteric viruses increased the number of virus-positive patients from three (37.5%) to five (62.5%) in outbreak OC01012 and from two (10.5%) to nine (47.4%) in outbreak OC01017. In outbreaks OC08028 and OC11021, all patients were virus-positive by investigation of other enteric viruses.

DISCUSSION

In this study, NoV and five other viruses were detected in fecal specimens from patients aged ≥18 years in oyster-associated gastroenteritis outbreaks, indicating the high prevalence of NoV infection from the consumption of oysters. Of these virus-positive samples, 14.7% contained both NoV and other viruses, suggesting the occurrence of mixed infections associated with contaminated oysters. In cases with single-virus infection, which were negative for other viral and bacterial pathogens, the virus was suspected as the causative agent of gastroenteritis.

AiV was detected frequently in oyster-associated gastroenteritis outbreaks [Yamashita et al., 2000; Le Guyader et al., 2008; Nakagawa-Okamoto et al., 2009]. The present study also showed that AiV was the most common virus after NoV, and 31.0% (9/29

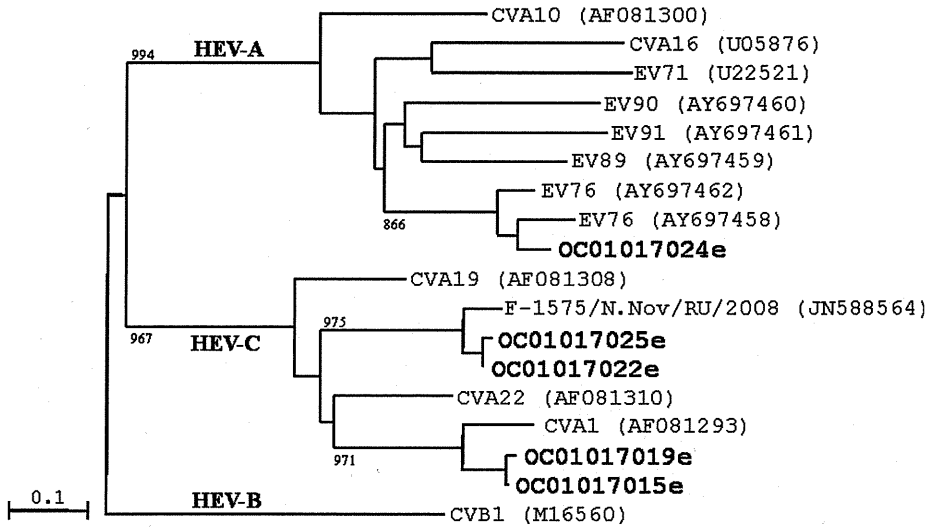

Fig. 2. Phylogenetic analysis of the partial viral protein (VP) 1 gene (approximately 300 nucleotide sequence) of human enteroviruses using the neighbor-joining method. Five enterovirus strains detected in this study are presented in boldface. The bootstrap values for the genetic group are indicated on the branches. The scale shows the number of substitutions per site. The GenBank accession numbers for the reference strains used in this analysis are shown in parentheses. CVA, coxsackievirus A; EV, enterovirus; CVB, coxsackievirus B. CVB1 was used as the outgroup.

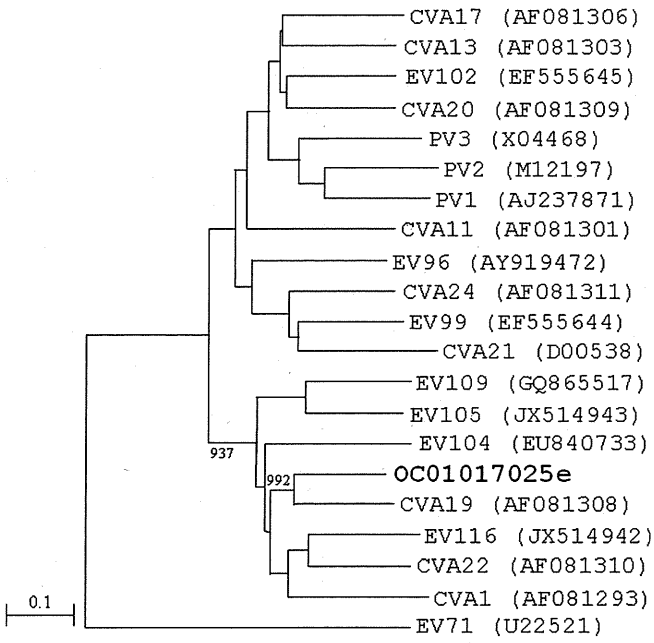

Fig. 3. Phylogenetic analysis of complete VP1 gene sequences of human enterovirus C using the neighbor-joining method. An enterovirus strain, OC01017025e, detected in this study is presented in boldface. The bootstrap values for the genetic cluster related with OC01017025e strain are indicated on the branches. The scale shows the number of substitutions per site. The GenBank accession numbers for the reference strains used in this analysis are shown in parentheses. CVA, coxsackievirus A; PV, poliovirus; EV71, enterovirus 71. EV71 was used as the outgroup.

strains) of AiV-associated outbreaks were single infections, indicating that this virus is a causative agent of gastroenteritis. However, further studies are needed to clarify the importance of AiV in human gastroenteritis because very few studies have examined this virus. SaV is considered as a causative agent of acute gastroenteritis in children and adults [Hansman et al., 2007]. Recently, SaV-associated outbreaks in adults have been reported worldwide, mostly in healthcare settings [Pang et al., 2009; Svraka et al., 2010]. Furthermore, SaV-associated outbreaks after consumption of shellfish have occurred [Nakagawa-Okamoto et al., 2009; Iizuka et al., 2010], and the existence of SaVs has been confirmed in shellfish [Hansman et al., 2008; Ueki et al., 2010]. AstV is a common cause of gastroenteritis in children [Walter and Mitchell, 2003], but it is relatively uncommon in adults [Pager and Steele, 2002; Liu et al., 2010]. Although there are not many reports of AstV-associated food-borne outbreaks, some outbreaks related to the consumption of shellfish or contaminated water have been reported [Seymour and Appleton, 2001; Nakagawa-Okamoto et al., 2009]. In this study, AiV, SaV, and AstV were detected in several outbreaks and were associated with single infections. These findings demonstrate that it is necessary to examine for these three viruses in human gastroenteritis cases associated with oyster consumption when NoV has been excluded.

The detection of EVs from shellfish or fecal specimens of patients with shellfish-associated gastroenteritis has been reported in previous studies, but most of these EV strains were not characterized

TABLE III. Details of Viruses Detected in Four Selected Outbreaks

Outbreak	Number tested	Number of virus-positive patients (%)					Total number of virus-positive patients (%)
		NoV	AiV	AstV	EV	Others	
OC01012	8	3 (37.5)	2	0	0	0	5 (62.5)
OC01017	19	2 (10.5)	5	3	5	0	9[a] (47.4)
OC08028	4	2 (50.0)	2	0	0	0	4 (100)
OC11021	3	2 (66.7)	2	0	0	0	3[a] (100)

[a]Includes co-detections.

[Christensen et al., 1998; Le Guyader et al., 2000, 2008; Umesha et al., 2008; Benabbes et al., 2013]. Five EV strains in this study were characterized into three rare genotypes, CVA1, CVA19, and EV76. In Japan, the detection of CVA1 has been reported since 1982, and the most recent detection was in 2005 in the Infectious Agents Surveillance Report (IASR) by the National Infectious Diseases Surveillance Center (http://www.nih.go.jp/niid/ja/typhi-m/iasr-reference/230-iasr-data/2968-iasr-table-v-p.html). No detections of CVA19 and EV76 have been reported in the IASR since 1981. In previous reports [Oberste et al., 2005; Begier et al., 2008; Chitambar et al., 2012], these three EV types were detected in fecal specimens from patients with gastroenteritis. However, Begier et al. [2008] described that it was ambiguous whether CVA1 infection caused the illness, and there have been very few studies on these three EV types. There is a need more studies to determine whether these EVs are related to gastroenteritis or other illnesses. The detection of rare types of EVs from the patients with oyster-associated gastroenteritis in this study and from the oysters in a previous report [Choo and Kim, 2006] suggested that these rare types of EV circulate in human populations inconspicuously and one of their transmission modes could be the consumption of contaminated oysters.

Rapid identification of pathogens is important for the development of means for control and prevention. The present study is useful for establishing an efficient approach for the identification of viral pathogens in oyster-associated gastroenteritis in adults. Our proposed approach is as follows: the first examination should be for NoV, and second for AiV, SaV, and AstV when negative or low rate of positive in the examination of NoV. Other viruses should be examined in consideration of the description of the patient or the outbreak.

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Emergence of a novel GII.17 norovirus – End of the GII.4 era?

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In the winter of 2014/15 a novel GII.P17-GII.17 norovirus strain (GII.17 Kawasaki 2014) emerged, as a major cause of gastroenteritis outbreaks in China and Japan. Since their emergence these novel GII.P17-GII.17 viruses have replaced the previously dominant GII.4 genotype Sydney 2012 variant in some areas in Asia but were only detected in a limited number of cases on other continents. This perspective provides an overview of the available information on GII.17 viruses in order to gain insight in the viral and host characteristics of this norovirus genotype. We further discuss the emergence of this novel GII.P17-GII.17 norovirus in context of current knowledge on the epidemiology of noroviruses. It remains to be seen if the currently dominant norovirus strain GII.4 Sydney 2012 will be replaced in other parts of the world. Nevertheless, the public health community and surveillance systems need to be prepared in case of a potential increase of norovirus activity in the next seasons caused by this novel GII.P17-GII.17 norovirus.

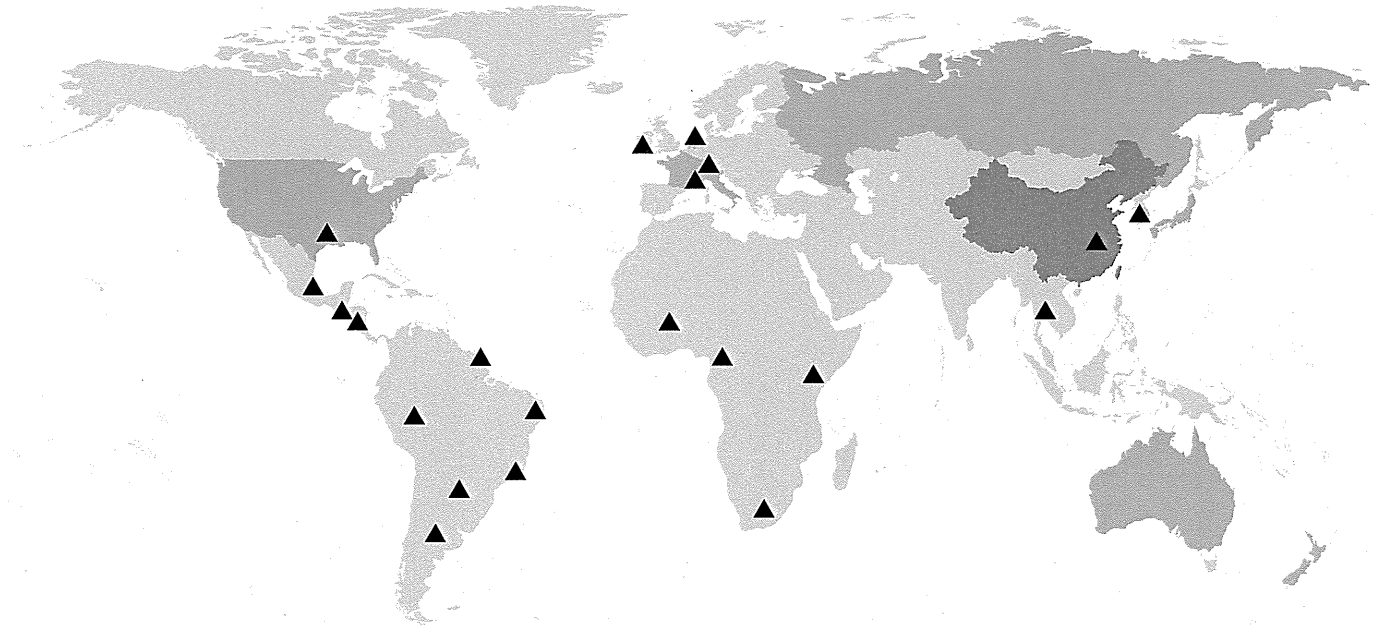
In this issue of *Eurosurveillance*, observations from Japan are reported on an unusual prevalence of a previously rare norovirus genotype, GII.17, in diarrheal disease outbreaks at the end of the 2014/15 winter season [1], similar to what was observed for China [2,3]. Norovirus is a leading cause of gastroenteritis [4]. Although the infection is self-limiting in healthy individuals, clinical symptoms are much more severe and can last longer in immunocompromised individuals, the elderly and young children [5,6].

The *Norovirus* genus comprises seven genogroups (G), which can be subdivided in more than 30 genotypes [7]. Viruses belonging to the GI, GII and GIV genogroups can infect humans, but since the mid-1990s GII.4 viruses have caused the majority (ca 70–80%) of all norovirus-associated gastroenteritis outbreaks worldwide [8-10].

GII.4 viruses can continue to cause widespread disease in the human population because they evolve through accumulations of mutations into so-called drift variants that escape immunity from previous exposures [11]. Contemporary GII.4 noroviruses also demonstrate intra-genotype recombination near the junction of open reading frame (ORF) 1 and ORF2, which is likely to foster the emergence of novel GII.4 variants [12]. In addition, the binding properties of GII.4 viruses have altered over time, resulting in a larger susceptible host population [13].

FIGURE 1

World map showing areas where GII.17 norovirus strains have been detected, 1978–2015



Emergence and geographical spread of GII.17 genotype noroviruses

Viruses of the GII.17 genotype have been circulating in the human population for at least 37 years; the first GII.17 strain in the National Center for Biotechnology Information (NCBI) databank is from 1978 [14]. Since then viruses with a GII.17 capsid genotype have sporadically been detected in Africa, Asia, Europe, North America and South America (Table, Figure 1). The virus appears to be clinically relevant, as it has been associated with acute gastroenteritis (AGE) in children and adults, and with chronic infection in an immunocompromised renal transplant patient [15] and a leukaemia patient (unpublished data). In the United States (US), only four GII.17 outbreaks were reported between 2009 to 2013 through CaliciNet, with a median of 11.5 people affected by each outbreak [16]. In Noronet, an informal international network of scientists working in public health institutes or universities sharing virological, epidemiological and molecular data on norovirus, GII.17 cases were also sporadically reported in Denmark and South Africa during this period [17].

More widespread circulation of GII.17 was first reported for environmental samples in Korea from 2004 to 2006. This information was published in a report in 2010 by the Korean Food and Drug Administration (KFDA) and was cited by Lee et al. [18], but the original document describing this finding is not publicly available and there are no matching clinical reports. From 2012 to 2013 a novel GII.17 virus accounted for 76% of all detected norovirus strains in rivers in rural and urban areas in Kenya [19]. In the winter of 2014/15, genetically closely related GII.17 viruses were first detected in AGE outbreaks in the Guangdong province in China in schools, colleges, factories and kindergartens [3]. Sequence analyses demonstrated that 24 of the 29 reported outbreaks during that winter were caused by GII.17. A large increase in the incidence of AGE outbreaks was also reported; 29 outbreaks associated with 2,340 cases compared with nine outbreaks and 949 cases in the previous winter when GII.4 Sydney 2012 still was the dominant genotype [3].

During the same winter there was also an increase in outbreak activity in Jiangsu province, which could be attributed to the emergence of this novel GII.17 [2]. This triggered us to investigate the prevalence of GII.17 in other parts of the world by means of a literature study and by inviting researchers collaborating within Noronet to share their data on GII.17. Currently, in Asia, in addition to Guangdong and Jiangsu [2,3], the novel GII.17 is also the predominant genotype in Hong Kong (unpublished data) and Taiwan [20], while in Japan, a sharp increase in the number of cases caused by this novel virus has been observed during the 2014/15 winter season [1]. Related viruses have been detected sporadically in the US [21] (http://www.cdc.gov/norovirus/reporting/calicinet/index.html), Australia, France, Italy, Netherlands, New-Zealand and Russia (unpublished data, www.noronet.nl) (Figure 1). In France the novel GII.17 virus appeared at the beginning of 2013,

Table A

Overview of detected GII.17 norovirus strains worldwide, 1978–2015

Country	Geographical spread GII.17[a]	Year[b]	ORF1	ORF2	Study population	Proportions of typed strains or outbreaks[c]	Suspected source of infection	Description of the sequence (size)	Accession number	References
French Guiana	Single location	1978	GII.P4	GII.17	Children with AGE	1 strain	–	Partial genome (7,441 bp)	KC597139, JN699043	[14]
Brazil	Rio de Janeiro	1997 (1994–2008)	–	GII.17	Children with AGE	3/52 strains	–	5'-end ORF2 (300 bp)	JN600531	[31]
Kenya	Nairobi	1999–2000	–	GII.17	HIV positive children with or without AGE	1/11 strains	–	5'-end ORF2 (309 bp)	KF279387	[32]
France	Briançon	2004	GII.P13	GII.17	Child with AGE	1 strain	–	Partial ORF1/2 (1,361 bp)	EF529741	Data not shown
Paraguay	Asuncion	2004–2005	–	GII.17	AGE in children (<5 years)	5/29 strains	–	3'-end ORF2 (255 bp)	KC736582, KC736580, KC736578, KC736569	[33]
Brazil	States of Acre (Brazil)	2005 (2005–2009)	–	GII.17	AGE	2/62 strains	–	3'-end ORF2 (215 bp)	JN587118 JN587117	[34]
United States	Houston	2005	–	GII.17	AGE evacuees hurricane Katrina	Predominant genotype in an outbreak	Sewage	ORF2 and 3 (2,459 bp)	DQ438972	[35]
Argentina	Single location (Argentina)	2005–2006	–	GII.17	River samples	1/33 strains	–	–	–	[36]
Brazil	State of Rio de Janeiro	2005–2006 (2004–2011)	–	GII.17	Outbreaks of AGE	3/112 outbreaks	–	3'-end ORF2 (214 bp)	KJ179752, KJ179753, KJ179754	[37]
Nicaragua	Léon	2005–2006	–	GII.17	AGE	1 strain	–	5'-end ORF2 (244 bp)	EU780764	[26]
France	Sommières	2006	GII.P13	GII.17	AGE	1 strain	Foodborne	Partial ORF1/2 (1,056 bp)	EF529742	Data not shown
Thailand	Lopburi	2006–2007	–	GII.17	AGE	2 strains	–	5'-end ORF2 (209 bp)	GQ325666, GQ325670,	[38]
China	Wuhan	2007 (2007–2010)	GII.P13	GII.17	AGE	1/488 strains	–	Partial ORF1/2 (1,096 bp)	JQ751044	[39]
Mexico	Mexico City	2007	–	GII.17	–	–	Waterborne	5'-end ORF2 (1,337 bp)	JF970609	NCBI[d]
Switzerland	Zürich	2008	–	GII.17	Renal transplant patient	1/9 strains	–	ORF2 (1,599 bp)	GQ266696	[15]
Nicaragua	Léon	2008	–	GII.17	AGE in children (<5 years)	2/38 strains	–	5'-end ORF2 (244 bp)	EU780764	[40]
South Korea	Seoul	2010 (2008–2011)	–	GII.17	AGE	1/710 strains	–	5'-end ORF2 (209)	JQ944348	[41]
Brazil	Quilombola	2009 (2008–2010)	–	GII.17	Children (<10 years)	2/16 strains	–	3'-end ORF2 (215 bp)	JX047021, JX047022	[42]

TABLE B

Overview of detected GII.17 norovirus strains worldwide, 1978–2015

Country	Geographical spread GII.17[a]	Year[b]	ORF1	ORF2	Study population	Proportions of typed strains or outbreaks[c]	Suspected source of infection	Description of the sequence (size)	Accession number	References
Cameroon	Southwestern region of Cameroon	2009	GII.P13	GII.17	Healthy children and HIV positive adults	4/15 strains	–	Partials ORF1/2 (1,024 bp)	JF802504–JF802507	[43]
Guatemala	Tecpan	2009	–	GII.17	Children after waterborne outbreak	1/18 strains	Waterborne	–	–	[44]
Burkina Faso	Ouagadougou	2009–2010	–	GII.17	AGE in children (<5 years)	1/36 strains	–	5'-end ORF2 (287 bp)	JX416405	[27]
Netherlands	Single location	2002–2007	–	GII.17	Nosocomial	3/264 strains	Nosocomial	–	–	[45]
South Korea	South Korea	2010	–	GII.17	Groundwater samples	2/7 strains	–	5'-end ORF2 (311 bp)	KC915021–KC915022	[18]
Ireland	Ireland	2010	–	GII.17	Influent waste water	4/24 strains	–	5'-end (302 bp)	JQ362530	[46]
South Africa	South Africa	2010–2011	–	GII.17	Waste water	9/69 strains	–	5'-end ORF2 (305 bp)	KC495680, KC495686, KC495672–KC495674, KC495664, KC495657, KC495655, KC495640	[47]
South Korea	Jinhae Bay	2010–2011	–	GII.17	Oysters	1 strain	–	–	–	[48]
Morocco	Oujda (Morocco)	2011	–	GII.17	AGE in children (<5 years)	1/42 strains	–	5'-end (205 bp)	KJ162374	[49]
South Africa	Johannesburg (South Africa)	2011	GII.P16	GII.17	AGE	–		Partial ORF1/2 (1,010 bp)	KC962460	[50]
Cameroon	Limbe	2011–2012	GII.P3	GII.17	Healthy adults and Children	4/100 strains	–	Partial ORF1/2 (653 bp)	KJ946403	[51]
Kenya	Kenya	2012–2013	–	GII.17	Surface water	16/21 strains	–	5'-end ORF2 (306 bp)	KF916584–KF916585, KF808227–KF808254	[19]
South Korea	Gyeonggi	2012	–	GII.17	AGE outbreak	1 strain	Waterborne	5'-end (205 bp)	KC413386 KC413399–KC413403	[22]
China	Guangdong province	2014–2015	–	GII.17	AGE outbreaks	24/29 outbreaks	–	5'-end (249 bp)	KP718638–KP718738	[3]
United States	Gaithersburg	2014	GII.P17	GII.17	AGE in child of 3 years	1 strain	–	Partial genome (7,527 bp)	KR083017	[21]
China	Jiangsu province	2014–2015	GII.P17	GII.17	Outbreaks of AGE	16/23 outbreaks	–	–	KR270442–KR270449	[2]

Table C

Overview of detected GII.17 norovirus strains worldwide, 1978–2015

Country	Geographical spread GII.17[a]	Year[b]	ORF1	ORF2	Study population	Proportions of typed strains or outbreaks[c]	Suspected source of infection	Description of the sequence (size)	Accession number	References
Japan	Japan	2014–2015	GII.P17	GII.17	Outbreaks of AGE	100/2,133 strains	-	Partial genome (7,534–7,555 bp)	AB983218, LC037415, LC043139, LC043167, LC043168, LC043305	[1]

AGE: acute gastroenteritis; HIV: human immunodeficiency virus; NCBI: National Center for Biotechnology Information; ORF: open reading frame.

[a] GII.17 detection location with study location between brackets (when different from GII.17 detection location).

[b] GII.17 detection year(s) with study years between brackets.

[c] Either the proportion of strains that was typed as GII.17 or the proportion of outbreaks that was caused by GII.17 is given.

[d] Information derived from the GenBank entry related to the accession number of the sequence.

but since then, it has not resulted in an increase in AGE outbreaks as observed in China, nor replaced the predominant GII.4 in the last seasons (data not shown).

Based on sequence analyses of the ORF1-ORF2 junction region, most diagnostic real-time transcription polymerase chain reactions (PCRs) will be able to detect this novel GII.17 virus, but it is not known whether the same holds true for immunoassays. However, only a small portion of norovirus outbreaks are typed beyond the GI and GII classification, therefore it is possible that GII.17 is more prevalent than we currently suspect.

Phylogenetic analyses and molecular characterisation of the novel GII.17 viruses

Phylogenetic analysis of the viral protein 1 (VP1) of GII.17 strains in the NCBI database demonstrated at least two clusters, with the novel Asian GII.17 strains grouping together with the GII.17 strains detected in the surface water in Kenya (Figure 2,[21]) and in an outbreak in 2012 in Korea [22]. Although the novel GII.17 clusters away from previously identified GII.17 strains, the amino acids changes in VP1 are not sufficient to separate it into a different genotype. For only a limited number of GII.17 strains the full VP1 has been sequenced, which demonstrated three deletions and at least one insertion compared with previous GII.17 strains (comprehensive alignments are given in Fu et al. and Parra et al. [2,21]). The majority of these changes could be mapped in or near major epitopes of the VP1 protein and potentially result in antigenic drift or altered receptor-binding properties [21]. Most publicly available GII.17 sequences only comprise the VP1, and most frequently the 5'-end of VP1 (C region), while most of the observed diversity within the GII.17 genotype is observed in the 3'-end of VP1 (D region) [23].

Previously, viruses with a GII.17 VP1 genotype contained a GII.P13 ORF1 genotype, although recombinants with an ORF1 GII.P16, GII.P3 and GII.P4 genotype have also been identified (Table). Sequence comparison showed that the ORF1 region of the novel GII.17 viruses was not detected before and cluster between GII.P3 and GII.P13 viruses [21]. Since this is the first orphan ORF1 sequence associated with GII.17, it has been designated GII.P17 according to the criteria of the proposal for a unified norovirus nomenclature and genotyping [24]. The novel GII.17 virus was termed Kawasaki 2014 after the first near complete genome sequence (AB983218) submitted to GenBank. Noronet provides a publicly available and widely used tool for the typing of norovirus sequences (http://www.rivm.nl/mpf/norovirus/typingtool). This typing tool was updated to ensure correct classification of both ORF1 and ORF2 sequences of the newly emerged GII.P17-GII.17 viruses.

The acquisition of a novel ORF1 could potentially result in an increase in replication efficiency and may – in part – explain the increase of the AGE outbreak activity. Histo-blood group antigens (HBGAs) function as (co-) receptors for noroviruses. Alpha(1,2)fucosyltransferase

FIGURE 2

Unrooted maximum likelihood phylogenetic tree based on the 5'-end of virus protein 1 (VP1) sequences (C region) of GII.17 noroviruses, available from the National Center for Biotechnology Information (NCBI)

The tree was estimated under the general time reversible model using PhyML. Bootstrap values above 70% are given. Sequences from Kenya are depicted in red and those from the recent outbreaks (2013–1015) reported in Asia in blue. The scale bar represents nucleotide substitutions per site.

2 (FUT2) adds an alpha-1,2 linked fucose on HBGAs, and individuals lacking the FUT2 gene are referred to as 'non-secretors', while those with a functional FUT2 gene are called 'secretors'. Non-secretors have been shown to be less susceptible to infection with several norovirus genotypes [25]. In studies investigating the genetic susceptibility to norovirus genotypes, a secretor patient with blood type O Lewis phenotype Le^{a-b+} and a secretor patient with blood type B Lewis phenotype Le^{a-b-} were positive for previously identified GII.17 viruses and no non-secretors were found positive [26,27], suggesting that there could be genetic restrictions for GII.17 viruses in infection of humans. How the observed genetic changes have affected the antigenic and binding properties of the novel GII.17 strains, and hereby the susceptible host population, remains to be discovered.

Public health implications

Based on the emergence and spread of new GII.4 variants, we know that noroviruses are able to rapidly spread around the globe [28,29]. The novel GII.17 virus has been detected in sporadic cases throughout the world, but until now it has not resulted in an increase

in outbreak activity or replacement of GII.4 Sydney 2012 viruses outside of Asia. Following the patterns observed in the past years for GII.4 noroviruses and based on the data from China and Japan, an increase in norovirus outbreak activity can be expected if the currently dominant GII.4 is replaced by GII.17. Another possibility – however – would be some restriction to global expansion, as has been observed previously for the norovirus variant GII.4 Asia 2003 [29]. Such restrictions could be due to differences in pre-existing immunity, but could also be the result of differences between populations in the expression of norovirus receptors [29]. Based on current literature on the novel GII.17 virus there is no indication that it will be more virulent compared with GII.4. Nevertheless, the public health community and surveillance systems need to be prepared in case of a potential increase of norovirus activity by this novel GII.17 virus.

Conclusions

Understanding the epidemiology of norovirus genotypes is important given the development of vaccines that are entering clinical trials. Current candidate vaccines have targeted the most common norovirus genotypes, and it remains to be seen if vaccine immunity is cross-reactive with GII.17 viruses [30]. Contemporary norovirus diagnostic assays may not have been developed to detect genotype GII.17 viruses since this genotype was previously only rarely found during routine surveillance. These assays need to be evaluated and updated if necessary to correctly diagnose norovirus outbreaks caused by the emerging GII.17 virus. Norovirus strain typing ideally should include ORF1 sequences and the variable VP1 'D' region as well as metadata on the host, like clinical symptoms, immune status and blood group. This will allow us to better study and monitor the genetic disposition, pathogenesis, evolution and epidemiology of this newly emerged virus.

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Conflict of interest

None declared.

Authors' contributions

MG, JB, HV: compiling the data, drafting the manuscript; AP, FB, KT, MC, JM, JN, GR, ML, LDR, NI JH, VM, KAB, JV, PW: collecting field data, critical review of the manuscript; MK: initiation of study, providing data, critical review of the manuscript.

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短　報

岡崎市におけるノロウイルス遺伝子型の6年間の特徴
—2007年4月～2013年3月—

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Key words: norovirus, epidemiology, phylogenetic analysis

序　文

ノロウイルス（NoV）は乳幼児から高齢者までの幅広い年齢層に感染し，急性の感染性胃腸炎を引き起こすウイルスであり，またウイルス性食中毒の主要な病原体である．NoVは遺伝子配列の相同性を基にgenogroup I～V（GI～V）の5つの遺伝子グループに分類され，そのうちGI，GIIおよびGIVがヒトに感染するが，GI，GIIが大勢を占める．これら遺伝子グループには多くの遺伝子型が存在し，近年はGII genotype 4（GII.4）が主流であり，さらにGII.4変異株の変遷が注目されている[1]．

今回，NoVの伝播経路の解明や感染症予防，食中毒防止対策の基礎データとするため，2007年4月から2013年3月までの6年間の岡崎市におけるNoV遺伝子型解析を実施したので報告する．

対象と方法

2007年度から2012年度までの市内における感染性胃腸炎集団発生事例や散発および集団発生の食中毒（疑いを含む）事例等においてNoV検査を実施した105事例を対象とした．

NoVの検査は，ウイルス性下痢症診断マニュアル第3版（国立感染症研究所）に準拠したReverse Transcription Polymerase Chain Reaction（RT-PCR）法またはリアルタイムPCRで実施した．NoVのカプシド領域遺伝子をG2-SKF/G2-SKRを用いたRT-PCRで増幅し，得られた増幅産物をダイレクトシーケンス法で塩基配列を決定後，近隣結合法で分子系統樹を作成した．

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結果および考察

調査した105事例のうち62事例（59.0%）がGII陽性，その内の51事例で遺伝子型が決定された．51事例中，GII.4が37事例（72.5%）と最も多く，次いでGII.6が4事例(7.8%)，GII.2，GII.12，GII.13，GII.3とGII.6が各2事例(3.9%)．また，GII.3およびGII.11が各1事例（2.0%）であった．年度別では2010年度を除き，各年度ともGII.4が最も多く検出され，全国的な流行と一致した[2]．2010年度はGII.4未検出であったが，国内の他機関からも同時期のGII.4流行が例年と比べて低調であったことが報告されている[3][4]．

分子系統樹解析により，各シーズンにおけるGII.4変異株の同定を試みた．GII.4変異株として2007年度および2008年度は全事例2006b，2009年度は2006bに加え2008aを検出した．2011年度は，2008bと2009aに加え，2012変異株（Sydney 2012）を1事例検出し，2012年度は全事例がSydney 2012であった(Fig. 1)．GII.4変異株の主流行タイプは2006bから2008a，2008b，2009a，そしてSydney 2012と移り変り，この傾向も全国の流行と一致した[5][6]．Sydney 2012は，北海道と大阪市で2012年1月に最初に検出され，10月以降全国に急速に広がったことが示唆されており[6]，愛知県においても2012年10月以降県内に侵入したと考えられている．ただし今回の調査から岡崎市において，2012年2月の1事例からSydney 2012が検出されており，2012年2月頃には既に県内にも侵入していたことが新たに確認された．

施設別にみると，飲食店はGII.2（8.3%），GII.4（75.0%），GII.12（16.7%），高齢者福祉施および障害者等支援施設はGII.4（100.0%），幼・保育園はGII.2（9.1%），GII.3（9.1%），GII.4（9.1%），GII.6（36.4%），GII.13（18.2%），GII.3とGII.6（18.2%），などであっ

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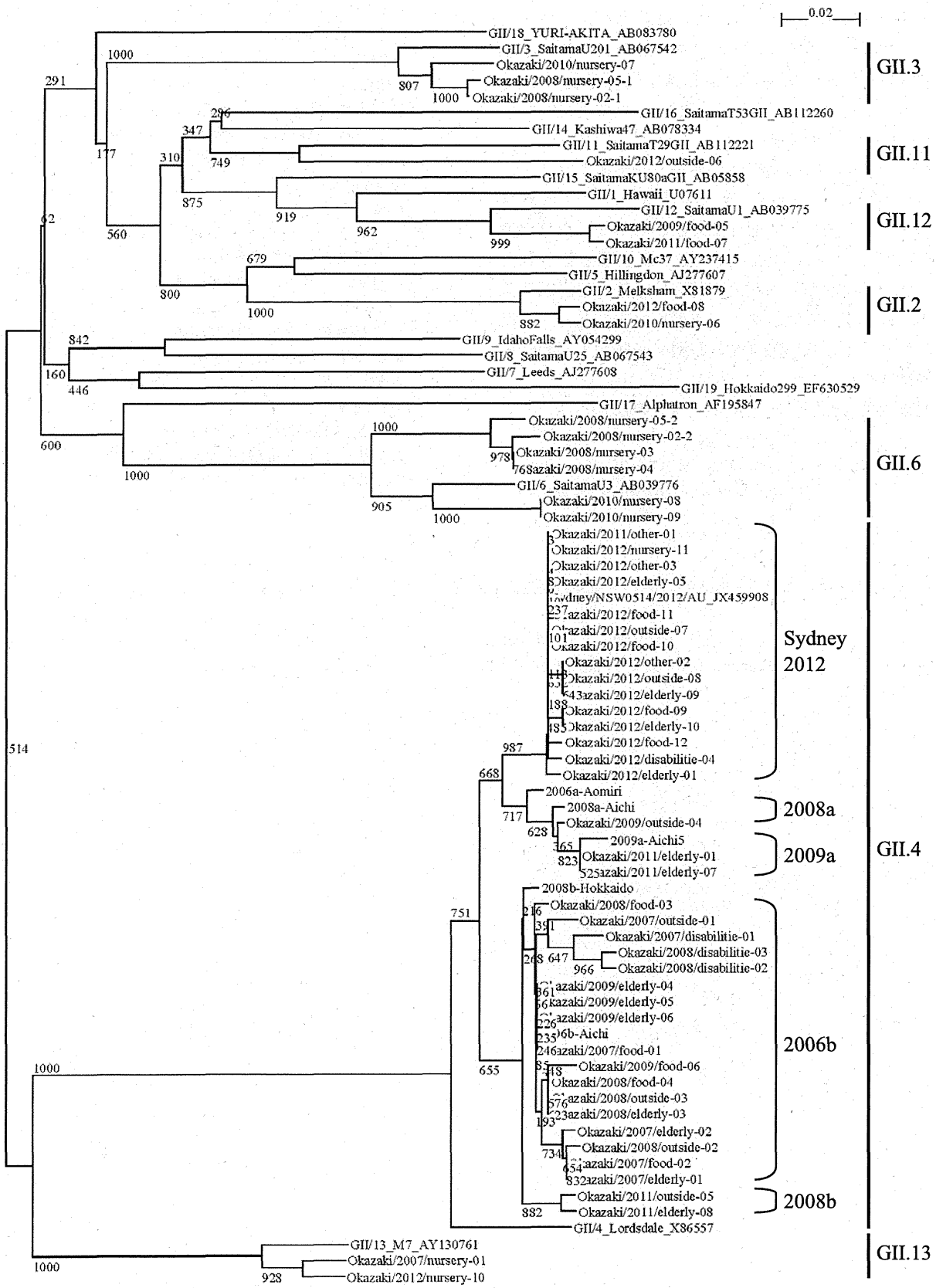
Fig. 1 Phylogenetic tree of norovirus GII based on the capsid nucleotide sequences in Okazaki City between April 2007 and March 2013.



た．飲食店，高齢者福祉施設などではGII.4が有意である一方，幼・保育園では多様な遺伝子型が検出され，他施設の検出傾向とは明らかに異なっていた．全国的にも幼・保育園はGII.4の検出率が低くなる年もあることから[2)4)]，幼・保育園児は多様な遺伝子型のNoVに感受性を有すると推察されるが，その要因については現時点では不明である．

今回の調査から，岡崎市において全国的な流行よりも早い時期でのSydney 2012の侵入や，幼・保育園における遺伝子型の多様性が確認され，幼・保育園児はNoV感染に対する感受性の高い集団と推察されたが，その感染源や感染経路の解明は今後の検討課題であり，遺伝子型の流行タイプを把握することで，集団感染および食中毒感染経路の解明や予防対策への活用が期待される．

利益相反自己申告：申告すべきものなし

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Characteristic of Norovirus Genotypes Detected in Okazaki City Between April 2007 and March 2013

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食品中の病原ウイルスの検出方法に関する研究

パンソルビン・トラップ法によって食品検体から検出されたノロウイルスの遺伝子解析法の開発

斎藤博之　　田中智之*1　　野田　衛*2

パンソルビン・トラップ法は，食品検体に含まれるウイルス粒子を黄色ブドウ球菌の表面に吸着させて回収することを基本原理としている。その性質上，抽出されたRNAには大量の黄色ブドウ球菌由来の遺伝子が混入し，遺伝子解析の障害となることがこれまでの研究で明らかとなった。黄色ブドウ球菌の遺伝子そのものは，極微量のウイルスRNAを安定的に保持するキャリアーとして働くため，検出感度に対してはプラスの効果が見込める。従って，それを排除するのではなく，遺伝子解析に影響が及ばないようにする解決策が求められた。本研究では，逆転写反応時にPCRで用いるものとは異なる専用プライマーを使うことで，遺伝子解析可能なPCR増幅産物を得ることに成功した。さらに，増幅プロセスにホットスタート&タッチダウンPCRを用いることで一層の改善が認められた。また，ノロウイルスGI.4，及びノロウイルスGII.4で汚染させたポテトサラダにおける本法の検出限界は，両者とも食品1g当たり35コピーであった。以上のことにより，黄色ブドウ球菌のキャリアーとしての長所を活かし，遺伝子解析の障害という短所を顕現させないようにするという目的が達成された。

1. はじめに

ウイルス性食中毒の対策として二枚貝の汚染実態調査や，調理従事者への衛生教育等が進められてきた[1,2]。しかしながら，原因として疑われる食品からのウイルス検出は，その作業の困難さからこれまでほとんど検討されてこなかったため，具体的な汚染ルートの解明に決め手を欠いていた。原因物質としてはノロウイルス（NoV）が大部分を占めているが，他にもサポウイルス(SaV)やアデノウイルス41型(AdV41)に代表される腸管系アデノウイルスも含まれている。さらに，平成22年3月に我が国におけるA型肝炎ウイルス（HAV）感染者の報告が急増するなど，食品中のウイルスを検出する方法の確立が急務となっている[3]。平成19～21年度に実施された厚生労働科学研究費補助金「食品中のウイルスの制御に関する研究」（H19-食品-一般-016）において，固形，液状，練り物，油物などの一般的な食品からNoVを検出する手法としてパンソルビン・トラップ法（パントラ法）を開発し，この問題を解決するための糸口を見出すことができた[4-13]。その際，各種ウイルスに対する抗体の安定供給が課題となっていたが，平成22年度の本研究事業において，市販のガンマグロブリン製剤を利用することで汎用化に成功した[14-17]。普及にあたって，本法の根幹をなすパンソルビンを製造・販売しているメーカーが1社しかないことから，在庫切れや製造中止などのリスクが問題となったが，自家調製プロトコルが完成したことで払拭された[18, 19]。一方で，実際の食中毒事例に用いられ，食品からのウイルス検出に成功したケースもあったが，非特異反応が多すぎることにより遺伝子解析が困難であるという問題が指摘された[20]。そこで本研究では，パントラ法で抽出されたRNAに対して遺伝子解析が可能となるような反応系の開発を行った。

2. 方法

2.1 研究材料

実験に用いる食品として，市販されている焼きそばとポテトサラダを用いた。また，検出対象となるウイルスとして，NoV-GI.4（AB685383），及びNoV-GII.4(AB293424)を用いた。

2.2 試薬類

2.2.1 食品洗滌液

Tris-HCl (pH8.4) – 0.5M NaCl – 0.1% Tween20

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を調製して使用した。

2.2.2 5%ガンマグロブリン製剤

米国 Baxter 社の 5%静注用ガンマグロブリン製剤「Gammagard」を用いた。Alfresa Pharma 社から購入した。

2.2.3　パンソルビン

黄色ブドウ球菌を熱処理してホルマリン固定したものの懸濁液で，メルク社から購入した。

2.2.4 フェノール系 RNA 抽出キット

TRIzol-LS (invitrogen)を使用した。

2.2.5 カラム方式の RNA 抽出キット

QIAamp Viral RNA Mini Kit (Qiagen)を使用した。

2.2.6　再懸濁液

2.2.5 の抽出キット添付の AVL 液を用いた。

2.2.7 逆転写反応エンハンサー

RTmate（ニッポンジーン）を使用した。

2.2.8 DNase I (RT Grade)及び RNase inhibitor

ニッポンジーンの製品を使用した。

2.2.9 アミラーゼ

前処理用：枯草菌由来 α-Amylase 粉末（和光純薬）を使用した。

後処理用：α-Amylase Ultrapure（ニッポンジーン）を使用した。

2.2.10 食品処理袋

サニスペックテストバッグ（アズワン）を使用した。

2.2.11 逆転写酵素

ReverTra Ace（東洋紡）を使用した。

2.2.12 ノーマル PCR 用酵素

Taq DNA Polymerase High Yield（グライナー）を用いた。

2.2.13 ホットスタート&タッチダウン PCR 用酵素

KOD FX Neo（東洋紡）を用いた。

2.2.14 逆転写反応に用いたプライマー

ランダムプライマー(9 mer, タカラバイオ)，G1SKR と G2SKR[21]，及び新規開発の逆転写反応専用プライマーPANR-G1 と PANR-G2 を用いた。PANR-G1 は表 1 に示した PANR-G1a と PANR-G1b を 1:1 で混合したものである。PANR-G2 は表 1 に示した PANR-G2a, PANR-G2b, 及び PANR-G2c を 2:1:1 で混合したものである。

2.3 パンソルビン・トラップ法の全体の手順

基本的な操作の流れを図 1 に示した。

2.4 ウイルスの検出

図 1 で得られた抽出液（60 μL）から 8.5 μL を取り，DNase I 及び α-Amylase Ultrapure を各 1 μL, RNase inhibitor を 0.25 μL ，5×逆転写 buffer（酵素に添付）を 4 μL 加えた後，蒸留水で反応量を 15.5 μL とし，37℃ 10 分，65℃ 5 分のインキュベーションを行った。その後，プライマー（前述のもの），dNTP，RTmate，及び逆転写酵素を追加して cDNA を合成した（反応容量 20 μL）。合成した cDNA 溶液を 5 μL 取り，COG1F[22] / G1SKR[21], または COG2F[22] / G2SKR[21]による 1st. PCR と，G1SKF/G1SKR または G2SKF/G2SKR による semi-nested RT-PCR を行った。この増幅反応において，通常用いられるノーマル PCR と，より特異性の高いホットスタート&タッチダウン PCR の比較を行った。反応温度条件は，次のとおりである。

【ノーマル PCR】
94℃4 分　1 サイクル
94℃30 秒－50℃30 秒－72℃30 秒　40 サイクル
72℃7 分　1 サイクル

表 1　逆転写反応専用プライマーの配列

名称	配列（5'→3'）	設定位置
PANR-G1a	GTBCKMACATCAGCAATCA	5800←5818
PANR-G1b	GGKTCAAGSRYCCTAACATCWGCAATGA	5800←5827
PANR-G2a	TCYARWKKYCTWACATCTAYAATYAYRTGGGGGAACAT	5502←5539
PANR-G2b	ARDGTCCTAACATCWATAATYAYATGAGGGAACAT	5502←5536
PANR-G2c	CTSACATCCACMAYYACRTGCGGRCACAT	5502←5530

・PANR-G1a と PANR-G1b の設定位置は、Norwalk68 株の配列に相当する塩基番号で表記した。
・PANR-G2a、PANR-G2b、及び PANR-G2c の設定位置は、Camberwell 株の塩基番号で表記した。
・PANR-G1a の配列中のアンダーラインで示した塩基（C）は LNA 修飾で合成した。

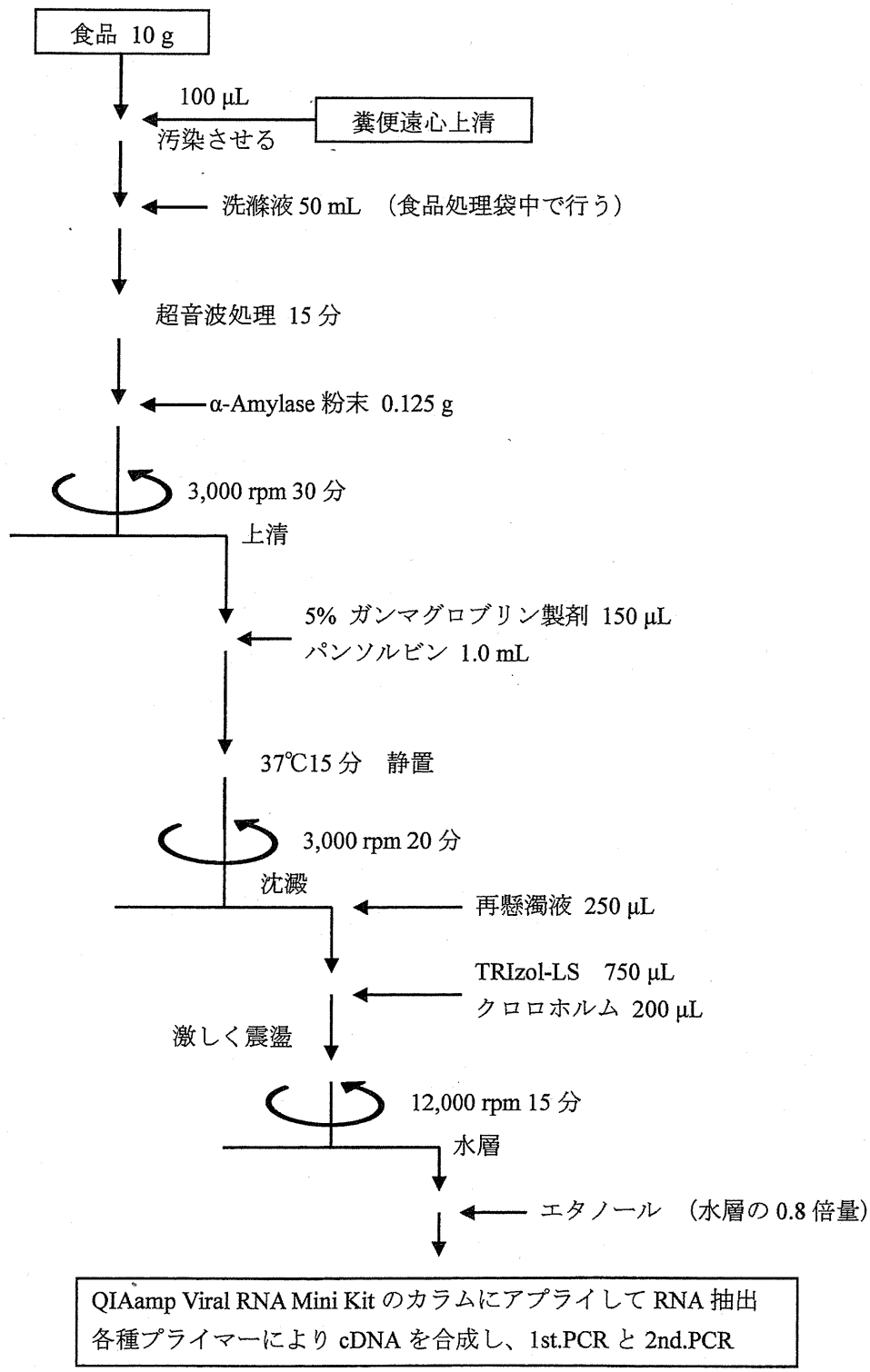

図1　パンソルビン・トラップ法の操作手順

【ホットスタート&タッチダウン PCR】
94℃4 分　1 サイクル
94℃30 秒－（55→50℃）30 秒－72℃30 秒 5 サイクル：下線部がタッチダウン設定
94℃30 秒-50℃30 秒-72℃30 秒 40 サイクル
72℃7 分　1 サイクル

PCR 産物のゲル電気泳動で予想位置にバンドが認められた場合は切り出してシークエンスを試みた。また，1st. PCR 産物に対して real-time PCR[22]を行い，NoV 特異的な増幅の有無を検討した。使用した real-time PCR 装置はロシュ製「LightCycler 350S」で，反応容量は 20 μL である。

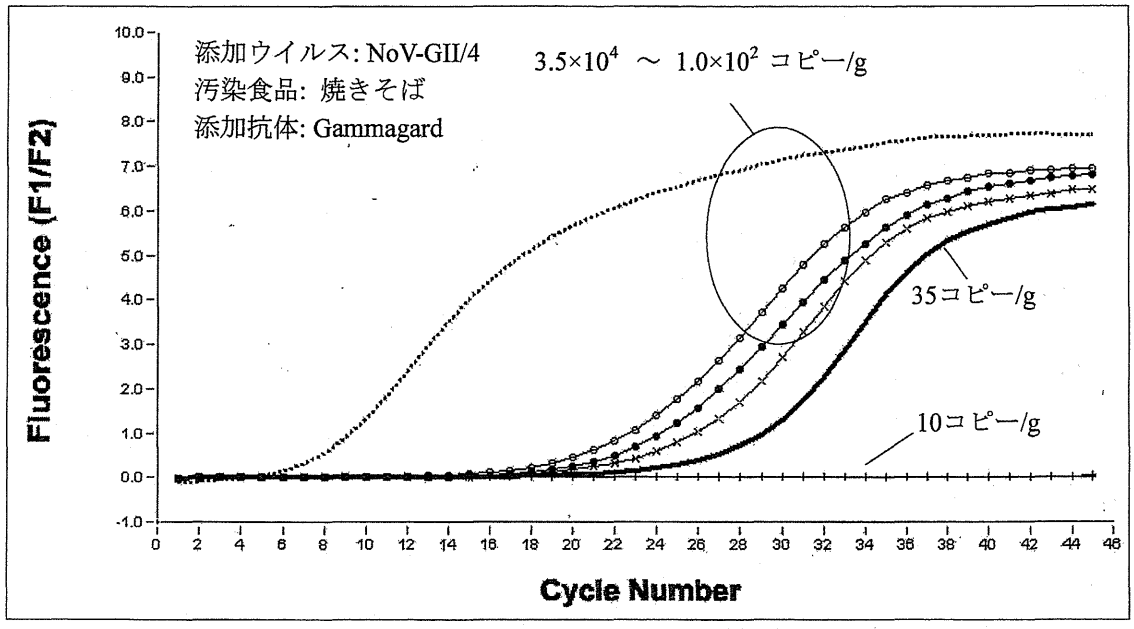

図 2　Nested real-time PCR の増幅曲線

・1st. PCR をプライマーCOG2F / G2SKR にて実施し、その増幅産物をプライマー・プローブセット COG2F / COG2R / RING2-TP を用いた real-time PCR で検出した。

3. 結果

3.1 遺伝子解析における問題点の把握

問題点を把握するための予備試験としてさまざまなレベルで汚染させた焼きそばから，Gammagard を用いて NoV 回収を行った際の増幅曲線を図 2 に示した。これによると，汚染度 35 コピー/g と 10 コピー/g で明確に陽性と陰性が分かれていることがわかる。一方，1st. PCR 産物をプライマー G2SKF / G2SKR による semi-nested RT-PCR で再増幅し，アガロースゲル電気泳動で確認したところ，汚染度 3.5×10^2 コピー/g までは 344 bps の増幅バンドが認められた（図 3）。しかし，1.0×10^2 コピー/g 以下ではバンドが不明瞭であった。明確なバンドが認められた 3.5×10^3 コピー/g のレーンからバンドを切り出してシークエンスを解析したところ，汚染に用いた NoV の塩基配列であることが確認できた。同様に 35 コピー/g のレーンから 344 bps 近傍に位置する部分のゲルを切り出し，そこに含まれる DNA 断片のシークエンスを解析したところ黄色ブドウ球菌（ブ菌）の 16s リボソームの塩基配列であった。

3.2 問題点の理論的検討

逆転写反応も PCR も，起点はハイブリダイゼーションであることから，反応系を単純すると 2 種類の分子の会合ということになる。従って，そこには会合定数（K）というものが存在し，さらにはそれぞれの分子の濃度が関係するため，質量作用の法則から，

$$K=\frac{[PT]}{[P][T]}$$

で表される。ここでは，[P]と[T]はそれぞれプライマーとテンプレートが反応系中で独立して存在している濃度，[PT]はそれらが会合して生じたハイブリダイゼーション分子の濃度とする。この式から，ハイブリダイゼーションが成立した分子の濃度を求めると，

$$[PT]=K[P][T]$$

となる。実際の反応に当てはめると，K がプライマーの特異性に相当し，理想的な特異反応ならば K が大きくなり，非特異反応ならば小さくなる。ところが，非特異反応において K が小さかったとしても，[T]が圧倒的に大きければ，結果として[PT]も大きくなる。本研究で直面している問題は，NoV の[PT]よりもブ菌の[PT]がはるかに大きいということに帰結する。食品汚染の NoV は「何コピー・・・」といった数字で論じられているのに対して，パントラ法で投入されるブ菌は肉眼で見える量であることから天文学的なコピー数になることが当然のごとく予想される。従って，個々のプライマーの特異性や

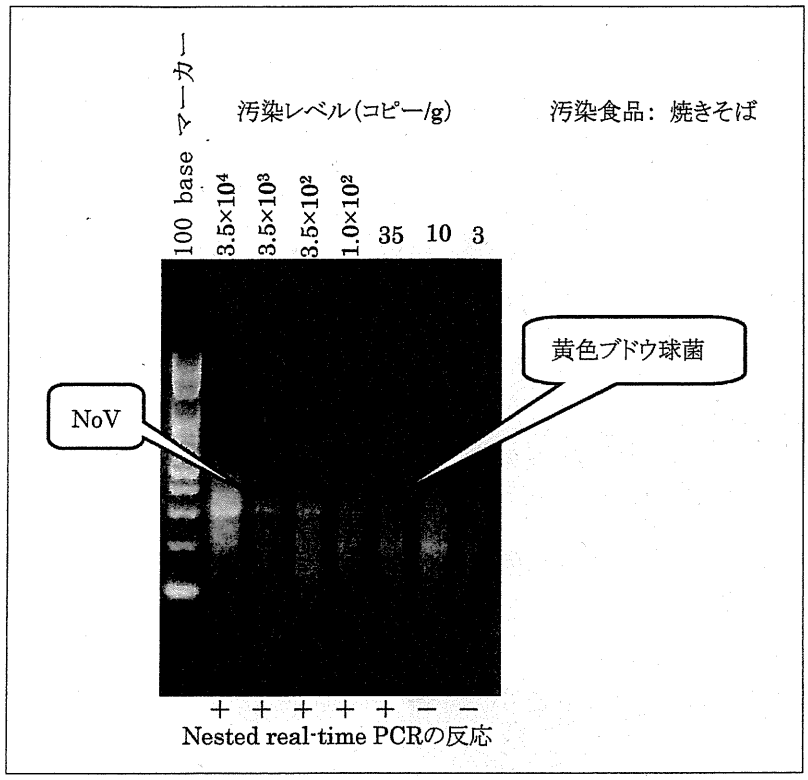

図3　Semi-nested RT-PCR 増幅産物の電気泳動パターン

・図2の1st. PCR 産物を、プライマーG2SKF / G2SKR を用いた2nd. PCR で再増幅した。

PCR の反応条件（いずれも K に影響）にのみ着目しても解決には至らない。例えば，非特異反応の割合が 1 万分の 1（K に相当）である一方で，量的な差が 1 億倍（[T]に相当）あったならば，プライマーの特異性が容易に相殺されてしまうことになる（NoV とブ菌の量的な差はもっと大きい）。

3.3 逆転写反応専用プライマーの設計

個々のプライマーの特異性やPCR の反応条件の改良では問題に対応できないことから，逆転写反応→1st. PCR→2nd. PCR の 3 段階の反応を一つのものとして俯瞰することで解決の糸口を見出した。すなわち，非特異反応にも種類があり，本来ならばそれぞれの反応段階における非特異反応の内容が違うはずである。ところが，実際にはNoV とブ菌の圧倒的な初期濃度差が解消されることなく最後まで反映されていた。そこで，逆転写反応に用いるプライマーの影響を比較しながら，最終的に NoV の遺伝子のみが検出される方法を模索した。

ランダムプライマーで逆転写反応を行うと，10^5 コピー/g のような高濃度汚染サンプルでは高い回収率が得られるものの，低濃度汚染になるにつれ急激に回収率が落ちて，PEG 沈澱法の方が好成績になるという逆転現象がすでに観察されている [9]。図 4 のレーン 3～6 に示したとおり，ポテトサラダをベースとした 100 コピー/g 以下の低濃度汚染サンプルでは，semi-nested RT-PCR を行ってもバンドが観察されず，semi-nested real-time PCR でも増幅が認められなかった。

G2SKR のような PCR と同じ NoV 特異的プライマーを使うと，ブ菌の遺伝子が最後まで混入することは予備試験の結果（図 3）と同様であった。ランダムプライマーで逆転写反応を行った場合と比べて，semi-nested real-time PCR では増幅が認められたが，電気泳動で明瞭なバンドが観察できず，遺伝子解析は困難であった（図 4 レーン 8～11）。ゲル上で薄く見えるバンドを切り出してシークエンスしたところ，レーン 8 と 9 はノイズが多すぎて判読不能であり，レーン 10 と 11 からはブ菌の 16 s リボソームの配列が検出された。

以上の結果から，逆転写反応の段階で PCR とは異なる専用プライマーを用いることが重要と