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## Prevalence and Genetic Diversity of Aichi Virus Strains in Stool Samples from Community and Hospitalized Patients<sup>∇</sup>

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**Aichi virus has been proposed as a causative agent of gastroenteritis. A total of 457 stool specimens from children hospitalized with acute diarrhea and 566 stool specimens from adults and children involved in 110 gastroenteritis outbreaks were screened for the presence of Aichi virus by reverse transcription-PCR (RT-PCR) amplification of the genomic region of the 3C and 3D (3CD) nonstructural proteins. Our results show a low incidence of Aichi virus in pediatric samples and the existence of mixed infections with other microbiological agents in some cases. From the outbreak survey, it appears that the presence of Aichi virus is an indicator of mixed infections causing gastroenteritis outbreaks and that it could be involved in half of the oyster-associated outbreaks. A second RT-PCR was developed to amplify a part of the VP1 gene. The phylogenetic analysis showed a good correlation between the two classifications based on 3CD and VP1 gene sequences and revealed the prevalence of genotype A in France. It also allowed us to partially describe an Aichi virus strain that could represent a new genotype, thus suggesting the existence of a certain diversity.**

Viral gastroenteritis is a common illness that affects humans of all ages. Group A rotaviruses, human caliciviruses including *Norovirus* and *Sapovirus*, adenoviruses, and astroviruses have been recognized as the main agents responsible for this disease. However, for many gastroenteritis cases no etiologic agent can be diagnosed, and it has been suggested that other viruses such as toroviruses, picobirnaviruses, parechoviruses, and picornaviruses are involved (4). Among these, Aichi virus was reported as the likely cause of oyster-associated nonbacterial gastroenteritis in a patient in Aichi, Japan, in 1989 (19). Aichi virus is a member of the *Kobuvirus* genus of the *Picornaviridae* family (14, 22). Its genome consists of a single-stranded, positive-sense RNA molecule of 8,280 nucleotides and a poly(A) tail. The single large open reading frame encodes a polyprotein of 2,432 amino acids that is cleaved into the typical picornavirus structural proteins VP0, VP3, VP1, and nonstructural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D (16, 22). Based on the phylogenetic analysis of 519-bp sequences at the 3C-3D (3CD) junction of 17 isolates, Yamashita et al. (23) have proposed that Aichi viruses be divided into two genotypes A and B with approximately 90% sequence homology.

Little is known about the epidemiology of Aichi virus. In Japan, the use of an enzyme-linked immunosorbent assay (ELISA) showed the presence of the virus in 18.8% of adult patients involved in gastroenteritis outbreaks (20). In another study Aichi virus RNA was detected by reverse-transcription PCR (RT-PCR) in 20.5% of Japanese adults involved in gastroenteritis outbreaks (23). In Germany, Aichi virus was de-

tected in stool samples of patients involved in an outbreak (11). This virus was also isolated from sporadic cases of gastroenteritis in children and adults in Asian countries (12, 21) and in Brazil (11). Moreover, one Japanese child suffering from lower respiratory tract disease was infected by the virus among 397 children consulting for various pathologies (20). In gastroenteritis outbreaks in Japan, seroconversion to Aichi virus was detected in 33 to 80% of the cases (19, 20). Few studies on the seroprevalence of antibodies to the Aichi virus strain have been conducted to assess the epidemiological importance of the virus: in Japan, seroprevalence in a panel of 833 people (age range, 7 months to more than 60 years) was estimated to be 55% (20), with an increase with age (from 7.2% for children aged 7 months to 4 years to over 80% for persons more than 35 years old). A German study showed a comparably high value of seroprevalence (76%) also with an increase with age (11) but with differences in age distribution: the majority of infections in Germany occur among children younger than 6 years old.

In this paper, we report a prevalence survey of Aichi virus strains in France in two populations including sporadic and epidemic cases of gastroenteritis: (i) children hospitalized for acute gastroenteritis between 2001 and 2004 and (ii) patients involved in gastroenteritis outbreaks from January 2006 to April 2007. This study is the first report of the presence of Aichi virus in France in gastroenteritis cases. In addition to using reverse transcription-PCR (RT-PCR) directed against a 519-bp sequence at the 3CD junction to detect the RNA in stool samples, we also developed another RT-PCR to amplify a part of the VP1 gene. The phylogenetic analysis of the strains based on both 3CD and VP1 gene sequences showed a good correlation between the two classifications. It also allowed us to partially describe an Aichi virus strain that could represent a new genotype of the virus.

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TABLE 1. Clinical and epidemiological data observed in the four children infected by Aichi virus

Sample no.	Patient age (mo)	Sex <sup>a</sup>	Clinical data					Epidemiological data			
			Severity score <sup>b</sup>	Bloody stool	Marbling	Antibiotic treatment	i.v. hydration <sup>c</sup>	Breast-feeding	Date of hospitalization	Foreign stay	Circle case <sup>d</sup>
Rn48	72.5	M	14	No	No	No	No	No	August 2002	Yes	Yes
Rn57	17.0	F	12	No	No	No	Yes	No	August 2002	Yes	No
R380	10.5	M	11	No	No	Yes	No	No	December 2002	No	No
R586	60.0	F	14	Yes	No	Yes	Yes	No	August 2003	No	No

<sup>a</sup> M, male; F, female.

<sup>b</sup> Vesikari scale.

<sup>c</sup> i.v., intravenous.

<sup>d</sup> Gastroenteritis cases diagnosed in the family circle.

## MATERIALS AND METHODS

**Stool samples.** Stool samples were collected from 457 of 552 children between 0 and 15 years of age who were hospitalized with acute diarrhea in Saint Vincent de Paul Pediatric Hospital in Paris from November 2001 to May 2004. For each child, clinical data were prospectively collected to grade the gastroenteritis episodes (based on a 20-point Vesikari severity score, bloody diarrhea, need for intravenous hydration, length of hospitalization, marbling, and breast-feeding). Stool samples were collected within 48 h of hospitalization to exclude nosocomial gastroenteritis. The bacteriological analyses were done on fresh samples. The stool samples were then stored at  $-20^{\circ}\text{C}$  and shipped to the virology laboratory, Reference Laboratory for Enteric Viruses (Center National de Reference), Dijon University Hospital, for retrospective viral investigation.

Stool samples were also collected by the Center National de Reference from 566 patients of all ages involved in 110 nonbacterial gastroenteritis outbreaks that took place all over France between January 2006 and April 2007. Thirty-seven of the outbreaks were food borne; of these 10 were associated with oyster consumption, and 4 were associated with the consumption of other seafood.

**RNA and DNA extraction.** Viral nucleic acid was extracted from 10% stool suspensions in phosphate-buffered saline (pH 7.5) by using either a QIAamp Viral RNA kit (Qiagen, Hilden, Germany) or the automated NucliSens easyMAG multiextraction system (BioMerieux) according to the manufacturer's instructions; samples were then stored at  $-40^{\circ}\text{C}$ .

**Enzyme immunoassays and RT-PCR techniques.** Stool samples were systematically screened for the presence of group A rotaviruses, astroviruses, adenoviruses types 40 and 41, human caliciviruses (noroviruses and sapoviruses), and Aichi viruses by an enzyme immunoassay (EIA) and/or RT-PCR. In some cases, enterovirus and hepatitis A viruses were also screened for.

Group A rotaviruses were detected by EIA with group-specific monoclonal antibodies as previously described (13). G- and P-typing of positive samples was done as described by Gouvea et al. (5) and Gentsch et al. (3).

Astroviruses and adenoviruses types 40 and 41 were detected with EIA kits, IDEIA Astrovirus (Dako Diagnostics Ltd.) and Adenoclone type 40/41 EIA (Meridian Diagnostics Inc.), respectively. Astrovirus-positive samples were confirmed by RT-PCR according to the method described by Noel et al. (8). Adenovirus-positive samples were confirmed by PCR according to the method described by Allard et al. (1).

Noroviruses and sapoviruses were detected by several RT-PCRs using different sets of primers (6, 7, 9, 17) allowing the amplification of fragments of the RNA polymerase and the capsid genes.

Enteroviruses were screened for by RT-PCR according to Chapman et al. (2).

Hepatitis A was detected by RT-PCR as described by Robertson et al. (15).

Screening for Aichi viruses was done by RT-PCR with the primer pair 6261 and 6779 described by Yamashita et al. (23), using a Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Briefly, the reaction was performed in 50  $\mu\text{l}$  of reaction mixture containing 29  $\mu\text{l}$  of sterile water, 10  $\mu\text{l}$  of 5 $\times$  Qiagen OneStep RT-PCR buffer, 2  $\mu\text{l}$  of deoxynucleoside triphosphate mix (containing a 10 mM concentration of each deoxynucleoside triphosphate), 2  $\mu\text{l}$  of Qiagen OneStep RT-PCR enzyme mix, 1.5  $\mu\text{l}$  each of 20  $\mu\text{M}$  primer, 1  $\mu\text{l}$  of 20 U/ $\mu\text{l}$  RNase inhibitor (Applied Biosystems), and 3  $\mu\text{l}$  of the extracted RNA. Cycling was performed in a Thermal Cycler 9700 (Perkin Elmer) as follows: 1 cycle of reverse transcription at  $50^{\circ}\text{C}$  for 30 min, followed by 1 cycle of initial denaturation at  $95^{\circ}\text{C}$  for 5 min and then 40 cycles of amplification with denaturation at  $95^{\circ}\text{C}$  for 30 s, annealing at  $55^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 1 min, with a final cycle of incubation at  $72^{\circ}\text{C}$  for 5 min. The amplification product obtained was 519 bp long at the junction between the C terminus of 3C and the N terminus of 3D.

A second primer pair was designed for RT-PCR based on the sequence of GenBank accession number AB010145 of the Aichi virus genome to specifically amplify a part of the VP1 gene. The RT-PCR was performed under the same conditions as those used for the polymerase amplification. This allowed the amplification of a 699-bp fragment. The oligonucleotide primer sequences were 5'-GGTGAATCCTTCAACGTACG and 5'-GCAAGAGAGCTGGAAGT for primers F2954 and R3636, respectively.

**Sequencing of the RT-PCR products.** RT-PCR-amplified DNA fragments of the expected sizes, as determined by agarose gel electrophoresis, were excised from the gel, extracted, and purified for sequencing with a QIAex II gel extraction kit (Qiagen).

Sequencing of the PCR products was performed with the same primers used for amplification by using an ABI Prism Big Dye Terminator cycle sequencing ready reaction kit on an automated sequencer (model 373A DNA sequencing system) (both from Applied Biosystems, Foster City, CA). The nucleotide sequences were determined at least twice in both directions.

**Characterization and phylogenetic analyses.** Alignments of the sequences with the sequences in the EMBL library were carried out by using the Fasta program, version 3, available from the European Bioinformatics Institute EBI (<http://www.ebi.ac.uk>).

For the phylogenetic analyses, sequence alignment and clustering were performed by the unweighted-pair group method using average linkages using Bionumerics software (Applied Maths).

**Nucleotide sequence accession numbers.** The nucleotide sequences reported here have been deposited in the GenBank sequence database under accession numbers DQ145759 to DQ145762 and EU159246 to EU159261.

## RESULTS

**Detection of Aichi virus in stool samples of hospitalized children.** A total of 457 pediatric stool samples were screened for the presence of Aichi virus by RT-PCR using the primers 6261 and 6779. Aichi virus RNA was detected in four samples (0.9% incidence). In samples R380 and R586, Aichi virus was associated in mixed infections with a rotavirus and a Shigella virus, respectively, while in samples Rn48 and Rn57, Aichi virus was the only microbiological agent identified.

Screening for other viruses showed that group A rotaviruses were the predominant virus detected (52.3%), followed by noroviruses (12.0%), enteric adenovirus (3.5%), astroviruses (1.5%), and sapoviruses (0.4%). Bacteria were isolated in 6.8% of the samples.

For the four samples infected by Aichi virus, the clinical and epidemiological data are summarized in Table 1. It has to be noted that for two children, the gastroenteritis episode occurred just after returning from a trip to Africa (Bamako, Mali) (sample Rn48) and to the Balearic Islands (sample Rn57).

**Outbreaks.** Of 566 stool samples tested with the primer pair 6261 and 6779, nine (1.6%) were positive for Aichi virus, which corresponds to six positive outbreaks out of the

TABLE 2. Outbreaks of gastroenteritis positive for Aichi virus

Outbreak no.	Epidemiology				RT-PCR result	
	Date	Setting	Mode of transmission	No. of cases/no. of exposed persons <sup>a</sup>	No. of positive samples/no. tested (%) <sup>b</sup>	Genotype
1	February 2006	Symposium	Oysters	11/80	2/4 (50)	A
2	February 2006	Private house	Oysters	15/?	3/6 (50)	A
3	December 2006	Nursing home	Oysters	21/~40	1/6 (17)	A
4	February 2007	Private house	Oysters	6/8	1/1 (100)	A
5	March 2007	Restaurant	Oysters	3/?	1/3 (33)	A
6	April 2007	Holiday center	Seafood	>16/340	1/16 (6)	A

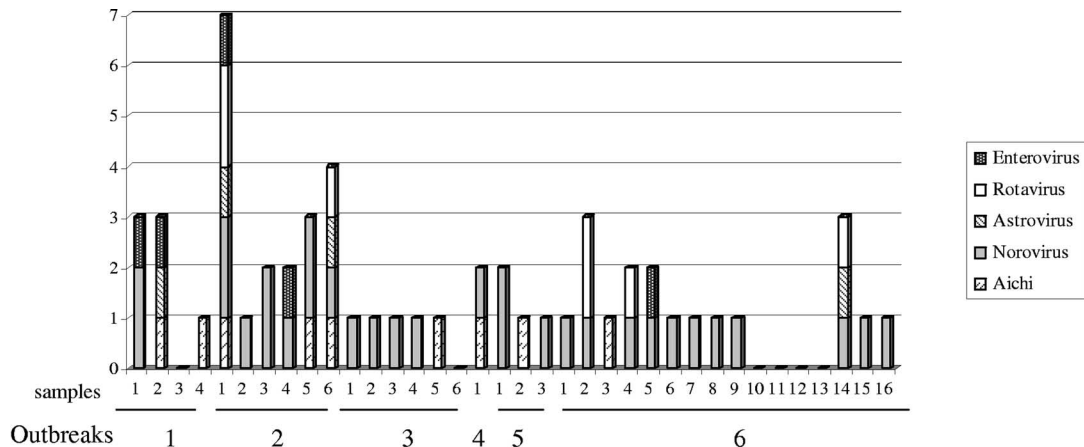
<sup>a</sup> ?, unknown.

<sup>b</sup> The values for the total group were 9/36 (25%).

110 analyzed. The incidence of the other enteric viruses has also been studied. Noroviruses are the predominant agent involved, with 109 strains isolated, followed by rotaviruses (12 strains), astroviruses (5 strains), enteroviruses (5 strains), and sapoviruses (1 strain). It has to be noted that the total number of strains isolated exceeds the number of outbreaks analyzed because of the presence of several mixed infections.

For the six Aichi virus-related outbreaks, the positive rates for Aichi virus varied considerably and ranged from 6 to 100% of the samples, with an average of 25% (9 positive samples out of 36 tested) (Table 2). These rates are related to the number of samples tested, which was different from the number of cases reported in each outbreak, except for outbreak 5. Five of the six outbreaks positive for Aichi virus were associated with oyster consumption, and one was associated with seafood other than oysters.

Figure 1 presents the distribution of the enteric viruses in each tested sample of six Aichi virus-related outbreaks. For four out of nine stool samples, Aichi virus was the only microbiological agent identified. Nevertheless, Aichi virus was never found as the only agent responsible for the outbreaks but was always in combination with at least one other virus. In outbreaks 3, 4, and 5, Aichi virus was associated only with noroviruses representing two, one, and three different genotypes, respectively. Outbreaks 1, 2, and 6 were due to a mix of numerous viruses: in outbreak 1, two genotypes of noroviruses were found along with astrovirus, enterovirus, and Aichi virus. Outbreak 2 was a mixed infection caused by noroviruses of six different genotypes, two strains of rotaviruses, and also astrovirus, enterovirus, and Aichi virus. In outbreak 6, norovirus was the predominant pathogenic agent found, represented by three different genotypes, in combination with two strains of rotavirus, astrovirus, enterovirus, and Aichi virus.



Outbreak n° 1: sample 2 = E1197 – sample 4 = E1202

Outbreak n° 2: sample 1 = E1203 – sample 5 = E1207 – sample 6 = E1208

Outbreak n° 3: sample 5 = E1588

Outbreak n° 4: sample 1 = E1818

Outbreak n° 5: sample 2 = E1859

Outbreak n° 6: sample 3 = E1867

FIG. 1. Distribution of enteric viruses in six Aichi virus-related outbreaks. The two samples specified below the figure for outbreak 1, the three samples specified for outbreak 2, and the four individual samples specified for outbreaks 3 to 6 represent the nine samples positive for Aichi virus as presented in the GenBank database.



**Characterization of the detected strains.** To genotype the four Aichi virus strains isolated in pediatric stool samples and the nine strains isolated in gastroenteritis outbreaks, the PCR products were sequenced with the same primer set, 6261 and 6779, and the partial polymerase sequences obtained were compared with sequences of the EMBL database. The phylogenetic analysis is depicted in Fig. 2. All samples but one were classified as genotype A. Their partial polymerase sequences demonstrate approximately 95% homology in nucleotides and 99% homology in amino acids. It has to be noted that sequences of both samples in outbreak 1 (E1197 and E1202) are 100% identical and so can be considered a unique strain: its polymerase sequence has been deposited in the GenBank database under accession number EU159246. Only E1197 appears in the phylogenetic tree. In the same way, in outbreak 2, samples 1 (E1203) and 5 (E1207) present 100% homology in their polymerase sequences, which have been deposited under accession number EU159247. Only E1203 is reported on the phylogenetic tree. In contrast, the partial polymerase sequence of sample 6 (E1208) in outbreak 2 presents a few differences in nucleotides (98.7% homology with samples 1 and 5) and has been deposited under accession number EU159248.

As shown in Fig. 2, sample Rn48 cannot be assigned to genotype A or B, previously established by Yamashita et al. (23), but must be assigned to a distinct, new genotype. The percentage of nucleotide identity of Rn48 with both genotypes A and B is approximately 87% (93% amino acid identity) whereas, based on the data given by Yamashita et al. (23), nucleotide identities among genotypes A and B are above 95% and 91%, respectively. It has to be noted that Rn48 is also very distant from the described bovine kobuvirus strain (accession number AB084788) (18), as are the other Aichi virus strains isolated in this study.

Specific amplification and sequencing of part of the VP1 gene were also performed with the primer set F2954 and R3636 on all of the samples in which Aichi virus was detected by screening of the polymerase region, except for samples R380 and E1859 because of a lack of stool. As observed with the polymerase sequences, both samples in outbreak 1 demonstrate 100% homology of the VP1 sequences, as do samples 1 and 5 in outbreak 2. The phylogenetic analysis was done by a comparison with four Aichi virus VP1 sequences available in the GenBank database (Fig. 3). The distribution of the strains is the same as that for the polymerase gene sequences. All but Rn48 are assigned to genotype A. Rn48 is distinct from both A and B genotype isolates. Nucleotide identity in genotype A is above 93%, whereas Rn48 is less than 82% identical to genotype A and 84% identical to genotype B. This corresponds to 97% identity of the deduced amino acid sequences for genotype A and 91% between Rn48 and genotypes A and B.

## DISCUSSION

This study presents the first isolation and characterization of Aichi virus in France. Four children hospitalized with acute diarrhea and nine patients involved in gastroenteritis outbreaks were diagnosed as infected with Aichi virus. With regard to the sporadic cases, the low incidence of 0.9% observed in pediatric cases is in accordance with a previous study of Yamashita et al. (21), who isolated Aichi virus from 5 of 222

Pakistani children presenting gastroenteritis symptoms. The low prevalence of Aichi virus was also documented in sporadic cases of gastroenteritis affecting adults and children in several Asian countries: 5 of 722 Japanese tourists returning from tours to Southeast Asian countries were positive for presence of Aichi virus (21). Recently, another study reported the presence of the virus in 28 of 912 fecal specimens from adults and children with diarrhea in Japan, Bangladesh, Thailand, and Vietnam (12).

These observations of sporadic cases of diarrhea (12, 21), taken together with studies of gastroenteritis outbreaks (11, 20, 23), led the authors to propose Aichi virus as a causative agent of gastroenteritis. This suggestion would be even better supported if no other common causative agents were found. In our study, in two fecal samples from hospitalized children, Aichi virus was the only microbiological agent detected among all of the known agents such as rotavirus, astrovirus, adenovirus, sapovirus, norovirus, and bacteria. Although the seroconversion was not documented in our study because of the lack of serum, the absence of other known pathogens in the stool samples collected during a short acute diarrhea episode strongly implicates Aichi virus in these cases. However, since in two samples Aichi virus was associated with another pathogen, the assertion that Aichi virus is the causative agent must be qualified. Rotavirus in one case and *Shigella* in the other case could have been the principal factors causing the gastroenteritis symptoms. Furthermore, Aichi virus has also been isolated in a child presenting a lower respiratory tract disease but no diarrhea (20). As noted by Oh et al. (11), further studies are therefore needed to obtain direct evidence of pathogenesis.

Aichi virus has been detected in gastroenteritis outbreaks in Japan (20, 23) and in Germany (11). The incidence of Aichi virus implicated in cases of gastroenteritis outbreaks in our study is much lower than the incidence reported by the Japanese studies. Yamashita et al. (20) found 13 stool samples that were positive for Aichi virus out of 69 examined by ELISA (18.8%), implicating Aichi virus in five outbreaks out of a total of nine (55%). By RT-PCR, 55 positive samples were reported out of 268 tested (20.5%) in 12 outbreaks out of 37 (32%) (23). In contrast, in our study we found only 9 positive samples among 566 examined (1.6% incidence), corresponding to 6 outbreaks out of 110 (5.4%). This difference might be due to the inclusion criteria of the outbreaks analyzed. Indeed, the study by ELISA was performed on stool samples from nine outbreaks of oyster-associated nonbacterial acute gastroenteritis (20). Also in the study of Yamashita et al. (23), of 37 outbreaks analyzed by RT-PCR, 21 were oyster associated, from which 11 were positive for Aichi virus. In contrast, in our study, only 10 outbreaks were food-borne outbreaks associated with oyster consumption and 5 of the 6 positive outbreaks were oyster associated. Thus, considering only the oyster-associated outbreaks, it appears that the numbers of outbreaks that were positive for Aichi virus are equivalent in the three studies: 55% (5/9), 52% (11/21), and 50% (5/10).

If we consider only the outbreaks that were positive for Aichi virus in the three studies, the detection rates of positive samples for each outbreak vary considerably: in the study by ELISA (20) the positive rate ranged from 13 to 60%, with a mean of 28% (13 of 47 patients). In the study by RT-PCR (23), the detection rate ranged from 14 to 82%, with an average of

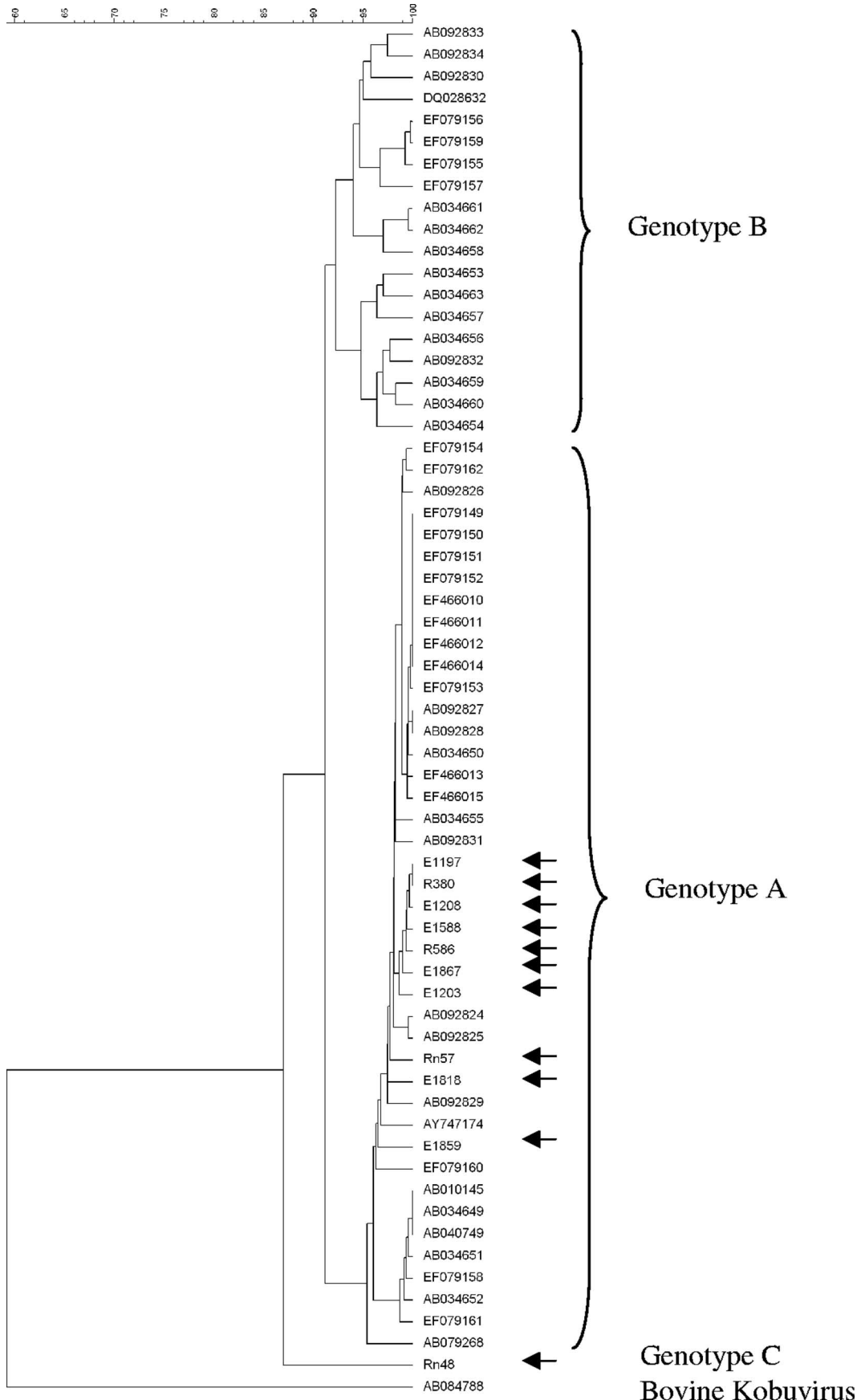


FIG. 2. Phylogenetic analysis of Aichi viruses based on comparison of 519-bp sequences in the 3CD junction region. The Aichi virus strains isolated in this study are indicated by arrows. The reference strains are identified by their GenBank accession numbers.

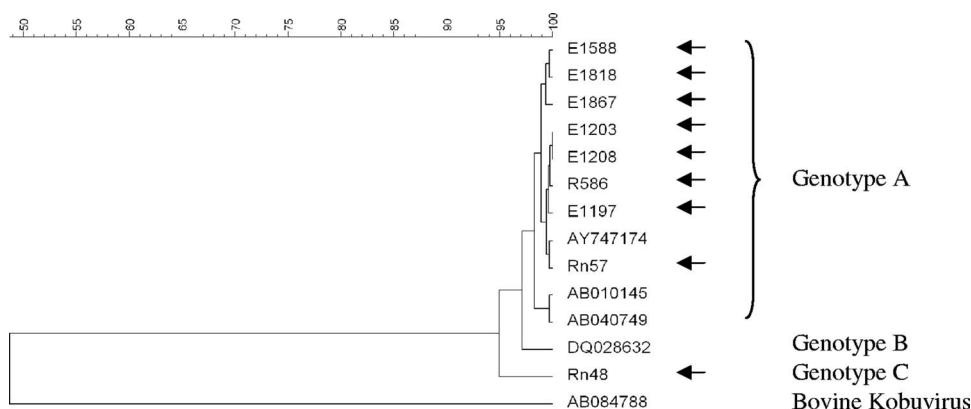


FIG. 3. Phylogenetic analysis of Aichi viruses based on comparison of 220-amino-acid sequences in the VP1 region. The Aichi viruses isolated in this study are indicated by arrows. The reference strains are identified by their GenBank accession numbers.

55% (55 of 99 patients). In our study, the positive rate ranged from 6 to 100%, with an average of 25% (9 of 36 patients). According to Yamashita et al. (23), high positive rates over 50%, as they observed in 10 of 12 outbreaks, could suggest the possibility that Aichi virus is an etiological agent of the outbreaks. However, these rates are related to the number of stool samples tested, which in our study was not always representative of the number of cases in the outbreaks. Indeed, finding one positive patient when only one is tested, as happened in outbreak 4, does not mean that the other five patients were also infected by Aichi virus. Our results should therefore be interpreted carefully; they indicate the presence of Aichi virus in gastroenteritis outbreaks but do not always represent the real rate of positive cases for Aichi virus. Moreover, even though some samples were infected by Aichi virus alone, it was never found as the only agent responsible for the outbreaks but occurred in combination with at least noroviruses and, in some cases, with rotaviruses, astroviruses, and/or enteroviruses as well. This contrasts sharply with the results of Oh et al. (11), who did not detect any viruses other than Aichi virus in the outbreak they reported. But it also has to be noted that Yamashita et al. gave no data about the potential presence of other viruses in the outbreaks they analyzed (21, 23). In view of our observations, and in contrast to previous suggestions (11, 20, 23), we cannot affirm that Aichi virus is the cause of gastroenteritis outbreaks but, rather, that its presence reveals the existence of mixed infections, as observed in oyster-related outbreaks.

As reported above, 50% of the oyster-associated outbreaks that we analyzed in this study turned out to be positive for Aichi virus. The same values were found by Yamashita et al. in two studies (20, 23), meaning that Aichi virus could be involved in half of the oyster-associated outbreaks. Moreover, except for one outbreak associated with seafood consumption, we were not able to detect Aichi virus in any of the other outbreaks that we tested, whether they were food-borne or person-to-person outbreaks. These observations corroborate the hypothesis of a correlation between Aichi virus and oyster pollution, as proposed by Yamashita et al. (23). However, the presence of Aichi virus in four pediatric samples of our study, as in other pediatric samples (11, 12) and in an outbreak not

associated with oyster consumption (23), also means that Aichi virus could be transmitted by other routes than via oysters.

So far, the presence of Aichi virus has essentially been reported in Asian countries, including Japan, Indonesia, Thailand, Malaysia, Pakistan, Bangladesh, and Vietnam (12, 20, 21, 23). Moreover, the presence of Aichi virus has been reported in Germany as well as in Brazil (11). Our results confirm the existence of Aichi virus in Europe (France and Balearic Islands) and extend the findings to Africa, since one child hospitalized for gastroenteritis had just returned from a trip to Mali. The phylogenetic comparison of Aichi virus sequences at the 3CD junction region showed that all but one of our samples can be assigned to genotype A. This observation, in addition to the description of a German isolate of genotype A (11), could suggest a prevalence of this genotype in Europe, as is the case in Japan (23).

In parallel, a phylogenetic analysis of the VP1 coding region was performed, since VP1 sequence comparisons have been suggested as valuable in picornavirus classification (10). The distribution of the strains according to the VP1 sequences appeared to be the same as that obtained using analysis of the 3CD region. All but one of the sequences were classified as genotype A. Nevertheless, it has to be noted that nucleotide identities within genotype A were lower for the VP1 region (93%) than for the 3CD region (95%), confirming the suggestion of Yamashita et al. (23) and Oh et al. (11) that sequence diversity in the P1 region may be higher than in the 3CD region. Despite this observation, the good correlation between the two phylogenetic analyses indicates that genotype classifications based on either the 3CD region or the VP1 region are valid.

Furthermore, the comparative study of the 3CD region revealed the existence of one strain that could not be assigned to genotype A or B but could represent a new genotype C. This classification was confirmed by the phylogenetic analysis of the VP1 region. Further studies are under way to get a complete genetic characterization of this strain. It has to be noted that this strain was isolated from a child returning from a trip to Africa. Since no study has been published yet on the prevalence of Aichi virus in Africa, additional studies will be required to learn about its epidemiology there and to determine

whether this genotype C is the most prevalent throughout the continent or in specific countries, as genotype A seems to be in Japan and in Europe.

Finally, Aichi virus remains rare, and its presence in gastroenteritis outbreaks can be considered as an indicator of mixed infections. The phylogenetic analysis of the isolated strains in this study shows, with the description of a new genotype, the existence of a certain degree of diversity.

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#### REFERENCES

- Allard, A., B. Albinsson, and G. Wadell. 2001. Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. *J. Clin. Microbiol.* **39**:498–505.
- Chapman, N. M., S. Tracy, C. J. Gauntt, and U. Fortmueller. 1990. Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. *J. Clin. Microbiol.* **28**:843–850.
- Gentsch, J. R., R. I. Glass, P. Woods, V. Gouvea, M. Gorziglia, J. Flores, B. K. Das, and M. K. Bhan. 1992. Identification of group A rotavirus gene 4 types by polymerase chain reaction. *J. Clin. Microbiol.* **30**:1365–1373.
- Glass, R. I., J. Bresee, B. Jiang, J. Gentsch, T. Ando, R. Fankhauser, J. Noel, U. Parashar, B. Rosen, and S. S. Monroe. 2001. Gastroenteritis virus: an overview. *Novartis Found. Symp.* **238**:5–19.
- Gouvea, V., R. I. Glass, P. Woods, K. Taniguchi, H. F. Clark, B. Forrester, and Z. Y. Fang. 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J. Clin. Microbiol.* **28**:276–282.
- Kojima, S., T. Kageyama, S. Fukushi, F. B. Hoshino, M. Shinohara, K. Uchida, K. Natori, N. Takeda, and K. Katayama. 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J. Virol. Methods* **100**:107–114.
- Le Guyader, F., M. K. Estes, M. E. Hardy, F. H. Neill, J. Green, D. W. Brown, and R. L. Atmar. 1996. Evaluation of a degenerate primer for the PCR detection of human caliciviruses. *Arch. Virol.* **141**:2225–2235.
- Noel, J. S., T. W. Lee, J. B. Kurtz, R. I. Glass, and S. S. Monroe. 1995. Typing of human astroviruses from clinical isolates by enzyme immunoassay and nucleotide sequencing. *J. Clin. Microbiol.* **33**:797–801.
- Noel, J. S., B. L. Liu, C. D. Humphrey, E. M. Rodriguez, P. R. Lambden, I. N. Clarke, D. M. Dwyer, T. Ando, R. I. Glass, and S. S. Monroe. 1997. Parkville virus: a novel genetic variant of human calicivirus in the Sapporo virus clade, associated with an outbreak of gastroenteritis in adults. *J. Med. Virol.* **52**:173–178.
- Oberste, M. S., K. Maher, D. R. Kilpatrick, and M. A. Pallansch. 1999. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. *J. Virol.* **73**:1941–1948.
- Oh, D. Y., P. A. Silva, B. Hauroeder, S. Diedrich, D. D. Cardoso, and E. Schreier. 2006. Molecular characterization of the first Aichi viruses isolated in Europe and in South America. *Arch. Virol.* **151**:1199–1206.
- Pham, N. T., P. Khamrin, T. A. Nguyen, D. S. Kanti, T. G. Phan, S. Okitsu, and H. Ushijima. 2007. Isolation and molecular characterization of Aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam. *J. Clin. Microbiol.* **45**:2287–2288.
- Pothier, P., and E. Drouet. 1987. Development and evaluation on a rapid one-step ELISA for rotavirus detection in stool specimens using monoclonal antibodies. *Ann. Inst. Pasteur Virol.* **13**:285–295.
- Pringle, C. R. 1999. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. *Arch. Virol.* **144**:2065–2070.
- Robertson, B. H., V. K. Brown, and B. Khanna. 1989. Altered hepatitis A VP1 protein resulting from cell culture propagation of virus. *Virus Res.* **13**:207–212.
- Sasaki, J., Y. Kusuhara, Y. Maeno, N. Kobayashi, T. Yamashita, K. Sakae, N. Takeda, and K. Taniguchi. 2001. Construction of an infectious cDNA clone of Aichi virus (a new member of the family *Picornaviridae*) and mutational analysis of a stem-loop structure at the 5' end of the genome. *J. Virol.* **75**:8021–8030.
- Vinje, J., and M. P. Koopmans. 1996. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in The Netherlands. *J. Infect. Dis.* **174**:610–615.
- Yamashita, T., M. Ito, Y. Kabashima, H. Tsuzuki, A. Fujiura, and K. Sakae. 2003. Isolation and characterization of a new species of kobuvirus associated with cattle. *J. Gen. Virol.* **84**:3069–3077.
- Yamashita, T., S. Kobayashi, K. Sakae, S. Nakata, S. Chiba, Y. Ishihara, and S. Isomura. 1991. Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. *J. Infect. Dis.* **164**:954–957.
- Yamashita, T., K. Sakae, Y. Ishihara, S. Isomura, and E. Utagawa. 1993. Prevalence of newly isolated, cytopathic small round virus (Aichi strain) in Japan. *J. Clin. Microbiol.* **31**:2938–2943.
- Yamashita, T., K. Sakae, S. Kobayashi, Y. Ishihara, T. Miyake, A. Mubina, and S. Isomura. 1995. Isolation of cytopathic small round virus (Aichi virus) from Pakistani children and Japanese travelers from Southeast Asia. *Microbiol. Immunol.* **39**:433–435.
- Yamashita, T., K. Sakae, H. Tsuzuki, Y. Suzuki, N. Ishikawa, N. Takeda, T. Miyamura, and S. Yamazaki. 1998. Complete nucleotide sequence and genetic organization of Aichi virus, a distinct member of the *Picornaviridae* associated with acute gastroenteritis in humans. *J. Virol.* **72**:8408–8412.
- Yamashita, T., M. Sugiyama, H. Tsuzuki, K. Sakae, Y. Suzuki, and Y. Miyazaki. 2000. Application of a reverse transcription-PCR for identification and differentiation of Aichi virus, a new member of the Picornavirus family associated with gastroenteritis in humans. *J. Clin. Microbiol.* **38**:2955–2961.