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Aichi virus infection in children with acute gastroenteritis in Finland

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SUMMARY

Aichi virus has been proposed as a novel causative agent of acute gastroenteritis. In addition to several Asian countries, South America and Africa, Aichi virus has also recently been found in Europe. Our objective was to study the causative role of Aichi virus in children with acute gastroenteritis in Finland. We analysed 595 stool specimens from infants in an efficacy trial of rotavirus vaccine and 468 stool specimens from children in a hospital-based epidemiological and aetiological study of acute gastroenteritis. The screening was done by nested reverse transcription–polymerase chain reaction amplifying a 519-bp segment and a 223-bp segment in the 3CD junction region of non-structural proteins. Aichi virus was detected in five stool samples (0·5%), of which four were co-infections with other gastroenteritis viruses. Two Aichi virus genotypes, A and B, were found. Aichi virus appears to be rare in children with acute gastroenteritis in Finland.

Key words: Gastroenteritis, virology.

INTRODUCTION

Acute gastroenteritis is a common cause of morbidity and mortality worldwide, especially in children. Major aetiological agents of viral gastroenteritis are group A rotaviruses, human caliciviruses, adenoviruses and astroviruses [1]. However, there remains a diagnostic gap for unknown viral agents, possibly including Aichi virus.

Aichi virus was first isolated in Japan from a patient with oyster-associated non-bacterial gastroenteritis in 1989 [2]. It was classified into the Picornaviridae family as a member of the *Kobuvirus* genus that also includes bovine kobuvirus and recently proposed porcine kobuvirus [3, 4]. The complete nucleotide sequence of Aichi virus was determined in 1998 [3].

A reverse transcription–PCR (RT–PCR) method for the genetic analysis of the 519-bp sequence in the junction of non-structural proteins 3C and 3D was developed by Yamashita *et al*. [5]. Based on the phylogenetic analysis of the 519-bp region, three genotypes have been proposed: genotype A is common in Japan and Europe [5–7], genotype B in Asian countries other than Japan and in Brazil [6, 8], and genotype C possibly in Africa [7].

Since its discovery in Japan, Aichi virus has also been detected in other Asian countries, South America, Africa and Europe [4, 6–11]. The first cases of Aichi virus in Europe were reported by Oh *et al*. in

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2006 from an outbreak of gastroenteritis in Germany [6]. In France, Ambert-Balay and co-workers found Aichi virus in children and adults hospitalized for gastroenteritis [7], and Le Guyader *et al*. from a gastroenteritis outbreak associated with oysters [11]. Aichi virus has commonly been found in mixed infections with other viruses and/or bacteria, and therefore, it has been difficult to conclude on its aetiological role in acute gastroenteritis [6, 7, 10]. Generally, studies show low incidence rates of the virus in gastroenteritis but high prevalence of antibodies in the population [7, 9, 12].

In 2005, Jokela *et al*. screened 68 stool specimens from Finnish gastroenteritis patients aged 3 days to 86 years by a multiplex RT–PCR, but no Aichi virus was found [13]. Thus, this report presents the first cases of Aichi virus infection in Finland.

METHODS

Clinical samples

Stool samples from two separate clinical studies were examined. The studies were approved by the Ethics Committee of Pirkanmaa Hospital District (reference numbers 00095M and 06064). A total of 595 stool specimens were analysed from infants participating in a community-based efficacy trial of RIX4414 human rotavirus vaccine between September 2000 and June 2002. The study design and details have been described elsewhere [14]. Of 595 samples, 393 were collected from the vaccine recipients and 202 from the placebo recipients during the follow-up. Children were vaccinated at ages 2 and 4 months and followed for two winter epidemic seasons of rotavirus up to the age of 20–24 months.

The second study was a hospital-based epidemiological and aetiological study of acute gastroenteritis in children conducted in Tampere and Kuopio University Hospitals from August 2006 to August 2008 (S. Räsänen *et al*., unpublished observations). Stool samples were collected in the emergency room or in the hospital ward from children aged <15 years with symptoms of acute gastroenteritis. A total of 468 stool specimens were available for this study, of these 400 were cases of acute gastroenteritis, 25 of fever and vomiting, 21 of respiratory infection, and 22 samples were from healthy children.

To assess the clinical severity of acute gastroenteritis we used a 20-point Vesikari score [15]. A score of <7 was prospectively defined as mild, a score of 7–10 as moderate, and a score of $\geqslant 11$ as severe.

RT–PCR method

Viral RNA was extracted from 10% stool suspensions in phosphate-buffered saline (PBS) by using either a Boom's silica method [16], or a QIAamp Viral RNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Stool suspensions were stored at -20 °C, and extracted RNA at -70 °C.

RT reactions were performed by means of random priming as previously described [17], except that the reaction contained 4 μl of 5 × first strand buffer (Invitrogen, USA), and the final concentration of dNTPs (Promega, USA) was 375 nM per nucleotide. Synthesized cDNA was stored at -20 °C.

Nested PCR was conducted with the primer sets amplifying the 3CD junction region as described by Yamashita *et al*. [5]. The first PCR was performed with primers 6261 and 6779 amplifying a 519-bp region between the C terminus of 3C and the N terminal of 3D. To amplify a 223-bp segment within the 3CD region, the second PCR included the primers C94b and 246k. In brief, 5 μl cDNA were added to the first PCR mixture (total volume of 25 μl) consisting of 12·5 μl sterile water, 2·5 μl GeneAmp® 10 × PCR buffer II (Applied Biosystems, USA), 1·5 mM GeneAmp $MgCl_2$ (Applied Biosystems), 225 μM of dNTPs (Promega), 0·5 μM of each primer, and 1·25 U AmpliTaq® DNA polymerase (Applied Biosystems). The cycling conditions run in GeneAmp PCR system 9700 or Thermal Cycler 2720 (both from Applied Biosystems) were as follows: primary denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 1 min, and final incubation at 72 °C for 5 min. Denaturation, annealing and elongation steps were repeated for 35 cycles.

Two microlitres of the first PCR reaction product were added to the second PCR mixture. The reaction was performed in total volume of 50 μl containing 29·6 μl sterile water, 10 μl of 5 × Green GoTaq® Flexi buffer (Promega), 1·5 mM GoTaq $MgCl_2$ solution (Promega), 150 μM of dNTPs (Promega), 0·5 μM of each primer, and 2 U GoTaq DNA polymerase (Promega). The cycling conditions run in GeneAmp PCR system 9700 or Thermal Cycler 2720 were as follows: primary denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 64 °C for 40 s, elongation at 72 °C for 30 s, and final incubation

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Table 1. *Clinical and epidemiological characteristics of samples positive for Aichi virus*

Sample no.	Age (months)	Sex*	Stool sample date	Severity score†	Mixed infections	Genotype
1	10	n.a.	June 2001	14	Rotavirus	A
2	17	n.a.	February 2002	9	Norovirus	A
3	49	M	February 2007	14	Norovirus	A
4	11	F	November 2007	16	Rotavirus	B
5	4	F	December 2007	6	None	B

* n.a., Not available; M, male; F, female.

† According to a 20-point Vesikari score [15]; a score of <7 is mild, 7–10 is moderate, $\geqslant 11$ is severe.

at 72 °C for 5 min. Denaturation, annealing and elongation steps were repeated for 30 cycles. The amplicons from both PCRs were analysed by agarose gel electrophoresis to confirm the correct size of the product.

Sequence analyses

PCR products from samples positive for Aichi virus RNA were excised from the gel, extracted and purified by QIAquick gel extraction kit (Qiagen). Sequencing was performed using the BigDye Terminator version 1.1 cycle sequencing kit and ABI Prism 310 Genetic Analyzer (both from Applied Biosystems) with primers C94b and 246k.

Sequence analysis and alignments were performed with the Sequencher version 4.1.4 program (Gene Codes Corporation, USA), and sequence homologies were evaluated using the basic local alignment search tool (BLAST) in GenBank. ClustalW2 [18], GeneDoc [19] and MEGA4 [20] programs were used to construct phylogenetic trees.

Detection of other gastroenteritis viruses

In addition, stool samples were screened for the presence of group A rotaviruses and human caliciviruses (norovirus and sapovirus), and for the vaccine efficacy trial also astroviruses, by modified PCR methods from protocols described earlier [21–23].

RESULTS

Of the 595 stool samples from the rotavirus vaccine efficacy trial, Aichi virus RNA was detected in two samples (0·3%), one from a placebo recipient (sample 1) and the other from a vaccine recipient (sample 2) (Table 1). Both samples were co-infected with either rotavirus (sample 1) or norovirus (sample 2). The severity of the disease is shown in Table 1.

Of the 468 stool samples analysed from the hospital-based epidemiological study, three samples were positive for Aichi virus (0·6% incidence). Aichi virus-positive samples were all detected in cases of acute gastroenteritis, and no Aichi virus was detected in 25 patients with fever and vomiting, 21 patients with respiratory infections, or in 22 healthy children. Samples 3 and 4 were co-infected with norovirus and rotavirus, respectively; however, sample 5 contained Aichi virus alone (Table 1). The severity for the mixed infections was scored as severe but the single mono-infection of Aichi virus was associated with only a mild disease.

Aichi virus genotyping was performed by sequencing the positive PCR products and comparing the sequences to the Aichi virus reference strains available in GenBank. The partial nucleotide sequences of 223 bp in the 3CD region were used to construct phylogenetic trees. The phylogenetic tree of Aichi virus nucleotide sequences detected from stool samples together with selected reference strains is shown in Figure 1.

Two Aichi virus genotypes, A and B, were found (Table 1). Genotype A was found in gastroenteritis infections in 2001–2002 as well as in 2007. However, two samples from 2007 were classified as genotype B.

DISCUSSION

This study describes the first cases of Aichi virus in children associated with acute gastroenteritis in Finland. Aichi virus was detected in five stool samples of 1063 patients (595 infant outpatients and 468 hospitalized children). The low incidence of 0·5% is in

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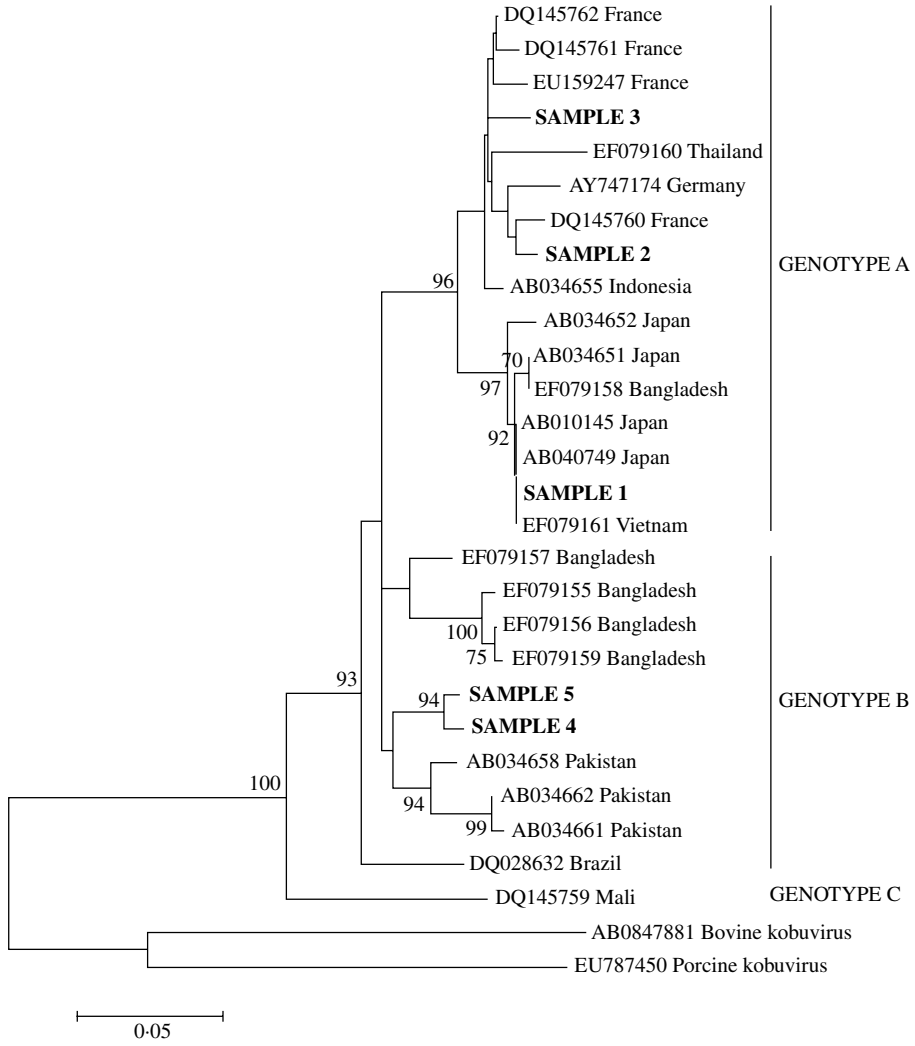

Fig. 1. Phylogenetic tree of Aichi virus nucleotide sequences detected in stool samples. The tree was constructed by comparing a 223-nt segment in the 3CD junction region using the neighbour-joining method in MEGA4. The strains detected in this study are identified by sample numbers 1–5, and the selected reference strains are marked by their accession numbers in GenBank followed by the country of origin. Bootstrap values (based on 1000 replicates) above 70 % are shown at the branch nodes.

accord with previous studies. Earlier, Aichi virus was isolated from 5/222 (2·3 %) Pakistani children, 5/722 (0·7 %) Japanese travellers returning from South East Asia, and 28/912 (3·1 %) gastroenteritis patients in Bangladesh, Thailand and Vietnam [4, 8]. Recently, 4/457 (0·9 %) hospitalized children in France, and 22/632 (3·5 %) Tunisian children were found to be infected with Aichi virus [7, 10].

In addition, our findings support the current observations that Aichi virus is found mostly in mixed infections [6, 7, 10]. When screened for other gastroenteritis viruses, noroviruses were most common (25·0 %) followed by rotaviruses (17·8 %), sapoviruses (6·6 %) and astroviruses (4·2 %) in 595 stool samples from infant outpatients (S. Q. Zeng, personal communication). Regarding 468 stool samples from hospitalized children, rotaviruses (38·9 %), noroviruses (27·1 %) and sapoviruses (1·1 %) were detected (S. Räsänen, personal communication). In the present data, 4/5 samples positive for Aichi virus were co-infected with either rotavirus or norovirus. Mixed infections with sapovirus or astrovirus were not observed. However, it is not possible to conclude whether Aichi virus was the aetiological agent causing symptoms of acute gastroenteritis in these cases.

Interestingly, the single Aichi virus monoinfection caused a mild disease. Similar observations were also noted previously, and it has been suggested that the

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number of Aichi virus infections is substantially underestimated due to subclinical symptoms or even an asymptomatic disease [12]. Nevertheless, a previous study in Tunisian children hospitalized for acute gastroenteritis showed no significant difference in severity of the monoinfections compared to mixed infections [10]. However, the rate of hospitalizations (3·6%) caused by Aichi virus monoinfections was higher than in other reports.

So far Aichi virus genotype A has been the only genotype found in Europe. However, in the present study, both genotypes A and B were found. Further investigations in other European countries are warranted to explore the extent of Aichi virus genotypes.

In addition to the cases of acute gastroenteritis, we investigated the prevalence of Aichi virus in a small number of patients with respiratory infections. To our knowledge, in addition to the study performed in Japan in 1990s [9], this is the second report to examine stool samples from patients with respiratory infections but no diarrhoea. Yamashita *et al*. [9] found Aichi virus in 1/95 patients having a lower respiratory tract disease. We found no Aichi virus associated with respiratory infections in 21 stool samples tested, nor in samples of fever and vomiting, or samples from healthy children.

In conclusion, Aichi virus was rarely detected in stool samples from infant outpatients and hospitalized children with acute gastroenteritis in Finland. Further studies in other populations are, however, justified in order to reveal the role of Aichi virus as a causative agent of acute gastroenteritis. The rate of symptomatic disease and the prevalence of antibodies to the virus in Finland warrant further elucidation. The true significance of Aichi virus as a human pathogen therefore remains to be investigated by future studies.

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DECLARATION OF INTEREST

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REFERENCES

1. **Glass RI, *et al.*** Gastroenteritis viruses: an overview. *Novartis Foundation Symposium* 2001; **238**: 5–19; discussion 19–25.
2. **Yamashita T, *et al.*** Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. *Journal of Infectious Diseases* 1991; **164**: 954–957.
3. **Yamashita T, *et al.*** Complete nucleotide sequence and genetic organization of Aichi virus, a distinct member of the Picornaviridae associated with acute gastroenteritis in humans. *Journal of Virology* 1998; **72**: 8408–8412.
4. **Yamashita T, *et al.*** Isolation of cytopathic small round virus (Aichi virus) from Pakistani children and Japanese travelers from Southeast Asia. *Microbiology and Immunology* 1995; **39**: 433–435.
5. **Yamashita T, *et al.*** 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. *Journal of Clinical Microbiology* 2000; **38**: 2955–2961.
6. **Oh DY, *et al.*** Molecular characterization of the first Aichi viruses isolated in Europe and in South America. *Archives of Virology* 2006; **151**: 1199–1206.
7. **Ambert-Balay K, *et al.*** Prevalence and genetic diversity of Aichi virus strains in stool samples from community and hospitalized patients. *Journal of Clinical Microbiology* 2008; **46**: 1252–1258.
8. **Pham NT, *et al.*** Isolation and molecular characterization of Aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam. *Journal of Clinical Microbiology* 2007; **45**: 2287–2288.
9. **Yamashita T, *et al.*** Prevalence of newly isolated, cytopathic small round virus (Aichi strain) in Japan. *Journal of Clinical Microbiology* 1993; **31**: 2938–2943.
10. **Sdiri-Loulizi K, *et al.*** Acute infantile gastroenteritis associated with human enteric viruses in Tunisia. *Journal of Clinical Microbiology* 2008; **46**: 1349–1355.
11. **Le Guyader FS, *et al.*** Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. *Journal of Clinical Microbiology* 2008; **46**: 4011–4017.
12. **Goyer M, *et al.*** Seroprevalence distribution of Aichi virus among a French population in 2006–2007. *Archives of Virology* 2008; **153**: 1171–1174.
13. **Jokela P, *et al.*** Detection of human picornaviruses by multiplex reverse transcription-PCR and liquid hybridization. *Journal of Clinical Microbiology* 2005; **43**: 1239–1245.

14. **Vesikari T, *et al.*** Efficacy of RIX4414 live attenuated human rotavirus vaccine in Finnish infants. *Pediatric Infectious Disease Journal* 2004; **23**: 937–943.
15. **Ruuska T, Vesikari T.** Rotavirus disease in Finnish children: Use of numerical scores for clinical severity of diarrhoeal episodes. *Scandinavian Journal of Infectious Diseases* 1990; **22**: 259–267.
16. **Boom R, *et al.*** Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology* 1990; **28**: 495–503.
17. **Pang XL, *et al.*** Multiplex real time RT-PCR for the detection and quantitation of norovirus genogroups I and II in patients with acute gastroenteritis. *Journal of Clinical Virology* 2005; **33**: 168–171.
18. **Chenna R, *et al.*** Multiple sequence alignment with the clustal series of programs. *Nucleic Acids Research* 2003; **31**: 3497–3500.
19. **Nicholas KB, *et al.*** Analysis and visualization of genetic variation. *EMBNEW News* 1997; **4**: 14.
20. **Tamura K, *et al.*** MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4·0. *Molecular Biology and Evolution* 2007; **24**: 1596–1599.
21. **Pang XL, *et al.*** Rotaviruses detected by reverse transcription polymerase chain reaction in acute gastroenteritis during a trial of rhesus-human reassortant rotavirus tetravalent vaccine: Implications for vaccine efficacy analysis. *Journal of Clinical Virology* 1999; **13**: 9–16.
22. **Farkas T, *et al.*** Genetic diversity among sapoviruses. *Archives of Virology* 2004; **149**: 1309–1323.
23. **Grimm AC, *et al.*** Development of an astrovirus RT-PCR detection assay for use with conventional, real-time, and integrated cell culture/RT-PCR. *Canadian Journal of Microbiology* 2004; **50**: 269–278.