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Molecular characterization of the first Aichi viruses isolated in Europe and in South America

Brief Report

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Summary. The occurrence of Aichi virus, a picornavirus associated with acute gastroenteritis, has so far only been described in Asian countries. This study reports the first finding of Aichi virus in clinical specimens from Germany and Brazil. The nucleotide sequences of both a German and a Brazilian isolate were determined, analyzed, and compared to known Aichi sequences. The German strain turned out to be a member of genogroup A, while the Brazilian belonged to genogroup B. For a primary assessment of the epidemiological importance of Aichi virus in Germany, a panel of 485 German serum samples was screened for antibody to Aichi virus, and a seroprevalence of 76% was found.

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Infectious gastroenteritis is a common illness that affects children and the elderly the most severely. Rotaviruses, adenoviruses, astroviruses, and caliciviruses have been established as the most important etiologic agents [1]. However, there remains a "diagnostic gap", which has been attributed to less explored viral pathogens. Among these, Aichi virus was first recognized in 1989 as the likely cause of oyster-associated gastroenteritis in a Japanese patient [9]. The presence of virus-specific antigen and of viral RNA was demonstrated in fecal specimens collected from Japanese gastroenteritis outbreaks; up to 32% of these were associated with Aichi virus. The virus has also been isolated from Pakistani children and from

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tourists returning from Southeast Asian countries, suffering from gastrointestinal symptoms, but so far has not been detected outside of Asia [3, 10, 11]. In Japan, a considerable prevalence of antibody to Aichi virus was found, suggesting that infections with this agent are quite frequent [10]. There is currently a lack of data regarding the seroprevalence in countries other than Japan.

The first genomic sequence was determined from a Japanese isolate in 1998 [12]. Complete sequences of other wild Aichi strains have not been published yet. The Aichi virus genome consists of a positive-sense, single-stranded, polyadenylated RNA molecule with a length of approximately 8.3 kb. An organization typical of picornaviruses is displayed, consisting of Vpg, 5′ UTR, leader protein, structural proteins, nonstructural proteins, and a 3′ UTR. In the family *Picornaviridae*, with an amino acid identity of 30–50% between genera, *Aichi virus* is a species of the novel genus *Kobuvirus* according to the International Committee on Taxonomy of Viruses [6]. Phylogenetic analysis based on a genome segment in the 3C/3D junction region revealed the occurrence of at least two genotypes termed A and B [13].

In this study, the nucleotide sequences of two Aichi virus isolates, one from Brazil and the other from Germany, were determined, analyzed and compared to known Aichi virus sequences.

Using electron microscopy (EM) and specific, newly established RT-PCR methods, Aichi virus was detected in three German stool samples and in five Brazilian samples. The German isolates were obtained from the fecal specimens of patients involved in an outbreak of acute gastroenteritis. For electron microscopy, the samples were diluted 1:5 in phosphate buffered saline (pH 7.2), homogenised and clarified by centrifugation (10 min, 2000 × g). Supernatants were concentrated by ultracentrifugation (60 min, 50.000 × g). Pellets were resuspended in distilled water, and specimens were stained with phosphotungstic acid (2%, pH 7.2) Electron microscopic examination revealed the presence of virus particles about 30 nm in diameter, with a surface morphology suggestive of picornaviruses. No other etiological agent, such as calicivirus, rotavirus, or astrovirus could be detected. The Brazilian Aichi virus was found when randomly chosen stool samples of Brazilian children suffering from diarrhea were screened using a virus-specific RT-PCR.

For molecular detection of Aichi virus, genomic RNA was obtained from 140 µl of the ultracentrifugates and from the Brazilian stool samples by application of a spin column technique according to manufacturer's instructions (QIAGEN, Germany). The RNA underwent a nested RT-PCR, which had been established beforehand with the Japanese reference strain (kindly provided by T. Yamashita) as a positive control. First-round amplifications were carried out with primers *Sense1*[1] (6290, 5′-ACA CTC CCA CCT CCC gCC AgT A-3′)[2] and *Antisense1* (6602, 5′-Agg ATg ggg Tgg ATR ggg gCA gAg-3′), which also functioned as an

[1]All nucleotide positions refer to the genomic Aichi virus cDNA clone (GenBank acc. no. AB040749).

[2]Reported by Yamashita et al. [13].

RT primer. Nested PCR was done using *Sense2* (6309, 5′-gTA CAA ggA CAT gCg gCg-3′) and *Antisense2* (6488, 5′-CCT TCg Aag gTC gCg gCR Cgg TA-3′). RT-PCR conditions were the same as described previously for astrovirus [4]. The nucleotide sequences of two viruses were determined from eight (German isolate) and four (Brazilian isolate) overlapping PCR fragments, which were cloned with the pGEM®-T Vector Kit according to manufacturer's instructions (Promega, USA) before they were sequenced as described previously [7]. Primers used for RT, PCR, and sequencing were derived either from the reference sequence or from sequence data obtained during the characterization of both isolates. Single-stranded DNA was synthesized with Superscript II, PowerScript™, or Thermo-X Reverse Transcriptases (Invitrogen, Germany; BD Clontech, Germany; Invitrogen, Germany) under cycling conditions as recommended by the respective manufacturer. PCR fragments were generated using either Takara, Phusion, or AccuPrime™ Pfx DNA polymerases (TaKaRa, Japan; Finnzymes, Finland; Invitrogen, Germany), and cycling conditions depended on the polymerase used. Briefly, the initial denaturation step was for 15 s–2 min at 95–98 °C, followed by 35 amplification cycles (95–98 °C, 10–15 sec; 42–55 °C, 30 sec; 68–72 °C, 5 min) and a final extension step of 5 min at 68–72 °C. Sequence alignments, phylogenetic analysis, and estimation of genetic distances were performed using the Phylogeny Interference Package (PHYLIP) as included in the BioEdit program, version 5.0.9 [2].

Regarding the three German ultracentrifugates, Aichi virus RT-PCR was positive, and subsequent sequencing of the amplicons confirmed classification as Aichi virus. One was randomly chosen for further sequence analysis. Five of the Brazilian fecal specimens, all of which had been obtained from the same geographical region (Goiânia, Central Brazil), yielded positive RT-PCR results, with identical amplicon sequences. In addition to Aichi virus, astrovirus was found in four of the Brazilian samples; of these, one contained norovirus as well, but neither rotavirus nor adenovirus was found. Further sequence analysis was performed on the isolate from the mono-infected patient.

For both the German and the Brazilian Aichi virus isolates, sequences with a length of 8266 nucleotides (nt) were determined[3]. The German sequence contains a single open reading frame with a length of 7299 nt, encoding a putative polyprotein precursor of 2432 amino acids (aa), whereas the 7302 nt – ORF of the Brazilian isolate encodes a precursor protein of 2433 aa. This ORF is preceded by a 5′ UTR at least 730 nt in length (for the Brazilian isolate, 727 nt). The 3′ UTRs of both viruses measure 237 nt excluding the poly(A) tracts. Base compositions of the isolates were found to be similar, with A, 19.3% for the German isolate (19.9%, Brazilian isolate), C, 38.2% (37.9%), G, 21.3% (20.8%) and U, 21.2% (21.4%).

The polyprotein precursors of both new Aichi strains comprise a predicted leader protein of 170 aa and putative VP0, VP3, and VP1 proteins with lengths of

[3]GenBank accession numbers AY747174 and DQ028632.

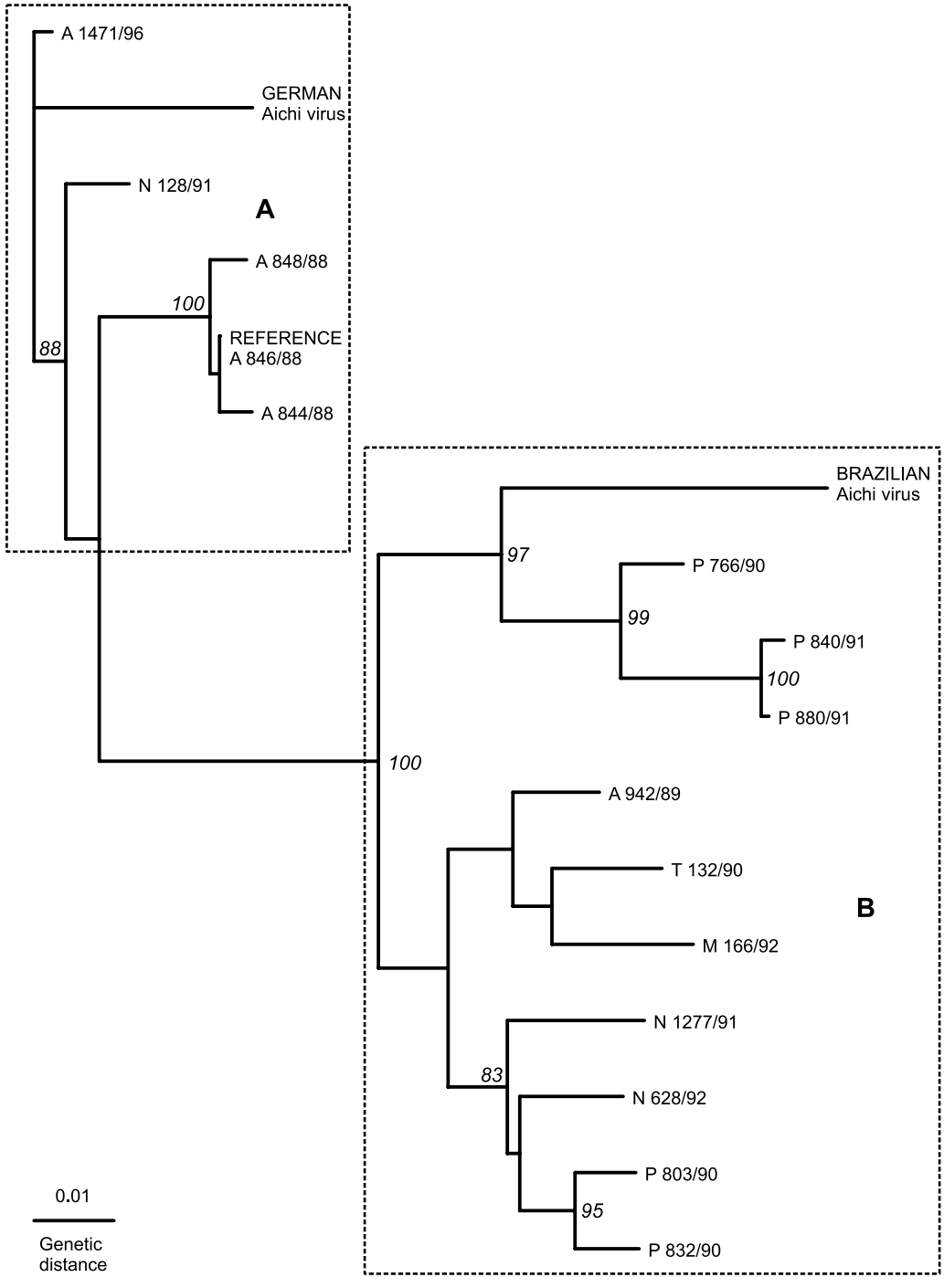

Fig. 1. Phylogenetic analysis of the new Aichi virus isolates from Germany and Brazil and 15 published Asian Aichi virus strains, including the reference strain, over a 519 nt segment in the 3C/D junction region. Bootstrap values >80% are shown. The known Aichi viruses are designated according to Yamashita et al. [13]

370 aa (Brazilian isolate: 371 aa), 223 aa, and 253 aa, respectively. Regarding the nonstructural proteins, lengths of 136 aa, 165 aa and 335 aa are predicted for 2A, 2B, and 2C, and of 95 aa (27 aa, 190 aa, 468 aa) for 3A (3B, 3C, 3D).

A first insight into the genetic classification of the isolates was obtained through phylogenetic analysis over a 519 nt segment located in the 3CD junction region of the genome. Published sequence data of the reference strain and other Asian Aichi virus isolates were included in the analysis. Construction of a phylogenetic tree demonstrated that the German isolate is assigned to the previously established genotype A, whereas the Brazilian virus clusters with isolates of genotype B (Fig. 1). The lowest percentage of nucleotide identity (88%) was found between the two new isolates.

Two Aichi virus genomic sequences have so far been published, that of the reference strain and that of the infectious cDNA clone derived from it [8, 12]. The latter one (GenBank acc. no. AB040749), which is commonly used for reference, displays three single-site nucleotide deletions that result in frameshifts and an amino acid sequence alteration corresponding to positions 38 to 65 of VP0. VP0 regions of both the German and the Brazilian isolate and of a bovine kobuvirus characterized previously [15] were analogous to the infectious cDNA clone. To evaluate the relevance of the described nucleotide deletions, further studies, including the verification of the published reference strain sequence should be done.

Sequences of the new Aichi virus isolates were compared to the Japanese reference sequence and a bovine kobuvirus sequence using the ClustalW algorithm. Over the entire length of the open reading frame, the German and the Japanese Aichi sequence (genotype A) shared a nucleotide identity of 95%, which corresponds to 97% identity of the deduced amino acid sequences. The type A isolates were 89–90% identical with the type B isolate on the nucleic acid level (96% on the amino acid level). All human viruses differed from the nucleotide sequence of bovine kobuvirus by at least 39%. With regard to the structural protein genes, intertype nucleic acid identity was slightly lower over the putative VP1 section of the genome (86–87%) than over the VP0 and VP3 sections (88–89% and 88%, respectively).

Concerning the nonstructural proteins of both new Aichi virus strains, amino acid sequence analysis revealed the presence of functional motifs conserved between all Aichi viruses published so far. The GPPGTGKS motif located in the 2C region of the genome defines the nucleotide-binding domain of the putative helicase. In the 3C region, the GLCGS motif considered to form part of the active site of Aichi virus 3C protease was found, as was the catalytic triad, consisting of histidine (aa 41), aspartate (aa 83), and cysteine (aa 142). The characteristic KDELR, YGDD, and FLKR motifs of the RNA dependent RNA polymerase were identified in the 3D region of the viruses.

To obtain a first insight into the importance of Aichi virus in Germany, 485 randomly chosen sera from German individuals were analysed employing microneutralization tests that included Vero cells and Aichi strain A846/88 (kindly provided by T. Yamashita). After inactivation (56 °C, 30 min), 50 µl of the

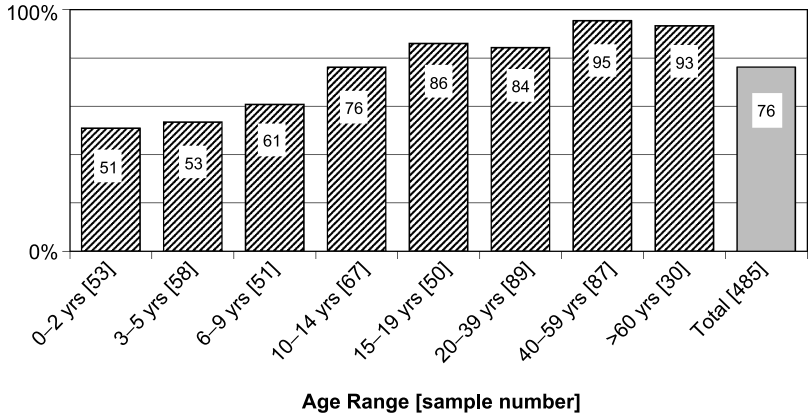

Fig. 2. Seroprevalence of Aichi virus antibodies in a panel of 485 randomly chosen German sera. Percentage of positive samples in each age group and in the total number of samples are given

respective serum dilution (1:4 to 1:512) was mixed with 50 µl of 100 tissue culture infective doses ($TCID_{50}$) of virus, followed by incubation (37 °C, 5% CO_2, 3 h) and the addition of 100 µl of Vero cell suspension (2×10^5 cells/ml). After six days of incubation, the neutralizing titer was determined. 76% of all specimens displayed an antibody titer of >8, which was considered positive. Seroprevalence rises from 51% [0–3 yrs] to >90% [>40 years] (Fig. 2).

To our knowledge, this is the first report of the isolation of Aichi virus in clinical specimens from Europe and South America. So far, occurrence of the virus has only been described in Asia, and recent efforts to detect the virus by multiplex PCR screening of 68 Finnish stool specimens failed [3]. Both the German and the Brazilian infections were associated with clinical gastroenteritis, which supports the notion of Aichi virus as a causative gastroenteritis agent [11, 13, 14]. However, mixed infections with noro- and astrovirus were found, and further studies are needed to obtain direct evidence for pathogenesis.

Yamashita et al. have proposed the designation of genotypes A and B based on sequence analysis of 519 nt at the 3CD junction region [13], and sequence information for genotype B viruses has so far been limited to this region. The characterization of the type B isolate from Brazil contributes significantly to the knowledge on genotype variation in other genomic regions.

Comparison of the new Aichi strains with the reference sequence revealed the VP1 coding region to be the most variable of the structural protein domains. Nucleotide identities in this region were lower (86–87%) than in the 3CD section (minimum 88%). This confirms the presumption of Yamashita et al., who suggested that sequence diversity in the structural protein coding regions may be higher than in the 3CD region. In this region, as well as over the whole length of the ORF, the genotype A sequences were more closely related to each other than to that of the Brazilian type B isolate. Our findings indicate that genotype classification over the 3CD junction region is representative; however, the VP 1 domain may offer a more distinct differentiation and therefore be even more appropriate.

Molecular characterization of new Aichi strains should include this genomic region. This may be of additional importance with regard to the identification of transmission chains and to potential intertypical recombinations.

Concerning the sequencing of the German and the Brazilian Aichi virus strain, it must be noted that the sequences determined here cannot be claimed to represent the complete genomes of the isolates, since the extreme 5′ ends are formed by an oligonucleotide complementary to positions 20 to 40 of the Aichi virus sequence published by Sasaki et al., who used a RACE approach to determine the true viral 5′ end [8]. However, the sequence information obtained here allows reliable conclusions on genome structure to be drawn and provides further knowledge on the genetic variation of Aichi viruses. As for the previously published kobuvirus sequences, the C content of both isolates (38%) is significantly higher than in the average picornavirus genome (28%) [5]. Our sequence data support the suggestion of Yamashita et al., who suspected a high C ratio to be a distinctive feature of kobuviruses [15]. We found that functional motifs characteristic of the putative viral helicase, protease, and RNA-dependent RNA polymerase were completely conserved between our isolates and all other published kobuvirus sequences, thus confirming the knowledge on the genomic organization of kobuviruses [8, 12, 15]. Even though evidence for recombination events cannot be drawn from the available sequence data on Aichi virus, including ours, potential Aichi virus recombinants can be identified using total sequence comparison in the future.

Screening of a large panel of randomly chosen sera [15] revealed a considerable prevalence of neutralizing antibodies to Aichi virus in German individuals (overall, 76%). Comparable values have been reported from Japan [10]. This indicates that infections with Aichi virus are common not only in Asian, but in European countries as well. Similar to data obtained from the Japanese seropanel, seroprevalence in Germany is age-dependent. Age distributions between the two countries vary significantly, though. The majority of infections in Germany appear to occur among children aged younger than 6 years, of whom around 50% have the acquired antibody. In contrast, the Japanese sera display a low prevalence of 7% at young ages [<5 yrs]; gastroenteritis due to Aichi virus was suggested to affect persons aged 15 to 34 years, up to 76% of whom were seropositive.

Our findings accent the specific genomic organization of Aichi virus and corroborate its classification as a distinct member of the family *Picornaviridae*. Aichi virus may well be an agent of considerable importance not only in Asian but also in South American and European countries, and further studies should be performed to assess its epidemiological relevance.

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