VI, 3. Molecular biology and epidemiology of Aichi virus and other diarrhoeogenic enteroviruses

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Introduction

In March 1989, an outbreak of gastroenteritis occurred in Aichi Prefecture, Japan. Out of 29 people who participated in the farewell party of a company, 21 complained of gastroenteritis symptoms such as diarrhea, stomachache, vomiting, and fever approximately 63 hours later. The probable cause of this outbreak was traced to raw oyster in vinegar dressing. Because the search for a bacterial cause remained negative, the feces and paired sera of 5 patients were transported to our laboratory. Spherical virus-like particles of approximately 30nm in diameter were detected by electron microscopy. In addition, agents cytopathic for BS-C-1 cells were also detected in 2 of the 5 fecal samples. Furthermore, seroconversion against these agents was confirmed in 4 of 5 serum pairs, and the antibody titer rises were confirmed against both fecal isolates using a neutralization test. It was conceivable that the two agents were the same virus and the causative agents of this gastroenteritis outbreak (Yamashita *et al.*, 1991). Rotavirus, adenovirus, Norwalk-like virus and astrovirus are well known to be causative agents of gastroenteritis (Ashley *et al.*, 1978; Blacklow 1988; Blacklow *et al.*, 1991; Cruz *et al.*, 1992; Cubitt, 1987; Cukor *et al.*, 1984; Dolin *et al.*, 1987). Our isolates were not related to such gastroenteric viruses or to enteroviruses which are common cytopathic agents in humans. Therefore, we called this agent "Aichi virus", a new type of virus (Yamashita *et al.*, 1993). Genetic analyses performed on Aichi virus lead to the conclusion that it should be classified as a new genus of the *Picornaviridae* family rather than a member of the other genera such as *Enterovirus*, *Rhinovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, or *Parechovirus* (Yamashita *et al.*, 1998), and recently this virus has been proposed to belong to a new genus, *Kobuvirus*, in the *Picornaviridae* family (King *et al.*, 1999; King *et al.*, 2000). "Kobu" means bump or knob in Japanese, which is derived from the characteristic morphology of the virus particle.

Biophysical and biochemical properties

Aichi virus can be propagated in monkey kidney cells such as BS-C-1 and Vero cells (accompanied by a cytopathic effect characterized by detachment of the cells from the culture flask surface), but not in human cells such as HeLa, HEL, or RD cells nor in newborn mice. Aichi virus can be stabilized by the addition of IUDR to the culture medium. Its infectivity withstands treatment with a combination of chloroform and acid pH (3.5), and with heat at 50°C for 30 min; however, the virus is thermolabile at 60°C for 30 min. The buoyant density is 1.36 g/ml as determined by cesium chloride gradient ultracentrifugation (Yamashita *et al.*, 1991). Aichi virus, negatively stained with 2% phosphotungstic acid (pH7.2) for 2 min, is spherical, measuring approximately 30 nm in diameter, and has a rough surface which is different from that seen in an enterovirus, astrovirus, or calicivirus (Fig. 1). Three capsid proteins of 42, 30, and 22 kDa could be observed in SDS-PAGE. The 42-kDa protein corresponds to VP0, and no VP4-VP2 cleavage occurs. The 30- and 22-kDa proteins correspond to VP1 and VP3, respectively (Yamashita *et al.*, 1998).
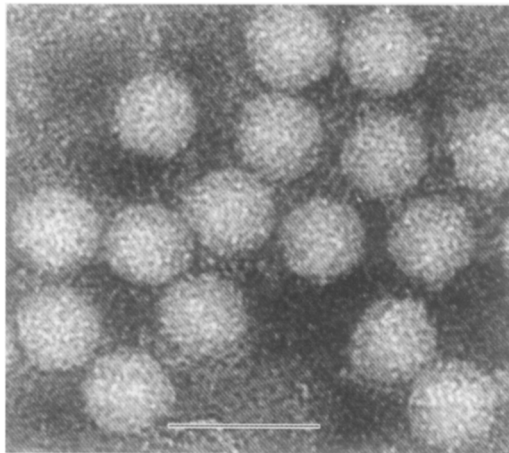
Fig. 1. Electron micrograph of Aichi virus after staining with 2% phosphotungstic acid (pH7.2) for 2 min. Calibration bar: 50 nm

Aichi virus genome

The virion of Aichi virus contains a single-stranded RNA molecule as the genome. The RNA genome consists of 8,280 nucleotides (nt), excluding a poly(A) tract. A large open reading frame with 7,299 nt that encodes a potential polyprotein precursor of 2,432 amino acid (aa) has been found and is preceded by 744 nt and followed by 237 nt and a poly(A). The 42 nt at the 5' end of the genome form a stable stem-loop

structure and play an essential role in the formation of virus particles as well as in RNA replication (Sasaki *et al.*, 2001). Although the precise secondary structure of the 5'NTR has not been defined, the location of the pyrimidine tract and the initiator methionine suggests that the IRES of Aichi virus is similar to that of aphtho-, cardio- and hepatoviruses. The 3' NTR of the Aichi virus genome (237 nt) is by more than 100 nt longer than that of the encephalomyocarditis virus (EMCV) which until then possessed the longest 3'NTR in the *Picornaviridae* family. Whether the 3'NTR of Aichi virus consists of three double-stranded hairpin stems, as seen in EMCV, two stems, as seen in Poliovirus 1 and Foot-and-mouth disease virus (FMDV), or a single stem, as in human rhinovirus and hepatitis A virus (Auvinen and Hyypiä, 1990; Poyry *et al.*, 1996), has not yet been determined (Yamashita *et al.*, 1998).

The organization of the deduced amino acid sequence indicated in Fig. 2 is analogous to that of the other picornaviruses. A leader (L) protein, consisting of 170 aa, is present upstream of VP0. The length of the L protein is a little shorter than that of FMDV (217 aa) and more than twice as long as that of EMCV (67 aa). However, neither the catalytic dyad (Cys and His) conserved in a papain-like thiol protease and found in the FMDV L protein (Gorbalenya *et al.*, 1991; Piccone *et al.*, 1995) nor a putative zinc-binding motif, Cys-His-Cys-Cys, found in EMCV or Theiler's murine encephalomyelitis virus (Chen *et al.*, 1995), could be identified. The function of the Aichi virus L protein is unknown at the moment. The homology of Aichi virus structural proteins (VP0, VP3, and VP1) with corresponding polypeptides of other picornaviruses varies between 19 and 32%. The dendrogram based on structural proteins is depicted in Fig. 3, indicating that the Aichi virus should be separated from the known genera of the *Picornaviridae* including entero-, rhino-, cardio-, aphtho-, hepato-, parecho-, erbo-, and teschoviruses. The nucleotide sequences compared in Fig. 3 have been obtained from DDBJ, EMBL, and GenBank: bovine enterovirus 1 (BEV-1), D00214; Coxsackievirus A16 (CV-A16), U05876; Coxsackievirus B3 (CV-B3), M16572; Enterovirus 70 (EV-70), D00820; Poliovirus 1 (PV-1), J02281; Human rhinovirus 2 (HRV-2), X02316; Human rhinovirus 14 (HRV-14), X01087; Encephalomyocarditisvirus (EMCV), M81861; Theiler's murine encephalomyelitis virus (ThV), M20301; Foot-and-mouth disease virus O (FMDV-O), X00871; Equine rhinitis A virus (ERAV), L43052; Equine rhinitis B virus (ERBV), X96871; Porcine teschovirus (PTV), AJ011380; Hepatitis A virus (HAV), M14707; Avian encephalomyelitis-like virus (AEV), AJ225173; Human parechovirus 1 (HPeV-1), L02971; and Aichi virus (AiV), AB 040749.

The 2A protein of picornaviruses is known to have a cis-acting proteolytic activity and has been classified into two types. In entero- and rhinoviruses, 2A protein functions autocatalytically to cleave the P1 polyprotein at its own N-terminus and mediates the cleavage of the p220 component of the *cap*-binding complex eIF-4γ, leading to the shutoff of host cellular protein synthesis. Like in 3C protease, a catalytic triad conserved in trypsin-like proteases has been identified (Bazan and Fletterick, 1988; Yu and Lloyd, 1992). In aphtho- and cardioviruses, on the other hand, 2A mediates the cleavage at its own C-terminus and the autocleavage motif, NPEG, is conserved in the C-terminus of the 2A protein (Donnelly *et al.*, 1997; Ryan and Drew, 1994). In the Aichi virus 2A protein, neither the critical GXCG motif of trypsin-like protease nor the NPEG motif could be found.
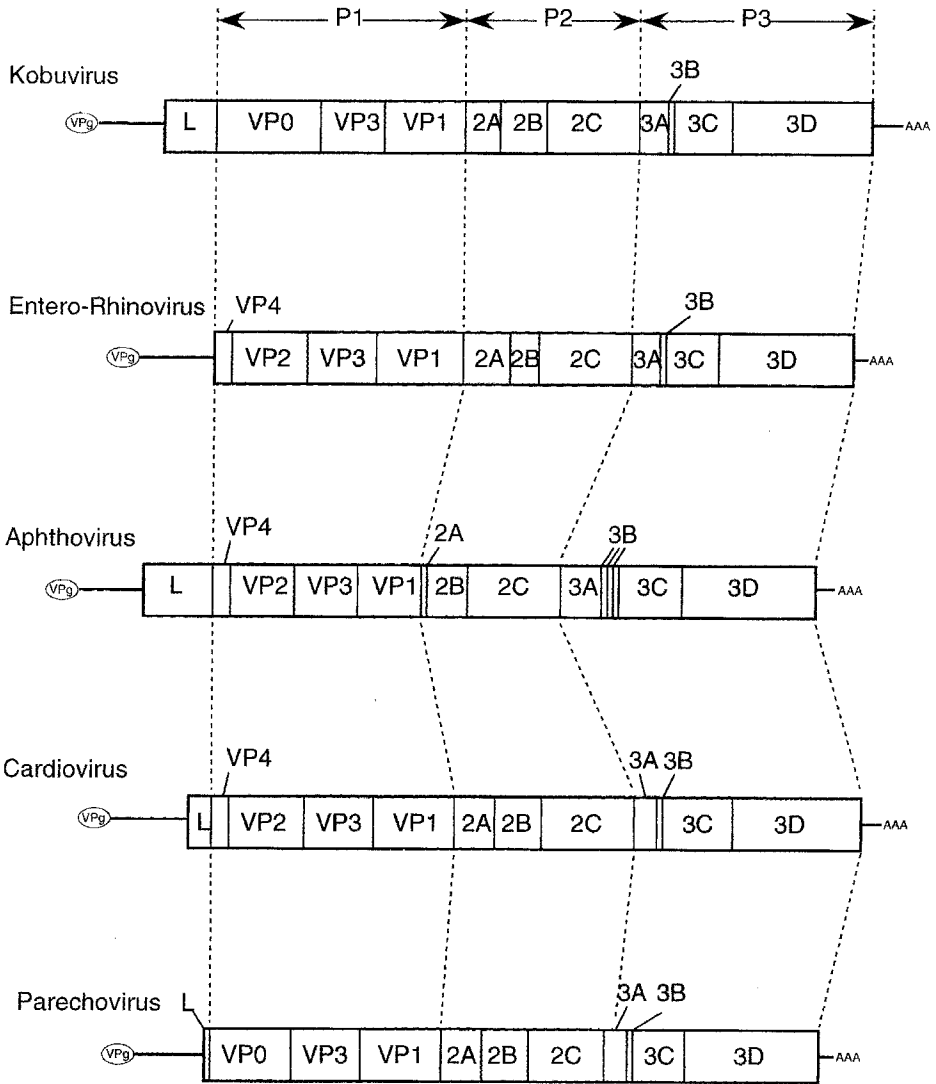
Fig. 2. Genome organization of Aichi virus and comparison of the structure among Picornaviruses. The genome organizations have been shown according to the L434 system. P1 represents viral structural proteins. P2 and P3 represent nonstructural proteins. P1 proteins of human parechovirus produce 12-aa leaders.

Recently, it has been reported that the 2A protein of Aichi virus as well as those of human parechoviruses and avian encephalomyelitis virus contain conserved motifs that are characteristic of a family of cellular proteins involved in the control of cell proliferation

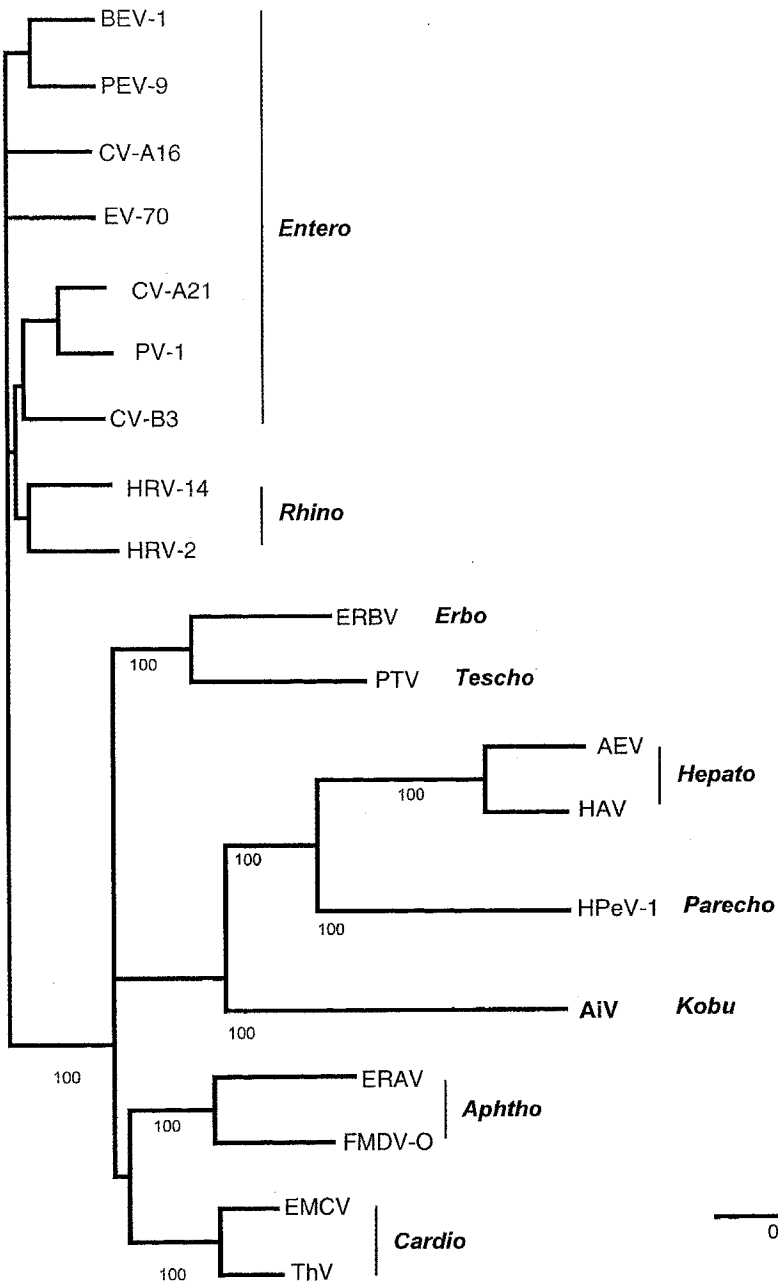Fig. 3. Relationships between Aichi virus and other picornaviruses based on amino acid differences of structural proteins. The dendrogram was generated by evolutionary distances computed by UPGMA. The current picornavirus genera are indicated. Bootstrap values are indicated at branch roots.

(Hughes and Stanway, 2000). Amino acid sequences of the 2C, 3C, and 3D regions are well aligned with corresponding sequences of other picornaviruses. Although the function of 2C protein has not been completely elucidated, a highly conserved motif (GxxGXGKT, X: uncharged, x: nonconserved amino acid) in the nucleotide binding domain of the putative picornavirus helicase is found in the Aichi virus 2C protein. The 3B protein (VPg) of Aichi virus (27 aa) is by 3 to 7 aa longer than those of other picornaviruses. A tyrosine residue is conserved at the third N-terminal amino acid position as observed in other VPg proteins of picornaviruses. The 3C protein that participates in most of the cleavages of picornavirus polyproteins contains a catalytic triad formed by histidine, aspartate/glutamate and cysteine. These amino acids are conserved in all picornaviruses, and they are seen in Aichi virus 3C at positions 42, 84 and 143, respectively. A motif GXCGG conserved at the C terminus of enterovirus and rhinovirus 3C proteins is considered to form part of the active site, and this motif is present but altered to GxCGS in Aichi virus (x, nonconserved amino acid). An identical change has been observed in cardioviruses. A histidine residue that probably participates in the substrate binding pocket in the trypsin-like protease (Boniotti *et al.*, 1994; Bazan and Fletterick, 1988) is found at aa position 161 of the Aichi virus 3C protein. Highly conserved motifs (KDELR, YGDD and FLKR) in picornavirus 3D are found at aa residues 160-164, 328-331 and 377-380, respectively, in the Aichi virus 3D protein (Yamashita *et al.*, 1998).

Molecular epidemiology

The epidemiology of Aichi virus as a medically important pathogen has not been well defined. Aichi virus was isolated in Vero cells from 7 (12.5%) of 56 patients in 6 gastroenteritis outbreaks, 5 (0.7%) of 722 Japanese travelers returning from tours to Southeast Asian countries and complaining of gastrointestinal symptoms at the quarantine station of Nagoya International Airport in Japan, and 5(2.3%) of 222 Pakistani children with gastroenteritis (Yamashita *et al.*, 1993; Yamashita *et al.*, 1995). In the ELISA, 15 (26.8%) of 56 stool samples from adult patients in six oyster-associated gastroenteritis outbreaks were found to be positive for Aichi virus. Seroconversion against Aichi virus was also observed in 20 (47%) of 43 patients involved in these five outbreaks by neutralization test using paired sera (Yamashita *et al.*, 1993). Based on these results, Aichi virus was recognized to be one of the causative agents of human gastroenteritis.

Seventeen Aichi virus isolates, obtained in Vero cells as described above, were examined for variation, based on their reactivity with a monoclonal antibody raised against the standard strain (A486/88) and on reverse transcription (RT)-polymerase chain reactions (PCR) of three genomic regions. The RNA sequences of these 17 isolates were determined over 519 nucleotides at the putative junction between the C-terminus of 3C and the N-terminus of 3D. The analyses revealed an approximately 90% homology between these isolates that were then divided into two groups: group one (genotype A) included 6 isolates from the 4 outbreaks and one isolate from a traveler; group two (genotype B) included one isolate from the other outbreak, 4 isolates from returning travelers, and all of the isolates from the Pakistani children (Yamashita *et al.*, 2000).

Based on these results, a primer pair was devised for amplification and detection of Aichi virus RNA in fecal specimens. The Aichi virus RNA was detected in 54 (55%) of 99 fecal specimens from patients in 12 (32%) of 37 outbreaks of gastroenteritis in Japan. Of these 12 outbreaks, 11 were suspected to be due to infections with genotype A viruses (Table 1). Aichi virus RNA was also detected in 11 (1.9%) of 567 travelers returning with diarrhoea from India, Nepal, Thailand, Indonesia, Singapore, and Vietnam between 1996 and 1998 (Table 2). Of the 11 isolates, 7 were of genotype A, but slightly different from isolates identified in Japan between 1987 and 1991, as indicated in Fig. 4. The other 4 isolates were of type B, and were also different from isolates from Pakistani children and Japanese travelers identified between 1989 and 1992. These results indicated that circulating Aichi viruses differ, both geographically and temporally. The nucleotide sequences described above were deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB092824 to AB092834. For the comparison in Fig. 4, the following nucleotide sequences were obtained from DDBJ, EMBL, and GenBank: A846/88, AB040749; N128/91, AB034655; A1156/87, AB079268; N1277/91, AB034654; P803/90, AB0324659; A942/89, AB034653; M166, AB034657; P766/90, AB034658.

Table 1

Outbreaks of gastroenteritis positive for Aichi virus by RT-PCR in Japan, 1987-1998

Outbreak			RT-PCR	%	Genotype (n)[a]
No.	Yr	Likely source	No. of fecal specimens positive/no. tested		
1	1987	Oysters	5/9	55.6	A (3)
2	1988	Oysters	5/7	71.4	A (3)
3		Oysters	9/11	81.8	A (3)
4	1989	Oysters	4/5	80.0	A (2)
5		School excursion	9/14	64.3	A (3)
6	1990	Oysters	2/4	50.0	B (2)
7		Oysters	6/11	54.5	A (2)
8	1991	Oysters	3/6	50.0	A (2)
9	1994	Oysters	2/14	14.3	A (2)
10	1997	Oysters	5/8	62.5	A (3)
11	1998	Oysters	2/4	50.0	A (2)
12		Oysters	2/6	33.0	A (2)
Total			54/99	54.5	A (27), B (2)

[a] Genotypes were determined in a limited number of fecal samples.

Table 2

Detection of Aichi virus by RT-PCR in travelers returning to Japan, 1996-1998

Travel destination	No. of travelers tested	No. of travelers positive by RT-PCR	%
Indonesia	176	3	1.7
Thailand	145	2	1.4
Singapore	16	2	12.5
India	8	2	25
Vietnam	7	1	14.3
Nepal	4	1	25
Others*	211	0	0
Total	567	11	1.9

*:The Philippines, Malaysia, Hong Kong, Taiwan, South Korea, Maledives, China, Egypt, Cambodia, Mexico

Antibody prevalence levels by age in Japan are very low in childhood, and rapidly rise during adolescence and young adulthood, reaching about 80% of the population by middle age (Yamashita *et al.*, 1993). This age-related seroprevalence has not significantly changed during the last 25 years in Japan as shown in Table 3. This observation demonstrates that there are likely to be many asymptomatic infections and that this is not a new but a newly discovered virus. Antibody to Aichi virus could be detected using a neutralization test and an ELISA. These methods were used for identification of Aichi virus infection in paired serum samples. The diagnosis of IgM and IgA responses will be useful in a single serum sample of a patient infected with Aichi virus. The prevalence of fever in patients with IgM response is significantly higher than in patients without (Yamashita *et al.*, 2001). Antibody to Aichi virus has not been detected in monkey, cattle, horse, pig, dog, cat, and rat sera.
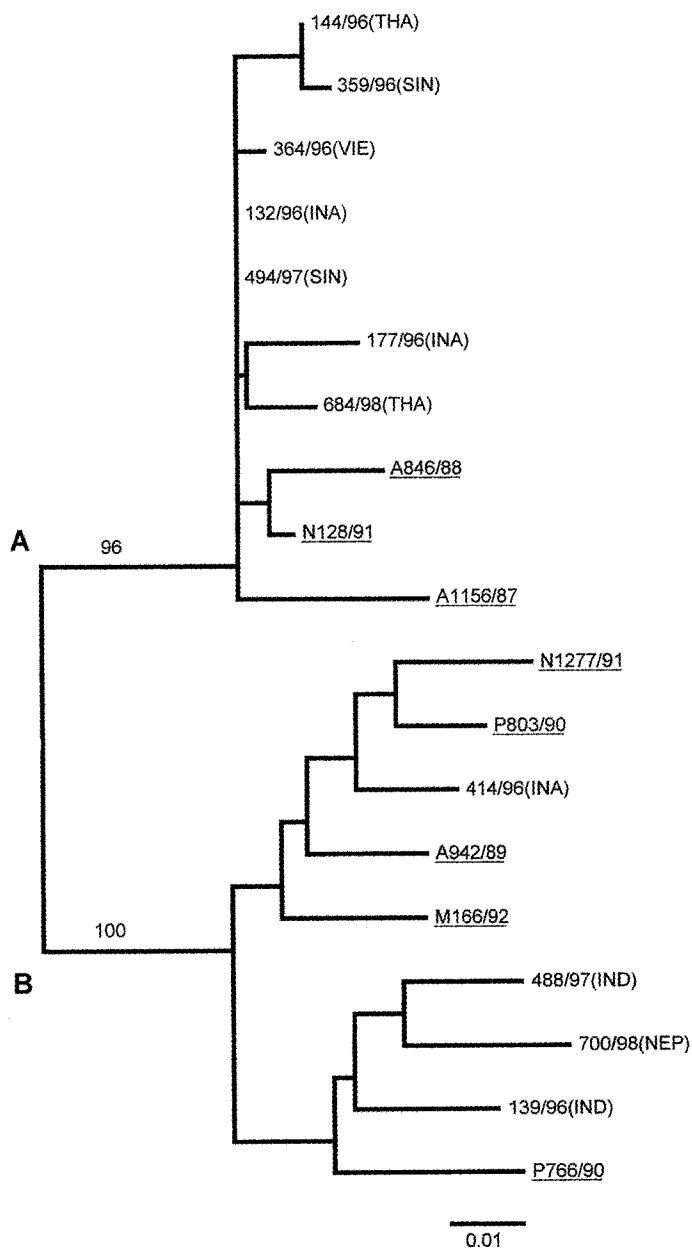
Fig. 4. Dendrogram showing the different sequences of PCR products in the 3CD regions between representative Aichi virus isolates (underlined; Yamashita *et al.* 2000) and stool samples of Japanese travelers returned from tours to India (IND), Nepal (NEP), Indonesia (IND), Singapore (SIN) Vietnam (VIE), and Thailand (THA) between 1996 and 1998. Bootstrap values are indicated at branch roots.

Table 3

Prevalence rates for antibody to Aichi virus in different age groups in Japan, 1973-1998

Age group (yr)	Positive rate (No. of serum samples positive / no. tested) in		
	1973	1989	1998
0-4	7.7 (4 /52)	7.2 (9 /125)	14.3 (3 /21)
5-9	35.7 (10/28)	17.8 (18 /101)	34.8 (8 /23)
10-14	55.0 (11/20)	31.9 (15 / 47)	41.2 (7 /17)
15-19	61.5 (8 /13)	50.0 (30 / 60)	65.2 (15/23)
20-29	67.6 (23/34)	62.5 (75 /120)	72.0 (18/25)
30-39	90.0 (9 /10)	79.2 (95 /120)	87.5 (21/24)
40-49	100 (4 / 4)	81.7 (98 /120)	100 (23/23)
50-59		85.5 (100/117)	100 (25/25)
60-64		87.0 (20 / 23)	100 (9 / 9)

Other diarrhoeogenic enteroviruses

Enteroviruses replicate in the gastrointestinal tract, and the resulting infection is usually symptomless. The clinical expressions of infection, when they do occur, range from paralysis to febrile illnesses. Gastrointestinal upset is commonly reported among associated symptoms in infections by a number of enteroviruses in which other clinical features predominate. In many outbreaks, the enterovirus reported might merely have been a passenger virus unrelated to the illness (Melnick, 1996). Several investigators have reviewed diarrhoeogenicity. There were some reports of enterovirus-associated gastroenteritis. However, these reports were not proof of enteroviruses diarrhoeogenicity (Madeley, 1990). Some enteroviruses have been documented in relation to outbreaks of diarrhea, but diarrhea was not invariably present in those infected. It is ironic that enteroviruses, named after the major site of replication, ie, the gastrointestinal tract, rarely cause gastroenteritis. Enterovirus-associated gastroenteritis may occur in the course of infection associated with another clinical picture (Moore and Morens, 1984).

In an epidemic outbreak of diarrhea in rural southern India, however, the peak of the epidemic curve was associated with echovirus 11 infections. The isolation of the echovirus 11 from 14 of 18 patients with diarrhea and the serological response established that an epidemic of echovirus 11 infections was present in the village at the same time as the wave of the epidemic of diarrhea (Patel et al., 1985). In a bone-marrow transplantation unit of a hospital, 7 of 14 transplant recipients were

infected with coxsackievirus A1 during a 3-week period. Diarrhea and mortality were significantly associated with infection (Townsend *et al.*, 1982). A further prospective study in the same unit yielded 37 enteric pathogens from 31 of 78 patients. Coxsackieviruses were found in the stools of four patients, occurring as the sole viral pathogen in two infections correlated with the occurrence of diarrhea and abdominal cramps (Yolken *et al.*, 1982).

The enteroviruses previously classified as echovirus 22 and 23, now constitute a new genus, *Parechovirus*, within the *Picornaviridae* family because of molecular characteristics considerably different from those of typical enteroviruses (Hyypiä *et al.*, 1992; King *et al.*, 2000). Human parechovirus 1 (echovirus 22) is often isolated from children with diarrhea and from patients during gastroenteritis outbreaks. A review of previously published studies concerning the epidemiology of echovirus 22 infections showed that gastroenteritis and respiratory infections are the most common symptoms observed in parechovirus 1 infection (Ehrnst and Eriksson, 1993; Joki-Korpeea and Hyypiä, 1998).

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