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Prevalence of Newly Isolated, Cytopathic Small Round Virus (Aichi Strain) in Japan

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Cytopathic small round virus (Aichi strain), isolated from a patient with oyster-associated gastroenteritis, showed no reaction in the polymerase chain reaction method for enteroviruses or in the enzyme-linked immunosorbent assay (ELISA) for the five serotypes of astroviruses. Our ELISA was sensitive in detecting the Aichi strain antigen in stool samples, but there was no reaction in this ELISA with any non-Aichi strains of enteric viruses, with such origins as enterovirus, rotavirus, Norwalk virus, calicivirus, or astrovirus. In the ELISA, 13 of 47 stool samples from adult patients in five of nine oyster-associated gastroenteritis outbreaks were positive, but only 1 of 397 pediatric stool samples in Aichi Prefecture was positive. The prevalence rate for Aichi strain antibody was found to be 7.2% for persons aged 7 months to 4 years. The prevalence rate for antibody to Aichi strain increased with age, to about 80% in persons 35 years old. On the basis of the results of the present study, it was hypothesized that Aichi strain could be a new type of small round virus that mainly produces diarrhea in patients in the 15- to 34-year-old age group, 50 to 76% of whom possess neutralizing antibody.

In Japan, acute epidemic gastroenteritis associated with eating raw oysters has been reported every winter, and the number of outbreaks has ranged from 9 to 19 per year for 1987 to 1989. Many of these outbreaks have been thought to be caused by small round viruses (SRV), on the basis of electron microscopic studies (7). The Norwalk-like virus, one of the most frequently observed SRV, has been confirmed as an important pathogen in epidemic gastroenteritis and has been investigated for pathogenesis as well as biophysical, biochemical, and genetic characteristics (5, 6, 10, 17). Other SRV, such as calicivirus and astrovirus, have also been investigated (3, 4, 8, 13). However, there have been cases in which the type of SRV, obtained from stool specimens of patients with gastroenteritis in Japan, could not be classified as a Norwalk-like virus, a calicivirus, or an astrovirus.

Recently, we reported a cytopathic SRV (Aichi strain) isolated with BS-C-1 cells from patients in outbreaks of oyster-associated nonbacterial gastroenteritis in Aichi Prefecture, Japan (23). This Aichi strain was about 30 nm in diameter, had a distinct ultrastructure resembling that of an astrovirus, but differed from other enteric viruses, such as enterovirus, Norwalk virus, and calicivirus, in biophysical, biochemical, and immunological characteristics. In that investigation, we did not test the reactivity of the new Aichi strain with an astrovirus by immunological methods.

Seroconversion against the Aichi strain was observed in the neutralization test for 18 patients in five of seven outbreaks. However, we were only able to isolate three strains (including the Aichi strain) in two outbreaks. Viral isolation required a great deal of time (about 4 to 6 weeks), and the sensitivity of the BS-C-1 cell line in detecting the virus was open to question.

In this report, we describe the difference between Aichi strain and astrovirus in an enzyme-linked immunosorbent assay (ELISA). We also report the development of a monoclonal antibody against Aichi strain for detection of the antigen in stool samples. Furthermore, the prevalence rate for antibody against Aichi strain in healthy adults and children in Aichi Prefecture was estimated by use of a neutralization test.

MATERIALS AND METHODS

Viruses and antisera. Aichi strain, isolated from a patient with gastroenteritis, was grown in Vero cells, and guinea pig immune serum for the virus was prepared as previously described (23). All 66 types of enteroviruses, 3 types of reoviruses (types 1, 2, and 3), and 14 types of adenoviruses (types 1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 19, 31, 40, and 41) were obtained from the National Institute of Health, Tokyo, Japan. HeLa, HEL, RD-18S, and Graham 293 cells and suckling mice were used to grow these viruses. A stool sample containing human calicivirus was obtained from S. Nakata, Sapporo Medical College, Sapporo, Japan. Stool samples containing Norwalk virus were tested in an ELISA by K. Numata, Sapporo Medical College. Stool samples containing astrovirus, rotavirus, and hepatitis A virus were tested in an ELISA at the National Institute of Health or our laboratory as described elsewhere (8, 19, 25). Astrovirus type 2 that had been cultivated in CaCo-2 cells, rabbit hyperimmune serum for the virus, and monoclonal antibody 8E7 were obtained from R. I. Glass, Centers for Disease Control, Atlanta, Ga.

Stool and serum samples. Adult stool specimens were collected 2 to 5 days after the onset of symptoms from 69 of 81 subjects in nine outbreaks of oyster-associated nonbacterial acute gastroenteritis in Aichi Prefecture between 1987 and 1991. Child stool specimens were collected as described elsewhere (24) from 397 patients who visited six pediatric clinics in Aichi Prefecture and were diagnosed with gastro-

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intestinal symptoms, upper or lower respiratory tract syndrome, exanthema subitum, aseptic meningitis, encephalitis, febrile illness, ileus, pharyngoconjunctival fever, or Kawasaki disease between 1987 and 1990. Child stool samples were also collected from 112 healthy children attending kindergarten in 1989. Fecal extracts were prepared as 10% homogenates in veal infusion broth with 0.5% bovine serum albumin, penicillin (200 U/ml), and streptomycin (200 μg/ml). These samples were centrifuged at 10,000 × *g* for 20 min, and the supernatants were collected for virus assay. Monolayers of BS-C-1 or Vero cells in test tubes were used for viral isolation.

Paired serum samples were collected from 59 of 81 subjects in the nine outbreaks mentioned above. Acute-phase serum samples were collected 2 to 5 days after the onset of symptoms. Convalescent-phase serum samples were collected 2 weeks after the acute-phase samples. To determine seroconversion in acute- and convalescent-phase serum samples from these patients, a neutralizing test was performed. Other serum specimens were obtained from ill children (aged 7 months to 14 years) on admission to the hospital for conditions other than gastrointestinal symptoms and from healthy adults (aged over 18 years) on admission to the Japan Blood Center. Antibody titers to 100 50% tissue culture infective doses ($TCID_{50}$) of Aichi strain were measured with the neutralizing test.

Polymerase chain reaction. RNA extraction from all enteroviruses and Aichi strain was done by the method of Rotbart (21). In brief, cultivated viruses were centrifuged at 10,000 × *g* for 20 min, and the supernatants (10 ml) were recentrifuged at 100,000 × *g* for 3 h. The pellets were suspended in 50 mM Tris-hydrochloride (pH 7.5)–50 mM EDTA–50 mM NaCl containing 0.5% sodium dodecyl sulfate and centrifuged at 10,000 × *g* for 20 min. The supernatants were phenol-chloroform extracted, chloroform extracted, and ethanol precipitated in 3 M ammonium acetate. Each nucleic acid was suspended in a reverse transcription mixture containing oligo(dT) and a random primer (Boehringer GmbH, Mannheim, Germany) and incubated for 90 min at 37°C. A polymerase chain reaction (PCR) mixture containing primers (5′-CCTCCGGCCCCTGAATGCGGCTAAT and 5′-ACCGACGAATACCACTGTTA) was added directly to each reverse transcription mixture, and amplification was performed with a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) for 30 cycles (each cycle consisted of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min). Analysis of the amplification product was performed by agarose minigel electrophoresis, and the product was confirmed as a distinct band 154 bases long.

Preparation of the monoclonal antibody against Aichi strain. Six-week-old BALB/c mice were infected subcutaneously with 10 μg of purified Aichi strain–Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) and, 1 month later, intravenously with 10 μg of purified antigen as a booster. Lymphocyte hybridomas were prepared 4 days later by fusing spleen and NS-1 myeloma cells by use of polyethylene glycol (Koch-Light, Colnbrook, England) as described elsewhere (11, 20). Hybridomas secreting Aichi strain antibodies were identified by an indirect fluorescent-antibody test in which culture fluids were reacted with infected Vero cells cultivated on multiwell glass slides and with fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G (IgG) (heavy and light chains) goat serum (Cooper Biomedical, West Chester, Pa.). These hybridomas were cloned two times by limiting dilution. Aichi strain antibody-secreting hybridomas (10^7 cells) were injected into pristane (Aldrich Chemical Co., Milwaukee, Wis.)-primed BALB/c mice. The immunoglobulin class of the monoclonal antibody was determined by the Ouchterlony method with anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 serum (Cooper Biomedical).

Sandwich ELISA. The ELISA used for detecting astrovirus was performed as described by Herrmann et al. (8).

The ELISA that we used to detect Aichi strain antigen was the same as that used for astrovirus by Herrmann et al. (8) and Moe et al. (18). In brief, an anti-Aichi strain monoclonal antibody and an anti-*Rickettsia tsutsugamushi* monoclonal antibody (22), each diluted 1:10,000 in phosphate-buffered saline (PBS), were used as capture and control antibodies, respectively. After a second coating, a 100-μl sample each of a stool suspension or a cell-cultured virus was added and incubated overnight at 4°C. After a wash, 100 μl of guinea pig antiserum to Aichi strain, diluted 1:10,000 in PBS-Tween 20 with 1% bovine serum albumin (BSA), was added. After incubation for 2 h at 37°C, the plates were washed, and 100 μl of peroxidase-labeled goat anti-guinea pig immunoglobulin G (Vector Laboratories, Burlingame, Calif.) in PBS-Tween 20 with 1% BSA was added to each well and incubated for 2 h at 37°C. For color development, *o*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) was used. After 30 min at room temperature, the reaction was stopped with the addition of 4 M H_2SO_4, and the degree of color development was measured by reading the optical density at 490 nm by use of a plate spectrophotometer (Corona Electric, Tokyo, Japan). To determine which stool samples were positive for Aichi strain antigen in the ELISA, we examined the relationship between the absorbance of the test wells and the absorbance of the control wells for each sample.

A competitive-inhibition ELISA was used to assay for the reactivity of Aichi strain with antibody to astrovirus and antibody to Aichi strain. The ELISA plate was coated with a 10,000-fold dilution of anti-Aichi strain monoclonal antibody; 1% BSA as a second coat and 100 ng of purified Aichi strain were then added. To each well of the plate was added a 20-fold or higher dilution of test serum, and the plate was incubated overnight at room temperature. After a wash, anti-Aichi strain guinea pig antibody was added to the plate, and the antibody reaction was determined as described above. Fifty percent binding inhibition was considered positive.

RESULTS

Reactivity of Aichi strain to enzymatic RNA amplification of enteroviruses. To evaluate the sensitivity of the polymerase chain reaction method, we tried to detect the nucleic acids of all 66 serotypes of enteroviruses. In a preliminary examination, 10^2 $TCID_{50}$ of cultivated enteroviruses per 25 μl could be detected only with ethidium-stained agarose gels and enterovirus-specific primers in the polymerase chain reaction. With this method, except for echovirus types 22 and 23, the remaining 64 serotypes of enteroviruses showed successfully amplified reaction products of the expected length (154 bases). However, Aichi strain (10^5 $TCID_{50}$/25 μl) was not detectable with the described primers (Fig. 1).

Reactivity of Aichi strain with astrovirus. Purified Aichi strain (10 μg) and stool samples positive for Aichi strain antigen (containing about 10 to 50 ng of viral protein) were tested for astrovirus. However, the astrovirus antibody did not react with either cultivated Aichi strain antigens or patient stool specimens. The reactivity of a rabbit antiserum

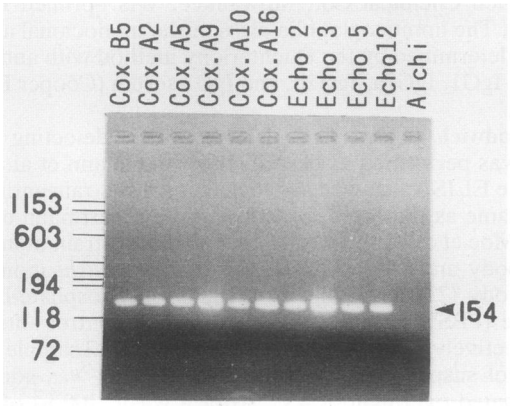

FIG. 1. PCR products of 10 enterovirus serotypes and Aichi strain in an ethidium bromide-stained 2% agarose gel. Band 154 bases long were found for all enteroviruses but not for Aichi strain. Numbers at the left indicate a sizing ladder. Cox, coxsackievirus; Echo, echovirus.

to astrovirus and Aichi strain was determined by a competitive-inhibition ELISA. The rabbit antiserum to Aichi strain showed a high titer (1:3,200) to the homologous antigen, but the antiserum to astrovirus (diluted 1:100) did not prevent the reactivity of Aichi strain in the ELISA.

Sensitivity of the ELISA for detecting Aichi strain antigen. The selected hybridoma, which produced an antibody reaction with Aichi strain, was designated clone Ai/2. The immunoglobulin subclass was immunoglobulin G2b. A total of 578 stool samples were tested to detect the antigen in the ELISA. Analysis of the relationship between test and control samples indicated that 14 samples were positive when a cutoff value set at three standard errors of prediction above the regression line was used (Fig. 2). Absorbance values (test − control samples) for positive samples ranged from 0.090 to 0.550. To determine the sensitivity of the ELISA, 100 to 0.1 ng of purified antigen of Aichi strain was tested to measure absorbance values. The result, presented in Fig. 3, indicated that this assay could detect 0.5 ng of viral protein.

The specificity of the ELISA was examined with representative types of enteric viruses. The results indicated that antibody reactivity did not occur with any non-Aichi strains of enteric viruses, with such origins as enterovirus (n = 66), hepatitis A virus (n = 2), adenovirus (n = 14), reovirus (n = 3), rotavirus (n = 3), Norwalk virus (n = 5), calicivirus (n = 1), and astrovirus (n = 6), under the conditions used for testing. Aichi strain (n = 6) showed a positive result.

Detection of Aichi strain antigen in oyster-associated gastroenteritis and pediatric stool samples. Aichi strain antigen was detected in 13 patient stool samples collected from five of nine oyster-associated gastroenteritis outbreaks and examined by the ELISA (Table 1). The amount of Aichi strain antigen in the stool samples was estimated to be 5 to 100 ng/ml by the assay. The detection rate in these five positive outbreaks ranged from 13% (2 of 16 patients) to 60% (3 of 5 patients), with a mean of 28% (13 of 47 patients). We had already tried, using BS-C-1 cells, to isolate the virus from 12 stool samples collected from three outbreaks (outbreaks 3, 4, and 7) in 1989, and we were able to detect three antigenically similar viruses containing Aichi strain antigen (23). In this study, using BS-C-1 or Vero cells, we tried to isolate the virus from 50 stool samples collected from another six outbreaks and 9 stool samples that had been negative in virus

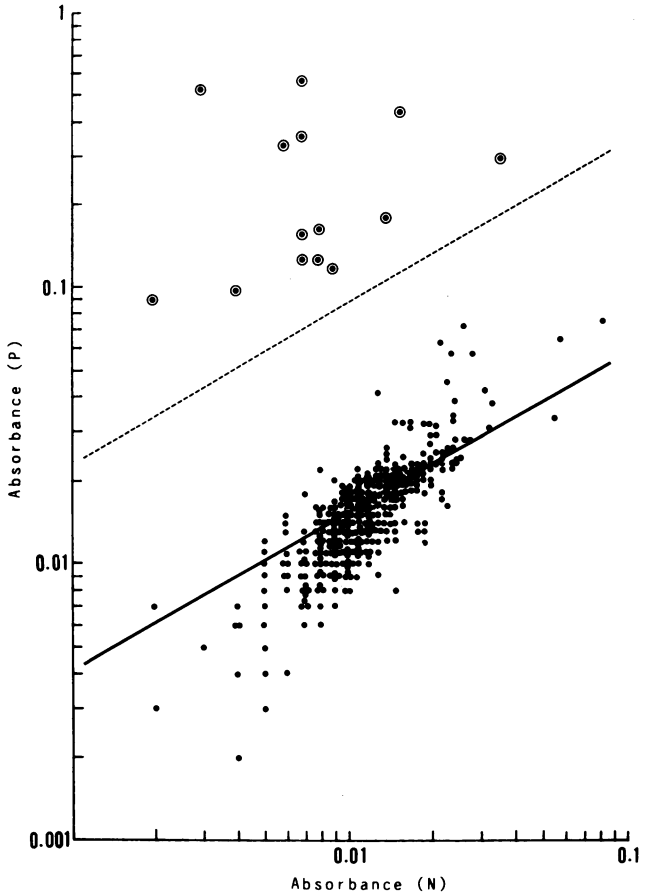

FIG. 2. Absorbance values for test wells (P) and control wells (N) for all stool samples tested. The solid line is a regression line calculated from the data, and the cutoff line (broken line) is set at three standard errors of prediction above the regression line. Symbols: ⊙, positive samples; ●, negative samples.

isolation in the last experiment. Of these 59 samples, 3 from outbreaks 2 and 3 were positive in virus isolation. All six stool samples positive in virus isolation were also positive in the ELISA (Table 1).

In the five outbreaks positive for Aichi strain, the ages of the 43 patients ranged from 18 to 56 years, and those under 34 years of age accounted for approximately 70%. Seroconversions, detected by increasing the neutralizing antibody titer four times or more, were found in 20 of 43 (47%) samples in the five outbreaks. The antibody was also found in 19 of 43 (44%) acute-phase serum samples from the five outbreaks. The antibody titers ranged from 1:8 to 1:32, and in 11 of the 19 (58%) samples, further confirmation was obtained by increasing the titer. On the other hand, seroconversions were not found in four outbreaks in which antigen was not detected in patient stool samples (Table 1).

For 397 pediatric stool samples, Aichi strain antigen was detected in only one patient diagnosed with lower respiratory tract syndrome (Table 2). Aichi strain antigen was not detected in 112 stool samples obtained from healthy children.

Prevalence of antibody to Aichi strain. The neutralizing antibody titer of convalescent-phase serum samples collected from patients in five outbreaks (outbreaks 1 to 5) ranged from 1:8 to 1:256 (n = 32; mean, $2^{5.44}$; standard

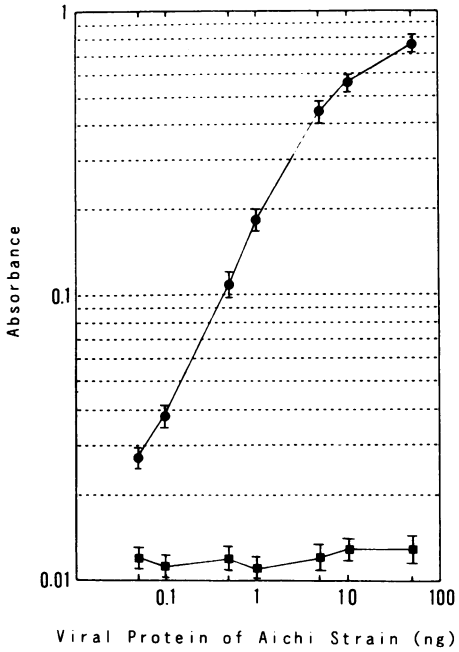

FIG. 3. Sensitivity of the ELISA for detecting Aichi strain antigen. Cesium chloride-purified Aichi strain grown in Vero cells was titrated, and samples exhibiting absorbance values higher than the cutoff value (more than 0.5 ng per well) were determined to be positive. Symbols: ●, test wells; ■, control wells.

deviation, $2^{1.25}$). Consequently, a neutralizing titer of ≥8 (mean − 2 standard deviations) was determined to be the cutoff value. Table 3 provides the distribution of different age groups for Aichi strain antibody detected by a neutralization test in people from Aichi Prefecture. Approximately 55% (460 samples) of sera collected from 833 people ranging in age from 7 months to over 60 years were found to have Aichi strain antibody. A low prevalence rate, 7.2%, was found for young children, ranging in age from 7 months to 4 years. However, the prevalence of antibody to Aichi strain increased sharply with age, to 68%, in persons in the 25- to 29-year-old age range. By 35 years of age, over 80% had acquired Aichi strain antibody.

TABLE 1. Aichi strain isolation, detection, and association with oyster-associated gastroenteritis outbreaks in Aichi Prefecture

Outbreak	Date (mo and yr)	No. positive/no. tested (%)		
		Tissue culture	ELISA	Seroconversion[a]
Positive				
1	March 1987	0/16 (0)	2/16 (13)	5/10 (50)
2	March 1988	2/11 (18)	5/11 (45)	6/13 (46)
3	March 1989	3/5 (60)	3/5 (60)	4/5 (80)
4	December 1989	1/4 (25)	1/4 (25)	2/6 (33)
5	January 1990	0/11 (0)	2/11 (18)	3/9 (33)
Negative				
6	February 1987	ND[b]	0/7 (0)	0/5 (0)
7	January 1989	0/3 (0)	0/3 (0)	0/4 (0)
8	December 1990	0/6 (0)	0/6 (0)	0/5 (0)
9	January 1991	0/6 (0)	0/6 (0)	0/2 (0)

[a] Seroconversions were detected by increasing the neutralizing antibody titer four times or more.
[b] ND, not done.

TABLE 2. Detection by the ELISA of Aichi strain antigen in 509 stool samples obtained from children

Clinical finding	No. tested	No. positive
Gastroenteritis	83	0
Upper respiratory tract disease	84	0
Lower respiratory tract disease	95	1
Exanthemata	58	0
Aseptic meningitis	34	0
Encephalitis	4	0
Febrile illness	13	0
Ileus	6	0
Pharyngoconjunctival fever	6	0
Kawasaki disease	14	0
None (healthy children)	112	0

DISCUSSION

In our previous report (23), the Aichi strain was found biophysically and biochemically identical to enteroviruses but serologically untypeable with regard to the previously reported 66 types of these viruses. Furthermore, the stability to heating at 50°C for 30 min and the molecular sizes of viral structural proteins were different from those of enteroviruses. In this report, we confirmed that isolated Aichi strain RNA was also different from that of enteroviruses on the basis of PCR results, which demonstrated genomic identity between enteroviruses. The ultrastructure studies demonstrated that the Aichi strain could be classified as an astrovirus. However, the Aichi strain could be cultivated without trypsin, whereas astrovirus requires trypsinization for growth in cells (15). The isolated Aichi strain (10 μg) and stool samples (10 to 50 ng) positive for the antigen in our ELISA did not react in the astrovirus ELISA, which could detect 5 to 15 ng of viral protein and which recognized five serotypes of human astrovirus (8, 18). It was estimated that the Aichi strain was less reactive with the antiastrovirus serum than the known astroviruses. In the ELISA for detecting Aichi strain antigen, there was no reaction with 66 different serotypes of human enteroviruses, Norwalk virus, calicivirus, and astrovirus. These observations suggested that the Aichi strain could be a new type of SRV or a different

TABLE 3. Prevalence rates for antibody to Aichi strain in different age groups

Age group (yr)	No. of serum samples examined	No. (%) positive for antibody to Aichi strain
0–4[a]	125	9 (7.2)
5–9	101	18 (17.8)
10–14	47	15 (31.9)
15–19	60	30 (50.0)
20–24	60	34 (56.7)
25–29	60	41 (68.3)
30–34	60	45 (75.5)
35–39	60	50 (83.3)
40–44	60	47 (78.3)
45–49	60	51 (85.0)
50–54	60	50 (83.3)
55–59	57	50 (87.7)
60–64	23	20 (87.0)

[a] From 7 months to 4 years old.

serotype of astrovirus. Up to now, it has been difficult to predict the genomic differences between Aichi strain and other SRV.

Our ELISA was able to detect rapidly more than 0.5 ng of viral protein in a stool sample, and it was a specific and sensitive method for distinguishing Aichi strain antigen from other SRV antigens in stool samples. The amount of Aichi strain antigen in the stool samples was estimated to be 5 to 100 ng/ml by our ELISA for five outbreaks (outbreaks 1 to 5). This range suggested a large increase in the number of viruses in the intestines of the patients and might be virulent enough to produce gastroenteritis. However, the positive detection rate for the antigen was relatively low, ranging from 13 to 60%, because the stool samples were collected from 2 to 5 days after onset of the illness, which meant that the peak period for viral shedding may have passed. The antigen detection rate could have been increased if the stool samples had been collected at a much earlier time, e.g., during the diarrhetic stage. The detection rate for Aichi strain antigen in stool samples by the ELISA was higher than the rate of isolation of the virus from 47 patients in five outbreaks. The application of the method to more cases of outbreaks of gastroenteritis in other areas of Japan and other countries would help reveal a more precise rate of prevalence of the virus in these outbreaks.

The ELISA technique was successfully used to detect the Aichi strain antigen in stool samples from oyster-associated outbreaks of gastroenteritis in adults. However, in pediatric samples, the antigen was detected for only one patient, who was diagnosed with lower respiratory tract syndrome. We were unable to identify the strain in patients with gastroenteritis. On the other hand, we demonstrated a seroprevalence of 32% by 14 years of age. It was estimated that many infected patients did not need medical treatment and that we would be unable to collect positive stool samples. The detection of Aichi strain antigen would be different from that of astrovirus antigen, which can be detected in stool samples from children with diarrhea (1, 2, 9, 14, 16). The relatively low prevalence rate for the distribution of antibody to Aichi strain in young children was also different from the results for astrovirus, which were reported by Kurtz and Lee (12). These observations support the results of the ELISAs, which suggested the antigenic difference between the Aichi strain and astrovirus.

We found that the prevalence of the neutralizing antibody increased with age and that almost 80% of all healthy adults acquired the neutralizing antibody in their sera by the age of 40 (Table 4). The increased prevalence in the young-adult age group may mean only that these subjects are more likely to be exposed to specific virus-containing foods (e.g., oysters) than infants and young children. For the Aichi strain related to five outbreaks, the age group of 18 to 34 years represented about 70% of the 43 patients. On the basis of this evidence, we hypothesized that most incidences of diarrhetic episodes would primarily occur in subjects in the 15- to 34-year age bracket, 50 to 76% of whom would have the neutralizing antibody.

The rise in serum antibody levels seen after the onset of Norwalk illness appeared to be a marker of infection in susceptible individuals, but it lacked a protective role. Norwalk illness commonly occurs in the presence of serum antibody (5). Seroconversion against Aichi strain occurred in 20 of 43 patients (47%) in the five outbreaks related to the virus. In these five outbreaks, 19 of 43 (44%) acute-phase serum samples were found to be positive for Aichi strain antibody and 11 of 19 (58%) samples were further confirmed as positive by an increasing antibody titer. However, 23 of 43 (53%) paired serum samples still had the same titer. These results suggested that the low titer of neutralizing antibody (1:8 to 1:32) did not play a role in the prevention of diarrhea and, in some cases of Aichi strain infection, there was no response to neutralizing antibody in the patients. On the other hand, it is possible for oysters to ingest several types of prevalent human enteric viruses at a time, so patients who did not exhibit evidence of Aichi strain may have been infected by another etiologic agent of gastroenteritis.

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