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Identification of Aichi Virus Infection by Measurement of Immunoglobulin Responses in an Enzyme-Linked Immunosorbent Assay

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Using inhibitory enzyme-linked immunosorbent assay, seroconversions to Aichi virus were detected in 24 (42.9%) of 56 patients with gastroenteritis in six outbreaks. Virus-specific immunoglobulin M (IgM) was detected in convalescent-phase sera from 7 of 24 patients. Of the other 17 patients, 12 developed a significant increase in both IgA and IgG levels and 5 developed a significant increase in IgG alone.

In 1989, a novel cytopathic small round virus (Aichi virus) was isolated from a patient with oyster-associated gastroenteritis using BS-C-1 cells (13). Seventeen strains of Aichi virus have been isolated from patients with gastroenteritis (12, 14). Genetic analyses of Aichi virus revealed that it should be classified into the *Picornaviridae* family but that it is different from any other genus such as the entero-, rhino-, cardio-, aphtho-, hepato-, and parechovirus groups (11). The *Aichi virus* is at present an unassigned species in the *Picornavirus* family (7). Recently, it has been proposed by the International Committee on Taxonomy of Viruses *Picornaviridae* Study Group that this virus be assigned to a new genus named *Kobuvirus* (6).

In an enzyme-linked immunosorbent assay (ELISA) for viral antigen detection, 13 (28%) of 47 stool samples from patients in 5 oyster-associated gastroenteritis outbreaks were shown to be positive (14). Recently, a reverse transcription-PCR was developed to detect Aichi virus RNA. The Aichi virus RNA was detected in 54 (55%) of 99 fecal specimens from patients in 12 (32%) of 37 outbreaks of gastroenteritis (15). Seroconversion, detected by increasing the neutralization antibody titer four times or more, has been found in 20 (47%) of 43 patients in five outbreaks (14). The serological test for Aichi virus was more sensitive than ELISA for detecting the viral antigen (15).

The presence of immunoglobulin M (IgM) is known to be evidence of a primary infection. To establish the diagnosis of acute or recent hepatitis A, blood samples were examined for the presence of IgM-specific anti-hepatitis A virus (5). The ELISA has been developed for quantifying serum IgA, IgG, and IgM responses to enterovirus infections (1). Diagnosis of an enterovirus infection can be presumptively made with a single sample by ELISA detection of a virus-specific IgM (8). It is also known that the duration of the serum IgM and IgA response following infection is less than that of the IgG response in Norwalk virus infection (2, 3, 4, 10).

In this study, paired sera collected from oyster-associated outbreaks were examined by an inhibitory ELISA and a neutralizing test for Aichi virus-specific antibody and the results were compared. An ELISA for Aichi virus-specific IgG, IgA, and IgM was also performed in order to distinguish the patients' immunological responses. Furthermore, we compared the clinical symptoms among patients with different immunological responses.

The purified antigen, guinea pig antiserum, and monoclonal antibody (Ai/2) for the standard strain of Aichi virus (A846/88) used in this study were as previously reported (14). Paired serum samples were collected from 56 patients in 10 outbreaks of oyster-associated nonbacterial acute gastroenteritis in the Aichi Prefecture, Japan (13, 14). To determine seroconversion in acute- and convalescent-phase serum samples from these patients, a neutralizing test was also performed. In brief, an eightfold or higher dilution of test serum was mixed with 100 50% tissue culture infective doses of Aichi virus, incubated at 37°C for 2 h and then at 4°C overnight, and inoculated into Vero cells cultivated in a 96-well tissue culture plate (Becton Dickinson Labware, Paramus, N.J.). The neutralization titer was determined by using the reciprocal of the highest dilution of test serum preventing a cytopathic effect of Aichi virus in Vero cells.

ELISA plates were first coated with Aichi virus-specific monoclonal antibody (Ai/2), blocked with 0.05% Tween 20–2% bovine serum albumin (Sigma, St. Louis, Mo.)–phosphate-buffered saline, and reacted with 10 ng of purified Aichi virus diluted in block buffer per well. Thereafter, a ≥10-fold dilution of test serum was applied and incubated overnight at 4°C. After that, anti-Aichi virus guinea pig serum was applied and reactivity was measured with horseradish peroxidase-conjugated anti-guinea pig serum and *O*-phenylenediamine (OPD) substrate. Inhibitory effect was expressed as a percentage of the optical density of a negative serum sample. The antibody titer was determined to be the reciprocal of the highest dilution of serum causing a 50% inhibitory reaction.

Each ELISA plate well was also coated with anti-human IgG, IgM, and IgA (Zymed Laboratories, South San Francisco, Calif.). After a blocking treatment, ≥50-fold-diluted test human sera were applied. Then, 10 ng of purified Aichi virus or an antigen-negative control was added to each well and incu-

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bated overnight at 4°C. After the plates were washed, Aichi virus-specific monoclonal antibody was applied and reactivity was measured with horseradish peroxidase-conjugated anti-mouse serum and OPD substrate. The endpoint titer was defined as the greatest dilution giving an optical density at 490 nm of >0.1 and a positive/negative ratio of >2.

Using the inhibitory ELISA, 33 of 56 patients with gastroenteritis in 6 of 10 oyster-associated outbreaks were found to be positive for the Aichi virus antibody in their acute-phase sera. The seroconversion to Aichi virus was detected in 24 (42.9%) of 56 patients (Table 1). These results were consistent with that of a neutralizing test performed in this study. However, 17 (70.8%) of the 24 patients had the antibody against Aichi virus in their acute-phase sera by ELISA compared with 12 (50%) patients by the neutralization test.

Aichi virus-specific IgM and IgA were detected only in the convalescent-phase sera of the patients, as shown in Table 1. Seroconversion patterns of the 24 patients were divided into three groups by specific Ig class responses to Aichi virus. Seven patients were observed to have high IgM levels in their convalescent-phase sera. In 12 patients, high IgA levels accompanied by high IgG levels were observed and 3 of them also had relatively low IgM levels. With the remaining five patients, only IgG responses could be observed in their convalescent-phase sera (Table 1). There were no Aichi virus-specific IgM and IgA responses in the acute-phase sera of any of the patients in this study. The detection of IgM and IgA responses in a single serum sample of a patient infected with Aichi virus is useful for diagnosis.

The clinical symptoms of each patient were obtained by telephone by the staffs of health care centers in Aichi Prefecture. Table 2 illustrates the main clinical symptoms such as diarrhea, abdominal pain, nausea, vomiting, and fever shown by 24 of 56 patients in six outbreaks who exhibited an Aichi virus-specific Ig response. Among the 24 patients, 14 (58.3%) complained of diarrheal episodes, 20 (83.3%) complained of abdominal pain, 22 (91.7%) exhibited nausea, 17 (70.8%) experienced vomiting, and 14 (58.3%) had fever. These percentages were different among the patient groups classified by Aichi virus-specific Ig classes in convalescent-phase sera. Based on the results of a χ^2 test, the prevalence of fever in patients with IgM responses (seven of seven) differed significantly among patients with Aichi virus-specific Ig responses ($\chi^2 = 5$, $0.05 > P > 0.02$). This result signifies that infection with the Aichi virus results in different symptoms according to patient condition, regardless of whether it is a primary infection or not. Many patients infected for the first time with Aichi virus may have a fever without gastroenteritis symptoms such as diarrhea. In our previous study, in which we used ELISA, Aichi virus antigen was detected in only one patient, who was diagnosed with a lower respiratory illness (14). Infections by certain enteroviruses, which are the largest group of *Picornaviridae*, can usually be characterized by different clinical manifestations, though often they produce no symptoms (8, 9). As one of these human enteric picornaviruses, Aichi virus may cause different clinical symptoms.

A neutralization test using paired sera is useful for detecting Aichi virus antibody and diagnosing the infection (13, 14). However, the technique is laborious and time-consuming. In this study, an inhibitory ELISA technique was successfully used to detect the seroconversion. This technique could be appropriate in laboratories where a neutralization test is not commonly performed. In this study, we could not detect any neutralizing antibody in the acute-phase sera of patients 37, 39, 40, 42, and 56, which were found to be positive by the inhibitory ELISA. The Ig class of these convalescent-phase sera was either A or G. This finding signifies that they were reinfected with the Aichi virus and would have possessed the Aichi virus antibody in acute-phase sera. Finally, only seven patients suspected of having their first infection by the IgM responses in

TABLE 1. Aichi virus-specific antibody responses of 24 patients

Outbreak	Yr	No. of patients tested	Patient no.	Phase[a]	Titer of antibody to Aichi virus[b]				
					NT	Inhibitory ELISA	ELISA for:		
							IgM	IgA	IgG
1	1987	10	1	a	<	<	<	<	<
			1	c	16	400	1,600	<	<
			3	a	8	100	<	<	<
			3	c	64	600	<	400	400
			8	a	<	<	<	<	<
			8	c	32	100	25,600	100	<
			9	a	<	<	<	<	<
			9	c	16	100	25,600	200	200
			10	a	<	<	<	<	<
			10	c	16	200	6,400	<	100
2	1987	13	14	a	<	<	<	<	<
			14	c	16	40	25,600	200	200
			20	a	<	<	<	<	<
			20	c	15	80	25,600	100	200
3	1988	13	24	a	16	100	<	<	<
			24	c	128	3,200	<	800	1,600
			26	a	16	100	<	<	<
			26	c	64	1,600	<	200	400
			27	a	16	200	<	<	<
			27	c	64	1,600	<	1,600	200
			28	a	16	200	<	<	<
			28	c	128	1,600	<	<	1,600
			29	a	8	10	<	<	<
			29	c	128	1,600	200	6,400	1,600
			31	a	16	200	<	<	<
			31	c	256	1,600	<	3,200	800
			32	a	8	200	<	<	<
			32	c	32	1,600	<	3,200	1,600
			33	a	8	100	<	<	<
			33	c	32	1,600	<	<	800
			36	a	8	200	<	<	<
			36	c	32	1,600	200	3,200	1,600
4	1989	5	37	a	<	100	<	<	200
			37	c	16	1,600	<	1,600	800
			39	a	<	10	<	<	<
			39	c	64	3,200	1,600	51,200	12,800
			40	a	<	200	<	<	<
			40	c	64	1,600	<	1,600	1,600
			41	a	<	<	<	<	<
			41	c	16	100	12,800	<	<
5	1989	6	42	a	<	40	<	<	<
			42	c	64	3,200	<	800	3,200
6	1989	9	48	a	8	200	<	<	<
			48	c	32	3,200	<	<	400
			49	a	32	1,600	<	<	200
			49	c	256	6,400	<	<	6,400
			56	a	<	40	<	<	<
			56	c	32	1,600	<	<	400

[a] a, acute phase; c, convalescent phase.
[b] NT, neutralization test. Values are reciprocals of the highest dilution of sera. A < sign indicates less than 1:8 (NT), 1:10 inhibitory ELISA), or 1:50 (ELISAs for IgM, IgA, and IgG).

TABLE 2. Comparison of clinical findings among three patient groups by Aichi virus-specific Ig class in six outbreaks

Symptom	No. of patients (%) (n = 56)	No. of patients (%) with Aichi virus-specific:			
		IgM (n = 7)	IgA and IgG (n = 12)	IgG (n = 5)	Ig (n = 24)
Diarrhea	40 (71.4)	4 (57.1)	6 (50.0)	4 (80.0)	14 (58.3)
Abdominal pain	40 (71.4)	7 (100)	8 (66.7)	5 (100)	20 (83.3)
Nausea	48 (85.7)	7 (100)	11 (91.7)	4 (80.0)	22 (91.7)
Vomiting	37 (66.1)	6 (85.7)	8 (66.7)	3 (60.0)	17 (70.8)
Fever	29 (51.8)	7 (100)	6 (50.0)	1 (20.0)	14 (58.3)

their convalescent-phase sera were found to be negative in acute-phase sera using an inhibitory ELISA (Table 1). Based on this result, we have concluded that inhibitory ELISA is a sensitive and reliable method to detect Aichi virus antibody.

In a previous study using ELISA (14), Aichi virus antigen was detected in 11 patients (patients 8, 9, 26, 27, 29, 31, 33, 37, 39, 41, and 42) (Table 1). Of them, three had an IgM response and eight had an IgA response. In this study, the percentage of patients showing an IgM reaction was 27% (3 of 11) and the percentage showing IgA and IgG reactions was 73% (8 of 11). On the other hand, Aichi virus could not be detected in 6 patients who had only an IgG response or in 32 patients without Aichi virus seroconversion. The result of the ELISA for Aichi virus detection was related to the sero-response of the specific Ig classes. It is hypothesized that the IgA response signifies a greater multiplication of the virus in a patient's intestine and the excretion of feces as a result of them.

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