APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1989, p. 2073–2078
0099-2240/89/082073-06$02.00/0

Vol. 55, No. 8

Ecology of *Vibrio mimicus* in Aquatic Environments

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Received 21 March 1989/Accepted 22 May 1989

An environmental study was done to examine the prevalence of *Vibrio mimicus* in some aquatic environments of Dhaka, Bangladesh, and of Okayama, Japan. Water samples from Dhaka environments and water and plankton samples from Okayama environments were quantitatively as well as qualitatively analyzed throughout the seasons for *V. mimicus*. The organism was isolated from Bangladesh environments throughout the year, whereas it was not isolated in Okayama when the water temperature fell below 10°C. Samples with as many as 9.0×10^2 CFU of *V. mimicus* per 100 ml of water in Dhaka and 1.5×10^4 CFU of *V. mimicus* per 100 ml of water in Okayama were detected during the study period. *V. mimicus* was not found in any environment with an average salinity of 10‰ or more. Brackish environments with an average salinity of 4‰ were observed to be the optimal natural condition for the pathogen. Using the API 20E system with the conventional test methods, we observed variations in biochemical properties within the *V. mimicus* species. This study reveals the inefficacy of the API 20E system to identify a significant percentage of *V. mimicus*. Therefore, in addition to the API 20E system, a salt tolerance test and a string test are recommended for identification of this species. Susceptibility testing of strains isolated from Okayama environments showed higher resistance to ampicillin and susceptibility to trimethoprim-sulfamethoxazole when compared with environmental isolates of *V. mimicus* from Bangladesh.

Vibrio mimicus has recently been established as a pathogenic member of the genus *Vibrio* (8). This newly described pathogenic species has been found to be the agent responsible for various types of human illness. Isolation of the pathogen from clinical samples has been made in different countries including the United States, Japan, Bangladesh, New Zealand, and Canada (8). Shandera et al. (18) isolated *V. mimicus* from patients recently exposed to seawater. Symptoms included diarrhea, nausea, vomiting, abdominal cramps with fever, and otitis. The organism has also been found to be associated with terminal ileitis (D. Watsky, Clin. Microbiol. Newsl. **5**:41–43, 1983) and traveler's diarrhea (4). Outbreaks of seafood-associated gastroenteritis caused by *V. mimicus* have been reported in Japan (14, 17). The organism has been recovered from aquatic environments of Toyama Prefecture and from raw fishes in Japan (13). Association of toxigenic *V. mimicus* with freshwater prawns has been described in Bangladesh (6). The pathogenicity of clinical isolates of *V. mimicus* from diarrheal patients is well documented by Sanyal et al. (16). Recently, we described the toxin production and toxigenic potential of *V. mimicus* in Bangladesh environments (3). In view of the pathogenicity of *V. mimicus*, it has been suggested that this pathogen should be included in differential diagnosis of acute gastroenteritis occurring after recent ingestion of seafood, especially raw oysters (18).

Previously we described the antibiotic susceptibility patterns of *V. mimicus* isolated from human and environmental sources in Bangladesh (5). Marked differences in the susceptibilities of organisms from the two sources were observed. However, the susceptibility pattern of *V. mimicus* from other environmental sources has not yet been described.

Although it is evident that the *V. mimicus* infections are frequently of environmental origin, the seasonal distribution, ecology, and difficulties associated with isolation and identification of the organism have not been well described. The present study was undertaken for the isolation, identification, and enumeration of *V. mimicus* through all seasons from freshwater, brackish, and saline aquatic environments of Okayama Prefecture, Japan. In addition, previous findings in Bangladesh environments are presented herein to compare the ecology and distribution of the organism in subtropical regions.

MATERIALS AND METHODS

Sampling sites and survey methods. The study included two different regional environments: Dhaka, Bangladesh, a subtropical region, and Okayama, Japan, a temperate region.

Surface water, sediments, and some aquatic plants (*Eichhornia crassipes*, *Pistia stratiotes*, and *Telenthera philexeroides*) were collected in Dhaka, Bangladesh, every fortnight from the Buriganga River at Babu Bazaar Point and the Dhanmondi Lake (map not shown). Samples from both of these freshwater environments were collected during the period from August 1984 to August 1985.

Surface water samples were collected monthly in Okayama, Japan, from October 1987 to January 1989 and plankton samples were collected from January 1988 to January 1989 at five selected sampling stations (Fig. 1). The study area included freshwater, brackish, and saline water environments.

Methods of isolation. Different assay methods were used for sample types collected from different environs. A gauze filtration technique, similar to that used for the detection of *Vibrio cholerae* in natural waters (20) was employed for the detection of *V. mimicus* in this study. Water samples (3 liters each) were passed through presterilized gauze (containing cotton and the gauze filter), and the retained materials were added to alkaline bile peptone broth (BPB) (pH 8.5), an enrichment medium (20). Water samples (50 ml each) were added directly to 25 ml of 3× BPB. Sediments from river and

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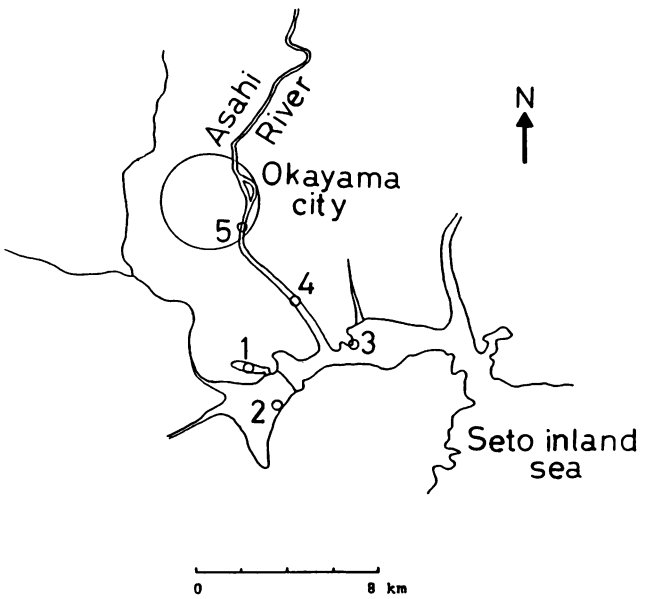

FIG. 1. Map of the study sites (○) at Okayama, Japan. Study sites: 1, Abe Pond; 2, Kojima Lake; 3, Kojima Bay; 4, Asahi River, downstream; and 5, Asahi River, upstream.

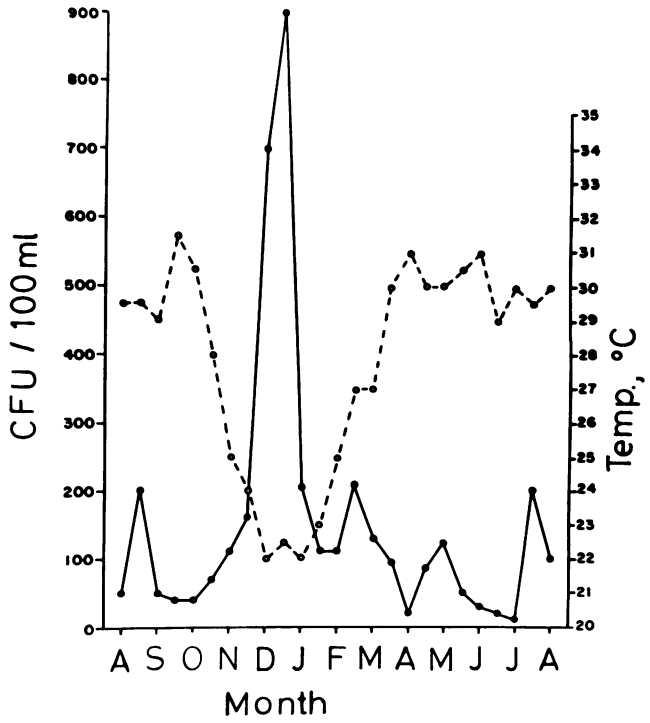

FIG. 2. *V. mimicus* counts (——) in the surface water of the Buriganga River (at Dhaka, Bangladesh) and water temperature curve (– – –) during the period from August 1984 to August 1985.

lake bottoms were collected with an ICDDR,B-constructed core sampler, and 50 g of each sediment sample was added to 3× BPB. Surface water samples (500 ml each) from all sampling stations were transported to the laboratory by keeping them inside an insulated box provided with ice packs. Aquatic plants were collected in presterilized polyethylene bags and transported to the laboratory, maintaining an ambient temperature. All samples were transported to the laboratory for processing within 2 h of collection. Roots of aquatic plants were cut asceptically for bacteriological analysis. About 10 g of the root system of each water plant was added separately to BPB. We cultured plant roots and sediments only to detect the possible presence of *V. mimicus* in such samples. Water samples (300 ml each) were passed through membrane filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and the filters with retained organisms were enriched in BPB. Ten liters of water from a depth of 5 m was collected, passed through a mesh net (pore size, 42 μm), and concentrated to a volume of 100 ml. Collected plankton samples were homogenized in a glass homogenizer. Crushed plankton samples (50 ml each) were added to 3× BPB. All the BPB was incubated at 37°C for 6 h. After incubation, loopful broth cultures were streaked onto thiosulfate-citrate-bilesalt-sucrose (TCBS) agar plates.

The water temperature and pH in Dhaka and the temperature, pH, and salinity of bodies of water in Okayama were also measured. We did not determine the salinity of waters from Bangladesh sites since we know from past experience that these freshwater environments contain less than 1.0‰ (around 0.20‰) salinity (11).

Water and plankton samples were subjected to 10-fold serial dilutions in normal saline and were spread in duplicate on TCBS agar plates. Duplicate volumes of 1, 10, and 100 ml of each water sample were filtered through membrane filters (pore size, 0.45 μm), and the filters with retained bacteria were cultured on the surface of TCBS agar plates. All the TCBS agar plates were incubated at 37°C for 24 h and non-sucrose-fermenting vibriolike colonies were picked for pure cultures on gelatin agar plates. After overnight incubation, the growth of each isolate was tested for oxidase, and oxidase-positive colonies were identified by biochemical testing (9) and by the API 20E system (15).

Only surface water and plankton samples were considered for quantitative estimation of *V. mimicus*. Other samples were qualitatively analyzed for the presence of *V. mimicus*. *V. mimicus* was identified according to the published criteria (8) and as described elsewhere (9). The number of colonies identified as *V. mimicus* were recorded, and the total numbers of *V. mimicus* per 100 ml of water or per 100 g of plankton samples were calculated.

Antibiotic susceptibility testing. *V. mimicus* strains isolated from the Okayama environments were tested for their susceptibilities to several commonly used antibiotics. We followed the standard agar disk diffusion method of Barry and Thornsberry (1), which is a modification of that described by Bauer et al. (2). Commercially available antibiotic disks obtained from BBL Microbiology Systems (Cockeysville, Md.) were used to test the susceptibility pattern. The antibiotic disks used included ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), streptomycin (10 μg), trimethoprim-sulfamethoxazole (1.25 and 23.75 μg, respectively) and tetracycline (30 μg). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the reference strains.

Clinical isolates. A total of 19 clinical isolates of *V. mimicus* were collected from patients attending the Dhaka hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh, as described previously (3).

RESULTS

V. mimicus was isolated from both sampling stations in all seasons in the aquatic environments of Bangladesh. In the

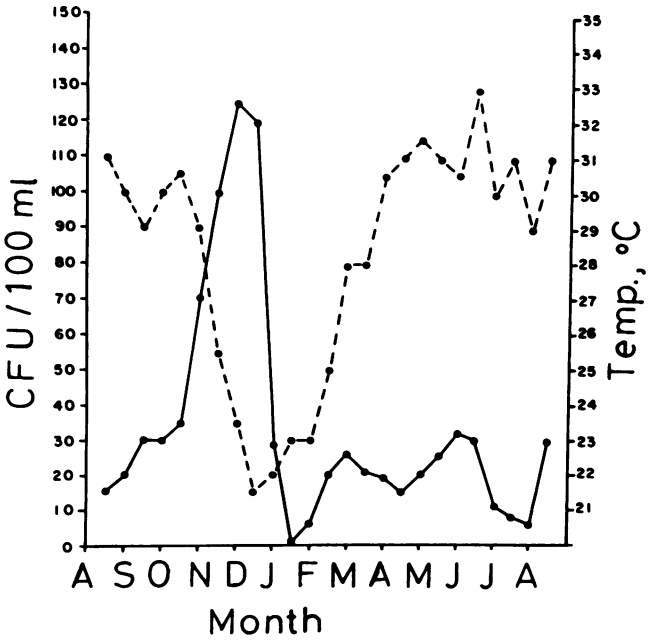

FIG. 3. *V. mimicus* counts (——) in the surface water of Dhanmondi Lake (at Dhaka, Bangladesh) and water temperature curve (– – –) during the period from August 1984 to August 1985.

Buriganga River water, the counts ranged from 10.0×10^0 to 9.0×10^2 CFU/100 ml (Fig. 2), and in the Dhanmondi Lake, it ranged from 0 to 125 CFU/100 ml of water (Fig. 3). *V. mimicus* was also isolated from roots of aquatic plants and sediments analyzed.

In the Okayama environments, *V. mimicus* was isolated from four of the five stations, with relatively higher counts in river samples. We did not find the organism in the Kojima Bay station. A direct relationship of the *V. mimicus* densities with water temperature was observed in this environment. The highest peak of 6.0×10^4 CFU/100 g of plankton and 1.5×10^4/100 ml of water was observed at one of the river points

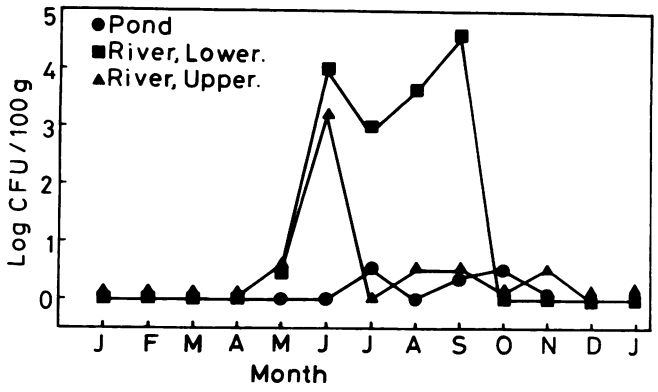

FIG. 5. *V. mimicus* in plankton samples from Okayama environments during the period from January 1988 to January 1989. *V. mimicus* was not found with plankton samples from sites 2 and 3 and thus is not shown in this figure.

in September 1988. *V. mimicus* appeared less frequently in the pond and the lake samples (Fig. 4 and 5). In plankton samples, *V. mimicus* was found only in the downstream site on the Asahi River, with significant densities especially during the warmer seasons (Fig. 5). The occurrence of the pathogen in plankton samples was not found to be uniform through all seasons in different stations, whereas a seasonality was observed in surface water samples (Fig. 4 and 5). The organism was not detected in samples from any of the sites between December and March, when water temperatures were below 10°C.

Of the 136 isolates of *V. mimicus* identified, variations in their biochemical behaviors were observed among the environmental isolates as well as clinical isolates. Again, the production of β-galactosidase (ONPG test) and gelatinase was noted with almost all the environmental isolates; clinical isolates were 74 and 90% positive, respectively, for these properties. Using the API 20E system with the additional conventional microbiological tests, we observed variations in biochemical properties within the *V. mimicus* species. The

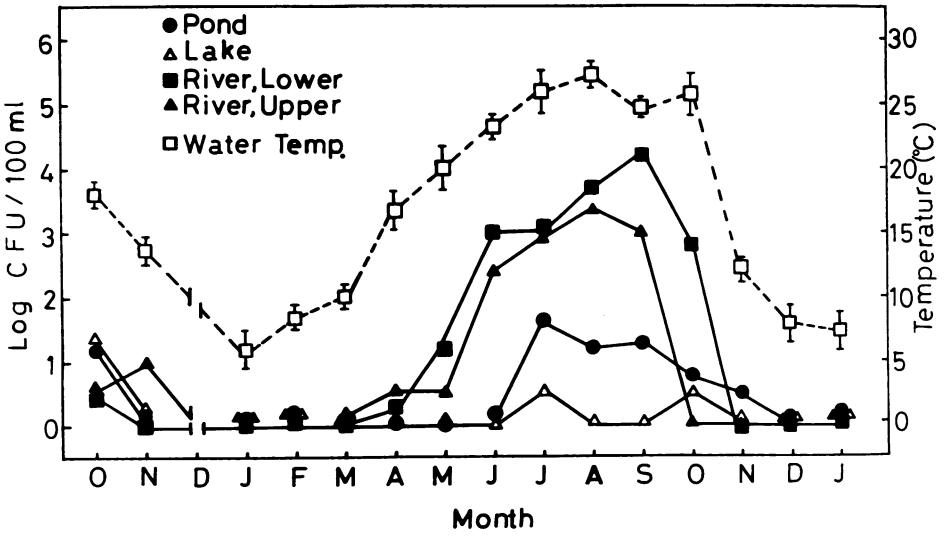

FIG. 4. Seasonal variations in recovery of *V. mimicus* from different study sites at Okayama, Japan, and the average water temperature of five stations. Samples were collected during the period from October 1987 to January 1989 but not in the month of December 1987. *V. mimicus* was absent from Kojima Bay (site 3 in Fig. 1) and thus is not shown in this figure.

TABLE 1. API 20E profile numbers obtained with *V. mimicus* isolates (n = 136)

Profile no.	Identification by API 20E system	Growth in peptone broth with the following concn of NaCl (%)					% of total isolates
		0	3	6	8	10	
5346104	*V. mimicus*	+	+	V[a]	−	−	44.85
5146104	*V. mimicus*	+	+	V	−	−	44.85
5144104	*V. mimicus*	+	+	V	−	−	0.74
4146104	*V. parahaemolyticus*	+	+	−	−	−	2.20
4346104	*V. parahaemolyticus*	+	+	−	−	−	1.47
4144104	*V. parahaemolyticus*	+	+	−	−	−	0.74
1246104	*V. mimicus*	+	+	+	−	−	2.94
1346104	*V. mimicus*	+	+	+	−	−	2.20

[a] V, Variable.

profile numbers in the API 20E system catalog were 5346104, 5146104, 5144104, 4146104, 4346104, 4144104, 1246104, and 1346104 (Table 1). *V. mimicus* isolates with profile numbers 4346104, 1246104, and 1346104 were isolated only from Okayama environments along with the other two predominating types, 5346104 and 5146104.

The water temperature in Dhaka stations ranged from 21.5 to 33.0°C (Fig. 2 and 3) and the pH ranged from 6.6 to 7.7 throughout the study period. In the Okayama stations, the water temperature ranged from 4.0 to 29.0°C (standard deviations of the values from the five stations shown [Fig. 4]). The mean pH and the salinity value for each station observed are as shown in Table 2.

Of the 58 strains of *V. mimicus* isolated from Okayama environments, all were found to be susceptible to chloramphenicol. Although a few strains showed an intermediate type of susceptibility, resistance to gentamicin, tetracycline, and trimethoprim-sulfamethoxazole was not observed with isolates from Okayama. Resistance to ampicillin (83%), streptomycin (17%), and kanamycin (3%) was observed (Table 3).

DISCUSSION

In the Dhaka environments, *V. mimicus* was found in abundance in all types of samples from both stations. In surface water, the peak was observed during winter months when the water temperature was around 20°C. It was observed that the water temperature in Dhaka stations ranged from 21.5 to 35.0°C (Fig. 2 and 3), comparable with the temperature observed during the warmer months in the temperate region. Therefore, a clear seasonality of the organism in these environs was not observed. Except for the counts during the winter months of this region, the differences in densities we encountered with *V. mimicus* counts in all seasons are not remarkable. However, this study at least describes the distribution and the occurrence of the possible highest density of this newly described pathogenic species in the aquatic environments of this region.

TABLE 2. Average pH and salinity of the sampling stations

Location and sampling station	pH	Salinity (‰)
Dhaka, Bangladesh		
Buriganga River	6.89	ND[a]
Dhanmondi Lake	7.01	ND
Okayama, Japan		
Abe Pond	8.00	<1.00
Kojima Lake	7.96	<1.00
Kojima Bay	7.60	13.50
Asahi River, downstream	7.50	3.90
Asahi River, upstream	7.38	2.00

[a] ND, Not determined.

TABLE 3. Antibiotic susceptibility pattern of *V. mimicus* isolated from aquatic environments of Okayama

Antibiotic(s) and amt (μg/disk)	% of *V. mimicus* isolates (n = 58)		
	Susceptible	Intermediate	Resistant
Ampicillin (10)	3	14	83
Chloramphenicol (30)	100	0	0
Gentamicin (10)	88	12	0
Kanamycin (30)	50	47	3
Streptomycin (10)	23	60	17
Tetracycline (30)	83	17	0
Trimethoprim (1.25)-sulfamethoxazole (23.75)	90	10	0

In the Okayama environments, the impact of seasonal changes was found to be quite different from that in the Dhaka environments. The winter of this region is much cooler and the autumn temperature is comparable with the winter temperature of the Dhaka environments. The peak densities of the pathogen are found in the summer months, and a direct relationship of the organism with water temperature (Fig. 4) is observed. In a study of *V. parahaemolyticus* in the Chesapeake Bay it has been observed that the pathogen appears in water samples only under warmer-water conditions and that the organism disappears from the water column during the winter (7). In our study, we observed the disappearance of *V. mimicus* with the fall in water temperature below 10°C. It seems therefore that like other species of the genus *Vibrio* (12), *V. mimicus* is also susceptible to low temperature and disappears from the water column with the fall in water temperature.

It has been observed in a laboratory study that the concentration of *V. cholerae* on the root system of water plants could be almost 300-fold greater than that in the surrounding water and that the rate of decrease in viable counts on plants was less than that in water samples (21). The positive role of water plants in the transmission of vibrios in the environment has also been suggested by investigators (21). We have not examined *V. mimicus* for such an adherence phenomenon. Since *V. mimicus* is a closely related species to *V. cholerae*, we have examined the possibility of its presence in root systems of aquatic plants. It is revealed from the present study that like *V. cholerae* (21), *V. mimicus* may also exist in association with aquatic plants in the environment. We did not quantitatively analyze the densities of *V. mimicus* in plant roots. However, the occurrence of the organism especially with *Eichhornia crassipes* (water hyacinth) is of significance in that this water plant is abundant throughout the seasons in Bangladesh and has a high rate of multiplication and therefore is conducive to the spread of the pathogen from one environment to another.

In the Okayama Prefecture, the distribution of *V. mimicus* among the three types of aquatic environments indicates the impact of salt concentration on the prevalence of the pathogen in the environment. In the freshwater environments, the Abe Pond and the Kojima Lake, the organism was found less frequently than on the Asahi River, downstream and up-

stream. We could not isolate the organism during the winter months; however, a clear seasonality was observed in the river stations. The absence of *V. mimicus* from the Kojima Bay station in all seasons may be due to the higher salinity of the environment. Although a high percentage of *V. mimicus* strains (49%) is able to grow in 6% NaCl (9), it is unknown why the organism is not found in environments with higher salt concentrations. Brackish water with an average salinity of 4.0‰ was found to be suitable for *V. mimicus*.

As with *V. cholerae* (11), it was thought that *V. mimicus* might be associated with plankton in the environment. The higher counts of *V. mimicus* with plankton samples in summer months in the Okayama environments indicated their possible association with plankton. It was observed that unlike *Pseudomonas* sp. or *E. coli*, *V. cholerae* can adhere to copepods in natural water and its survival period in water is extended in the presence of live copepods (11). Our aim in culturing plankton samples was to ascertain whether plankton samples yield higher counts of *V. mimicus* than that in water. However, the higher abundance of *V. mimicus* in water samples in different stations was also observed during the study period. It appears that *V. mimicus* was found only in one of the stations with significant densities during the warmer months (Fig. 5). The occurrence of *V. mimicus* in plankton samples (Fig. 5) was not uniform throughout the seasons in different stations compared with that in surface water samples (Fig. 4). It is therefore apparent that unlike *V. cholerae*, *V. mimicus* does not adhere to plankton samples in the environment. Microscopy of the samples revealed the presence of different kinds of phytoplankton in all the sites. We observed a very low count of copepods only in samples from Kojima Lake station (data not shown). It is important to note that we collected samples during the daytime, and therefore zooplankton was absent in most cases. Our findings should be considered from this point of view. Further study would be worthwhile, particularly collecting plankton samples at night or closer to the surface to examine the possible association of *V. mimicus* with zooplankton, especially copepods in their natural habitat.

We employed both the conventional microbiological test methods and the API 20E system for the identification of *V. mimicus*. Several investigators have used the latter system for the identification of the members of the family *Enterobacteriaceae* as well as for members of the *Vibrionaceae* (10, 15). However, the present study with *V. mimicus* showed the inefficacy of the API 20E system alone in identification of a remarkable number of strains. The API 20E system repeatedly failed to identify *V. mimicus* isolates that had been confirmed by the traditional test method. *V. mimicus* isolates that had been confirmed by the tube method in many cases were identified as *V. parahaemolyticus* by the API 20E system. Because the API 20E system gave such strains ONPG-negative profile numbers, *V. mimicus* turned out to be an excellent fit as a *V. parahaemolyticus* isolate (Table 1). The contradictory identification of *V. mimicus* using the API 20E system reflects the inefficacy of the system for the ONPG-negative strains. However, the salt tolerance test and the string test (19) results confirm the *V. mimicus* identification. Therefore, we recommend that diagnostic as well as environmental research laboratories using the API 20E system for vibrio identification employ the salt tolerance and string tests in addition. The present study describes *V. mimicus* isolates with different possible API 20E profile numbers, some of which require additional tests (Table 1).

We observed previously that clinical isolates of *V. mimicus* are mostly susceptible to commonly used antibiotics, while environmental isolates are not. A marked difference in the antibiotic resistance patterns of *V. mimicus* from human and environmental sources of Bangladesh was observed (5). Most environmental isolates were resistant to kanamycin, tetracycline, and trimethoprim-sulfamethoxazole, whereas the resistance to ampicillin was observed with a lower percentage of isolates (5). In the present study, environmental isolates from Okayama also showed higher resistance to some of the antibiotics than did clinical isolates; however, the pattern differed from that observed with the environmental isolates of *V. mimicus* from Bangladesh. Isolates from Okayama were found with higher resistance to ampicillin (83%) and susceptibility to trimethoprim-sulfamethoxazole, streptomycin, kanamycin, and tetracycline (Table 3) than that found with isolates from Bangladesh environments. These results suggest the existence of differences in the susceptibility patterns of clinical and environmental isolates, as observed previously (5). However, the variation in the resistance patterns of isolates from two environments is surprising and indicates the possibility of variation in drug resistance in isolates from different environments. The suspected impact of the environmental conditions on the drug resistance pattern of organisms needs further investigation.

ACKNOWLEDGMENT

A part of this work was supported by grant 016700 from the International Centre for Diarrhoeal Disease Research, Bangladesh.

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