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Distribution of *Aeromonas* Species in the Intestinal Tracts of River Fish

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Aeromonas **isolates were obtained from fish intestines, water, and sediments from an urban river and identified by the DNA-DNA microplate hybridization method. The isolates were *Aeromonas veronii* (22%), *Aeromonas caviae* (18%), *Aeromonas hydrophila* (13%), *Aeromonas sobria* (8%), *Aeromonas jandaei* (7%), and other *Aeromonas* spp. (33%). *Aeromonas* species occurred at high densities with high incidences, regardless of season. The results strongly suggest that aeromonads are indigenous in fish intestines, water, and sediments of rivers and have the potential to be predominant in aquatic environments.**

Aeromonads are known to be of great importance both economically and medically. Members of this genus are mainly distributed in freshwater and sewage and in association with aquatic animals are sometimes predominant components (3, 15–21). Additionally, they are known to cause a diverse spectrum of diseases in both warm- and cold-blooded animals (7, 12, 22). They sometimes cause serious damage in the aquaculture of freshwater fish (22). Although the role of aeromonads as gastrointestinal pathogens is still somewhat controversial, mounting evidence indicates that at least some strains are involved in diarrheal disease (7). This situation strongly suggests that ecological studies of *Aeromonas* species in aquatic environments are indispensable to efficiently control the diseases caused by these organisms.

However, it has been pointed out that there are still serious problems with species identification because of poor correlations between genotypes and phenotypes and a lack of reliable traits for species discrimination and delineation (7, 9). Recently, we encountered a similar phenomenon in that as many as 48% of the *Aeromonas* isolates were different from the type strains in one to three phenotypic characteristics (20). These results reveal that *Aeromonas* isolates are not correctly identified at the species level when only phenotypic characterization is used. Contrary to this, the DNA-DNA microplate hybridization technique, which was developed by Ezaki et al. (5), was recently proved to be a powerful tool for correctly identifying *Aeromonas* species and *Plesiomonas shigelloides* (19, 20). In addition, we reported that all of the *Aeromonas* isolates from the intestinal tracts of six species of freshwater-cultured fishes constituted five *Aeromonas* species: *Aeromonas caviae*, *Aeromonas hydrophila*, *Aeromonas jandaei*, *Aeromonas sobria*, and *Aeromonas veronii* (20). Therefore, in this study, we investigated the distribution of these five aeromonads in the intestinal tracts of freshwater fish, along with water and sediments of a river, by using the microplate hybridization method.

Fish specimens and water and sediment samples were collected monthly at a sampling site of the Hikiji River, Kanagawa, Japan, which is an urban river. The sampling site was located 8 km upstream of the river mouth, and samples were collected during April to October 1994. Twenty specimens of common carp (*Cyprinus carpio*; 1.1 to 6.9 kg of body weight), 13 of crucian carp (*Carassius carassius*; 136 to 780 g), and two of gray mullet (*Mugil cephalus*; 650 to 800 g) were caught by fishing. Concomitantly, seven surface water and seven sediment samples were collected with sterile glass bottles. All specimens and samples were kept on ice, immediately transported to the laboratory, and examined within 6 h of collection.

The intestinal content was aseptically removed from each fish specimen and homogenized in a ninefold volume of a diluent of phosphate buffer (pH 7.6) containing 0.1% Bacto Agar (Difco, Detroit, Mich.). Each sample thus prepared, along with the water and sediment sample, was serially diluted, and 0.05-ml portions of diluent were spread over two different media: Trypticase soy blood agar (BBL, Cockeysville, Md.) and MacConkey agar (Eiken, Tokyo, Japan). The inoculated agar plates were incubated aerobically at 25°C for 7 days. After incubation, the bacterial colonies were counted, and about 20 colonies were isolated at random from each plate and identified as described previously (17, 21). Gram-negative rod-shaped organisms with positive reactions for motility, oxidase, glucose fermentation, and resistance to a vibriostatic compound (O/129) were identified as belonging to the genus *Aeromonas*. In suitably diluted samples, colonies of *Aeromonas* species were counted and expressed as the number of CFU per milliliter or gram of material. The highest count obtained on the different agar media was regarded as the estimated viable count for that *Aeromonas* species.

A total of 906 bacteria belonging to the genus *Aeromonas* were isolated from the intestines of fish specimens and water and sediment samples and further examined genotypically by the DNA-DNA hybridization method. In addition, *A. caviae* ATCC 15468^T, *A. hydrophila* ATCC 7966^T, *A. jandaei* ATCC 49568^T, *A. sobria* ATCC 43979^T, and *A. veronii* ATCC 35624^T were used as reference strains.

DNAs were extracted from the bacterial strains examined and purified by using the salting out method of Miller et al. (11) with some modifications. After incubation on a Trypticase soy agar plate for 18 to 20 h at 25°C, bacterial cells (approximately 100 mg) were harvested and then suspended in 3 ml of lysis buffer (10 mM Tris-HCl, 2 mM sodium EDTA, 400 mM NaCl [pH 8.2]). The suspension was incubated at 60°C for 10 min with 200 μl of 5% sodium dodecyl sulfate (SDS) to inactivate DNase. The cells were digested overnight at 37°C with 500 μl of proteinase K (2 mg/ml; Merck, Darmstadt, Germany). After addition of 1 ml of saturated NaCl (approximately 6

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M), the mixture was shaken vigorously for 15 s and centrifuged at 3,500 × *g* for 30 min. The supernatant was transferred to a new test tube. A 400-μl portion of an RNase A (Sigma, St. Louis, Mo.) solution (1 mg/ml of 10 mM Tris HCl–15 mM NaCl [pH 7.5]) was added to the mixture, and this combination was then incubated at 37°C for 60 min. The mixture was further incubated at 37°C for 60 min with 500 μl of 10% SDS–50 μl of proteinase K (6 mg/ml), followed by being gently mixed with 1,338 μl of saturated NaCl. After centrifugation at 12,000 × *g* for 15 min, the supernatant was transferred to a new test tube and mixed thoroughly with an equal volume of isopropyl alcohol to precipitate DNA. The DNA was transferred to a new test tube, rinsed four times with 70% ethanol, and dried at room temperature.

Hybridization was performed according to the method of Ezaki et al. (5), with minor modifications (19). Briefly, heat-denatured DNA, which was extracted from each strain of five reference species and purified according to the manual of Ausubel et al. (1) with minor modifications (19), was immobilized in microdilution plates (Maxisorp F16; Nunc, Roskilde, Denmark). On the other hand, DNAs from 911 bacterial strains (906 isolates examined and 5 reference strains) and salmon sperm (Wako, Tokyo, Japan) as a control were biotin labeled with photobiotin (Vector Laboratories, Burlingame, Calif.). DNA-DNA hybridization was carried out at 50°C for 2 h. Streptavidin-conjugated β-D-galactosidase (Zymed Laboratories, San Francisco, Calif.) and 4-methylumbelliferyl-β-D-galactopyranoside (Sigma) were used as an enzyme and a substrate, respectively. After hybridization, fluorescence intensity was measured with a Titertek Fluoroskan (Flow Laboratories, McLean, Va.) at wavelengths of 355 nm for excitation and 480 nm for emission. The fluorescence intensity of the well of salmon sperm DNA was calculated as 0%, and the intensity of the well with the maximum fluorescence for each reference strain was calculated as 100%. The homology value of 70% was considered to be the cutoff value for DNA relatedness (5, 23). The previous report showed that *Aeromonas* species and some related bacteria are apparently able to be discriminated by the microplate hybridization method (20).

As a result, of 906 bacteria isolated from an urban river, 195 isolates (22%) were identified as *A. veronii*, 161 (18%) were identified as *A. caviae*, 116 (13%) were identified as *A. hydrophila*, 69 (8%) were identified as *A. sobria*, and 63 (7%) were identified as *A. jandaei*, although 302 isolates (33%) showed low homology values of less than 70% against all 5 of the reference strains and so were designated as other *Aeromonas* spp. in this study. Table 1 shows the distribution of these species in the fish intestines, water, and sediments collected from the Hikiji River. With the exception that no *A. hydrophila* isolate was recovered from the gray mullet, the five aeromonad species were isolated from all fish species and their environments in the Hikiji River.

Figure 1 shows the population density and incidence of *Aeromonas* species in the fish intestines, water, and sediments of the river. In the intestines of common carp, *A. caviae*, *A. hydrophila*, and *A. veronii* were detected in 17 to 18 of 20 specimens with mean viable counts of 5.9×10^5 to 2.3×10^6 CFU/g, while the other two species were detected in 2 to 9 specimens with 3.1×10^2 to 1.5×10^3 CFU/g. Of 13 crucian carp, 9 to 13 specimens harbored *A. caviae*, *A. hydrophila*, *A. jandaei*, and *A. veronii* at mean viable counts of 1.9×10^5 to 6.2×10^7 CFU/g, but only 6 specimens showed intestinal colonization by *A. sobria* (5.2×10^3 CFU/g). In gray mullet, *A. caviae*, *A. jandaei*, *A. sobria*, and *A. veronii* were isolated from one or two of two specimens, with mean viable counts of 7.4×10^1 to 4.8×10^5 CFU/g, but no *A. hydrophila* isolate was detected. In the river water, only *A. caviae* organisms were detected in all seven samples, with mean viable counts of 7.8×10^3 CFU/ml, while the other four species were present in one or two samples at less than 8×10^0 CFU/ml. Of seven sediment samples, *A. caviae* and *A. hydrophila* were isolated from six samples with mean viable counts of 1.2×10^5 to 1.3×10^5 CFU/g, whereas the other three species were detected in two or three samples with 4.1×10^1 to 3.5×10^2 CFU/g. Interestingly, other *Aeromonas* spp. were frequently recovered in 95% of the common carp specimens, 100% of the crucian carp and gray mullet specimens, 57% of the water samples, and 100% of the sediment samples. These facts demonstrate that the intestinal tracts of fish, water, and sediments collected from the Hikiji

TABLE 1. Distribution of *Aeromonas* species in fish intestines, water, and sediments from the Hikiji River

Specimen or sample (*n*)	No. of isolates found					
	A. caviae	*A. hydrophila*	*A. jandaei*	*A. sobria*	*A. veronii*	Other *Aeromonas* spp.
Common carp (20)	65	62	31	11	136	149
Crucian carp (13)	53	36	20	42	47	99
Gray mullet (2)	3	0	5	7	1	4
Water (7)	28	3	3	5	4	10
Sediment (7)	12	15	4	4	7	40
Total	161	116	63	69	195	302

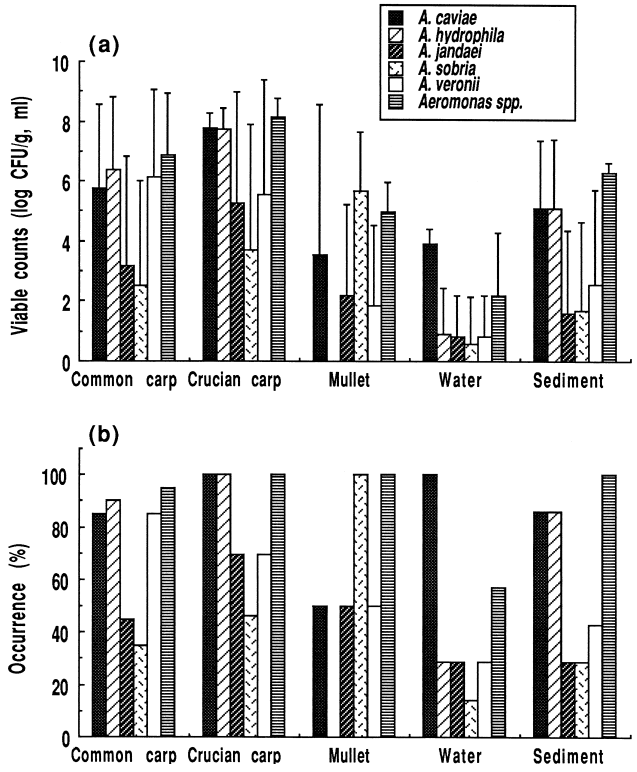

FIG. 1. Population densities (a) and incidences (b) of each *Aeromonas* species in fish intestines, water, and sediments from the Hikiji River. Error bars represent standard deviations of the means.

River are colonized by *Aeromonas* spp. other than the five species mentioned above. These aeromonads remain to be identified at the species level in the near future.

In the present study, the investigation was carried out during April to October 1994, when the water temperature ranged from 17.0°C (October) to 27.0°C (August). pH values ranged from 7.1 (September) to 7.7 (June), and salinities ranged from 0.082 (September) to 0.189 ppt (July). During this period, no significant variation in the viable counts and incidences of five *Aeromonas* species was observed, suggesting that these conditions are not stressful to the five mesophilic aeromonads in the Hikiji River.

The genus *Aeromonas* was proposed by Kluyver and van Niel in 1936 (2). In *Bergey's Manual of Systematic Bacteriology* (2), this genus consists of four species: *A. hydrophila*, *A. caviae*, *A. sobria* and *A. salmonicida*. Thereafter, an additional nine mesophilic species, *Aeromonas allosaccharophila*, *Aeromonas enteropelogenes*, *Aeromonas eucrenophila*, *Aeromonas ichthiosmia*, *A. jandaei*, *Aeromonas media*, *Aeromonas schubertii*, *Aeromonas trota*, and *A. veronii*, have joined the genus (7, 13, 14). *A. veronii* was originally described by Hickman-Brenner et al. (6) in 1987 as a new species that had previously been referred to by the Centers for Disease Control as enteric group 77. In their report, Hickman-Brenner et al. examined 11 *A. veronii* strains, of which at least 5 strains were isolated from patients who had been exposed to lake water or who had drunk untreated well water. On the other hand, *A. jandaei* was reported by Carnahan et al. (4) in 1991 as a new *Aeromonas* species which had been designated as DNA group 9 *Aeromonas* (7). In this report, the nine *A. jandaei* strains examined included two strains isolated from patients exposed to freshwater and one strain derived from a prawn. These facts strongly suggest that both *A. veronii* and *A. jandaei* are aquatic microorganisms, although there has been little information concerning their ecology in freshwater environments (8, 15). In the previous study (20), we found that 65 aeromonad strains isolated from freshwater-cultured fish included 2 strains each of *A. veronii* and *A. jandaei*. Additionally, the present study demonstrates that *A. veronii* and *A. jandaei* occurred in 19 and 27 of 35 fish specimens and in both 2 of 7 water samples and 2 and 3 of 7 sediment samples, respectively. Population densities of *A. veronii* and *A. jandaei*, respectively, have ranged from $<2 \times 10^3$ to 5.2×10^8 CFU/g and $<2 \times 10^3$ to 1.5×10^8 CFU/g in fish intestines, from $<2 \times 10^1$ to 8.0×10^2 CFU/ml and $<2 \times 10^1$ to 1.2×10^3 CFU/ml in water samples, and from $<2 \times 10^2$ to 1.6×10^6 CFU/g and $<2 \times 10^2$ to 1.2×10^6 CFU/g in sediment samples collected at the Hikiji River (Fig. 1). The densities and incidences of *A. veronii* and *A. jandaei* are comparable to those of *A. caviae*, *A. hydrophila*, and *A. sobria*, which are known as indigenous bacteria in aquatic environments (3, 15–21). These results strongly suggest that *A. veronii* and *A. jandaei* are also widely distributed in aquatic environments and that they possess the potential to be predominant in fish intestines, water, and sediments. However, in order to promptly and exactly anticipate and effectively prevent the outbreak of infectious diseases caused by *Aeromonas* species, further studies along these lines are needed.

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