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REGULAR ARTICLE

Distribution of *Aeromonas* species in environmental water used in daily life in Okinawa Prefecture, Japan

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Abstract
Objectives The genus *Aeromonas* is known to causes diseases such as food poisoning, sepsis, and wound infection. However, the mode of *Aeromonas* transmission from environment to humans is not clearly understood. To evaluate the health risks of *Aeromonas* spp. in environmental freshwater, the number, proportion and putative virulence factors of *Aeromonas* species were investigated in Okinawa Prefecture, Japan.
Methods Environmental freshwater samples were collected from three dams, two springs and three private wells. *Aeromonas* strains were identified by the biochemical method and the viable count was calculated. The production of extracellular enzymes and the virulence genes were investigated for possessing putative virulence factors using representative isolates.
Results At least seven species of already-known *Aeromonas* isolates as well as unidentified *Aeromonas* spp. with/without arginin dehydrolase (ADH) exist in water at these sites. *Aeromonas* spp. was found to exist at over 1000 CFU/100 ml in one spring and two wells. *A. veronii* biovar sobria and *A. jandaei* were the predominant species in dams, and *A. hydrophila* and/or *A. eucrenophila* were predominant in wells. Almost all the sampled *Aeromonas* species produced protease, gelatinase, lipase, esterase and DNase, but *A. caviae*, *A. caviae*-like bacteria, and *A. eucrenophila* had low hemolytic activity. Most sampled *A. hydrophila* strains possessed both aerolysin gene (*aer*) and hemolysin gene (*hly*A), but *A. caviae* and *A. eucrenophila* strains did not possess either gene.
Conclusions Since these results indicated that several *Aeromonas* species having potential pathogenicity exist in environmental water in Okinawa, surveys are recommended as a public health measure.

Keywords *Aeromonas* species · Environmental water · Distribution · Proportion · Putative virulence factors

Introduction

As recently as 27 years ago, only five species of the genus *Aeromonas* were recognized [1] but the classification is now subdivided, and at least 32 genospecies have been named [2]. Some species of the genus *Aeromonas* known to cause food poisoning, sepsis, wound infection and other diseases [3] have been found in human environments such as fresh water, coastal marine water, and soil [4]. Serious *Aeromonas* infections can occur in exposed patients with underlying diseases such as hepatitis, diabetes and/or malignant tumors [3, 5]. In addition, there have been reports of *Aeromonas* infections through trauma [6, 7] and near-drowning events [8] caused by recreational activities such as boating, fishing and diving.

Aeromonas spp. has also been isolated after natural disasters. High numbers (approximately 10^7 CFU/ml) of *Aeromonas* spp. were detected in floodwater samples during Hurricane Katrina in New Orleans in August 2005 [9]. *Aeromonas* species were found in 145 (22.6 %) of 641 isolates from patients with skin and soft-tissue infections during the tsunami in southern Thailand in December 2004 [10].

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Several *Aeromonas* species secrete a number of extracellular proteins that are known virulence factors, including amylase, chitinase, elastase, aerolysin, nuclease, gelatinase, lecithinase, lipase and protease, found in four species [11]. Sechi et al. [12] also characterized slime, hemolysin, gelatinase, and protease, and adhesion to epithelial cells, and the virulence genes such as the cytolytic enterotoxin (AHCYTOEN), type IV pilus (*Tap*), and bundle forming pilus (*Bfp*A and *Bfo*G) in clinical and environmental isolates of three *Aeromonas* species.

The mode of transmission of pathogenic *Aeromonas* from environment to humans is still not clearly understood. Since the detailed distribution and proportions of *Aeromonas* species in aquatic environments are also not known precisely, a survey was required to classify the species living in the actual environment. Knowledge of the concentrations and virulence factors of *Aeromonas* spp. is important also for management of *Aeromonas* infections but there have been few relevant investigations in aquatic environments.

To evaluate public health risks of *Aeromonas* spp. in the environment, we investigated the number and proportion of *Aeromonas* species in the aquatic environment to learn the extent to which *Aeromonas* species exist in dams, springs and private wells used in daily life in Okinawa Prefecture, Japan. Further, we investigated whether the *Aeromonas* isolates possess any putative virulence factors.

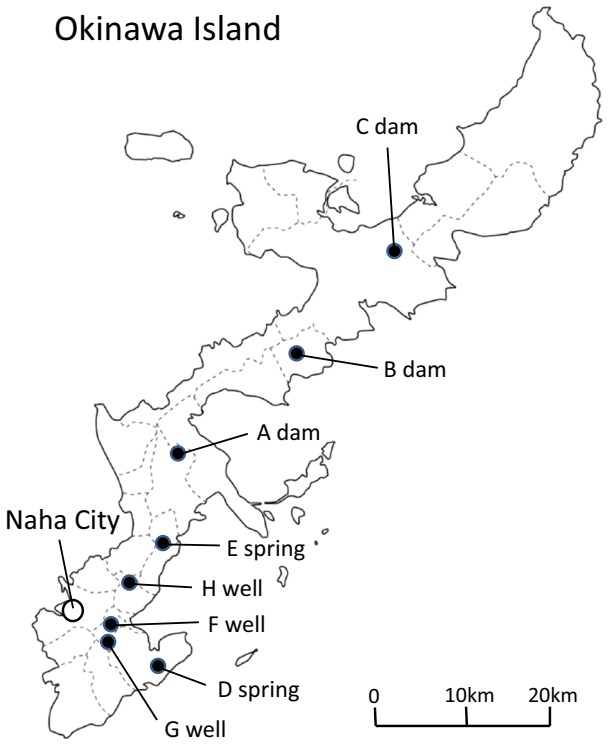

Fig. 1 Freshwater sampling points. Environmental freshwater samples were collected from three dams (**a**, **b** and **c**), two springs (**d** and **e**) and three private wells (**f**, **g** and **h**) of Okinawa Island in Okinawa Prefecture. Naha city is the prefectural capital of Okinawa Prefecture

Materials and methods

Isolation of bacteria and measurement of viable count

Okinawa Prefecture (population about 1,431,000) is located in the south-west part of Japan, in the subtropical zone. Environmental freshwater samples were collected from April to December 2009 at three dams, two springs and three private wells on Okinawa Island, the main island in Okinawa Prefecture (Fig. 1). The reasons for choosing the dams, springs, and wells as sampling points are as follows: (1) the water behind dams is used as drinking water after filtration and disinfection, (2) since the springs are also tourist resorts, visitors may consume the water, (3) water from the wells is used in personal daily life. Three samples each of 500 ml environmental freshwater were collected in sterilized bottles from a single point in Okinawa Island, respectively. The water temperature was measured at each sampling point. The measurement of viable count of *Aeromonas* spp. was performed as follows. One hundred ml and 10 ml of the water were centrifuged for 30 min at 1700*g* and resulting pellets were mixed with 1 ml of saline to produce 100 and 10 times concentrated samples, respectively. One hundred microliter samples of 100, 10 times, and the original concentration were each inoculated in triplicate using a spreader on Ryan's Aeromonas Medium (RAM: Oxoid Ltd., Hampshire, England) plates and Xylose Deoxycholate Citrate Agar (XDCA: self-compounded) plates. The agar plates were incubated overnight at 35 °C. Suspect *Aeromonas* colonies from segments (whole, 1/2, 1/4, or 1/8) of the RAM and XDCA plates containing up to 300 colonies per plate were transferred to nutrient agar plates and incubated at 35 °C overnight. These colonies were screened by oxidase test, catalase test and gram staining, and suspect colonies were characterized by resistance to vibriostatic agent O/129 disc tests (150 μg/ml; Oxoid Ltd, Hampshire, England) using triple sugar iron agar (TSI: Eiken Chemical Co., Ltd., Tokyo, Japan) and sulfide indole motility medium (SIM: Eiken Chemical Co., Ltd., Tokyo, Japan); Voges-Proskauer and methyl red tests; growth in 0 % NaCl; tests for esculin hydrolysis, citrate, urocanic acid, and DL-lactate utilization, presence of lysine decarboxylase, ornithine decarboxylase, and arginine dihydrolase; production of acid and gas from glucose, and acid production from lactose, sucrose, arabinose, myo-inositol, mannitol, salicin, cellobiose, rhamnose, and mannose. The strains were identified according to the biochemical scheme of Abbott et al. [13] and Bergey's

manual [14]. The viable count of *Aeromonas* spp. was calculated using the identified positive colonies from the direct agar plates. The total viable count in the environmental water was calculated by plating on trypticase soy agar (TSA: BD, Sparks MD, USA) regulated to 3 % agar concentration, using samples of 100, 10 times and original concentration. The most probable number (MPN) of fecal coliform group was calculated using EC broth (Eiken Chemical Co., Ltd., Tokyo, Japan) with 10 ml and 1 ml of the original water, and 1 ml each of samples diluted to 10 and 100 times.

Production of extracellular enzymes

The tests for β-hemolysin and protease were carried out with modifications by Khan et al. [15] using 5 % sheep blood cells and 3 % skim milk, respectively. The test for gelatinase was performed by the method of Frazier [16] using agar plates including gelatin. The test for lipase was performed by the method of Davis and Ewing [17] using agar plates including corn oil. The test for esterase was performed by the method of Sierra [18] using agar plates including Tween 80. The test for DNase was carried out using commercially available deoxyribonucleic acid (DNA) agar (Eiken Chemical Co., Ltd., Tokyo, Japan).

PCR assay

To extract DNA, a colony on a nutrient agar plate was suspended in 150 microliters of Tris–EDTA (TE) buffer and boiled for 10 min. The suspension was centrifuged at 17,000*g* for 10 min with refrigeration and the supernatant was used as template DNA for PCR. Primer set Aero-F (5′-gag cga gaa ggt gac cac caa gaa c-3′) and Aero-R (5′-ttc cag tcc cac cac ttc act tca c-3′) designed by Nam and Joh [19] were used to amplify a 417 bp aerolysin gene (*aer*) fragment. Another primer set H1 (5′-ggc cgg tgg ccc gaa gat acg gg-3′) and H2 (5′-ggc ggc gcc gga cga gac ggg-3′) designed by Wong et al. [20] were used to amplify a 597 bp hemolysin gene (*hly*A) fragment. A modified protocol for the amplification of the *aer* and *hly*A genes from *Aeromonas* isolates was used. To amplify the *aer* gene, 2.5 µl of the template DNA was mixed with 2.5 µl of 10× Taq reaction mixture, 2 µl each of the 2.5 mM dNTPs, 0.125 µl each of the 50 µM primers, 0.2 µl of 5 U/µl Taq polymerase and 17.55 µl of distilled water (Total 25 µl) in a 200 µl microtube. Each reagent used for the PCR mixture was from a commercially available PCR reagent set (TaKaRa Ex Taq: Takara Bio Inc., Shiga, Japan) except the primers, which were synthesized (Hokkaido System Science Co., Ltd., Hokkaido, Japan). Each microtube was heated as a pre-denaturation step at 94 °C for 5 min, and as a DNA amplification step for 35 cycles at 94, 60 and 72 °C for 40, 20 and 30 s, respectively, as a final extension step at 72 °C for 5 min in a thermal cycler (Program Temp Control System PC-320: ASTEC, Fukuoka, Japan). PCR products were separated by electrophoresis in 1.5 % agarose gels and stained with 1 µg/ml ethidium bromide. The products were then observed under UV light, and a single band at 417 bp was determined to be positive for *aer* gene. To amplify the *hly*A gene, a PCR mixture was prepared in the same way as the mixture for the *aer* gene except using half the primer volume. The amplification was performed as follows: Pre-denaturation at 94 °C for 2 min, DNA amplification for 30 cycles at 94, 68 and 72 °C for 40, 5 and 5 s, and final extension at 72 °C for 3 min. The verification of PCR products of 597 bp was performed by the above method.

Results

We investigated the viable count of *Aeromonas* spp. and other bacteria to understand the number of these bacteria in environmental freshwater being utilized in daily life (Table 1). *Aeromonas* spp. was found at over 1000 CFU/100 ml in environmental water at three points, E spring, F well and H well. Although the proportion of *Aeromonas* spp. in the total viable count was below 2.3 % in the other sampling points, it was 21.8 % in the E spring. The fecal coliform group was highest in the wells.

Regarding the prevalence of *Aeromonas* species in each sampling point, the unidentified *Aeromonas* spp. of arginin

Table 1 Viable count of *Aeromonas* spp. and other bacteria in water samples

	A dam	B dam	C dam	D spring	E spring	F well	G well	H well
Month of water sampling	Aug	Aug	Dec	Apr	Apr	Sep	Sep	Dec
Water temperature (°C)	32.0	31.4	18.0	22.5	20.5	26.5	27.0	22.8
Total viable count (CFU/100 ml)	7000	6000	9500	2000	11,000	61,000	6000	140,000
Fecal coliform group (MPN/100 ml)	4	<3.0	<3.0	<3.0	<3.0	7	4	43
Viable count of *Aeromonas* spp. (CFU/100 ml)	88	40	220	15	2400	1200	5	2200
Viable count of *Aeromonas* spp./Total viable count (%)	1.3	0.7	2.3	0.8	21.8	2.0	0.1	1.6

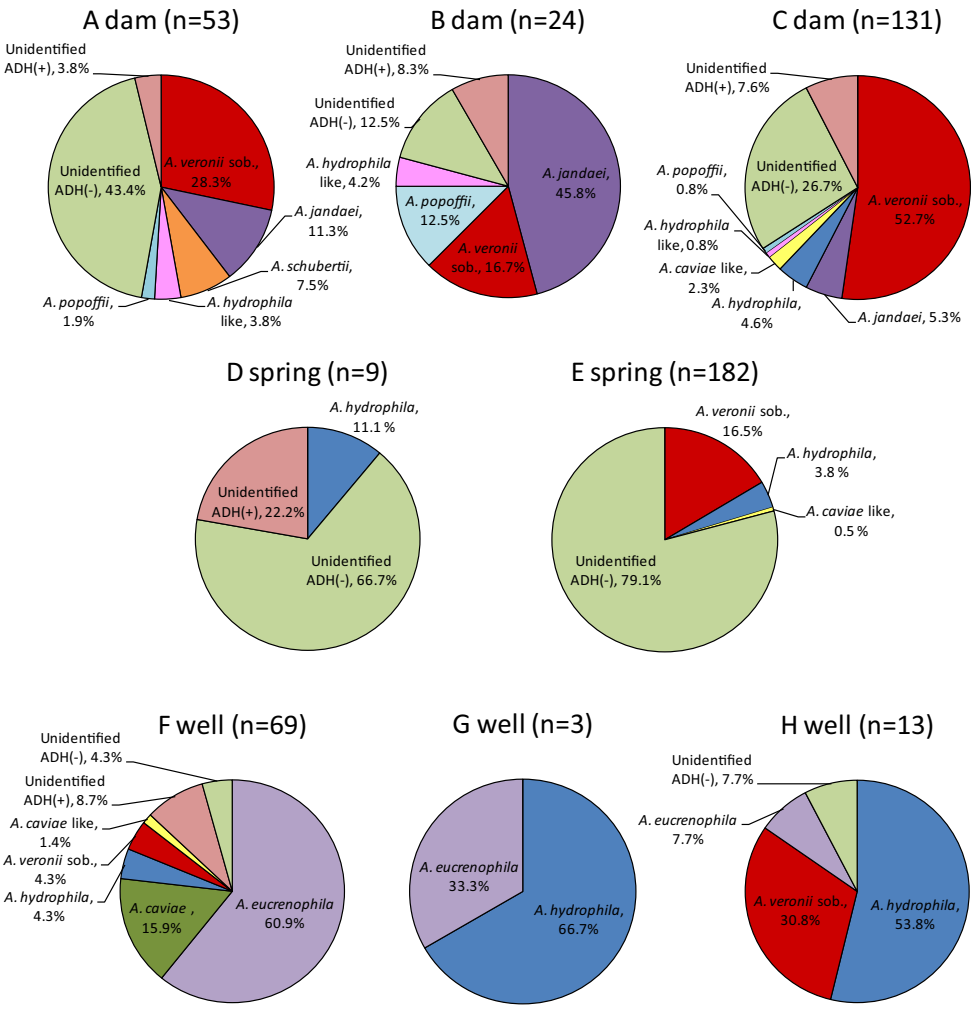

Fig. 2 Proportion of isolated *Aeromonas* species from direct plating. The proportion of *Aeromonas* species in each sampling point was investigated using direct plating, with colonies on 3 RAM and 3 XDCA plates using water samples of 100 times concentration except for 10 times sample of F well and original sample of H well. *A. veronii* sob. is *A. veronii* biovar sobria. *A. hydrophila*-like and *A. caviae*-like bacteria are difficult isolates that distinguishes *A. hydrophila* from *A. bestiarum* and *A. caviae* from *A. media*, respectively. Unidentified ADH(+) is the unidentified *Aeromonas* spp. of arginin dehydrolase (ADH) positive. Unidentified ADH(−) is the unidentified *Aeromonas* spp. of arginin dehydrolase (ADH) negative

dehydrolase (ADH) negative (−) was the predominant species in springs and *A. veronii* bv. sobria and *A. jandaei* were predominant in dams. In wells *A. hydrophila* and/or *A. eucrenophila* were the predominant species, with *A. eucrenophila* accounting for 60.9 % of all *Aeromonas* spp. in F well, a much higher frequency than was isolated at other points (Fig. 2). Although unidentified *Aeromonas* spp. of ADH(−) was the most frequent isolate from A dam, they were classified into seven types by biochemical patterns (data not shown), indicating the presence of seven distinct unidentified strains of *Aeromonas* spp. Likewise, in C dam the unidentified *Aeromonas* spp. of ADH(−) included 11 types of biochemical patterns, and the unidentified *Aeromonas* spp. of ADH(+) included six types (data not shown). One of the three types of biochemical character pattern of unidentified *Aeromonas* spp. ADH(−) was 33.3 % at the D spring, and one of three types of unidentified *Aeromonas* spp. ADH(−) was 78.0 % at the E spring (data not shown). The total result of isolated *Aeromonas* species found by direct plating is shown in Table 2.

We isolated 565 *Aeromonas* strains from water samples from dams, springs and wells. The already-known isolates included at least seven species of *A. hydrophila*, *A. caviae*, *A. eucrenophila*, *A. veronii* bv. sobria, *A. jandaei*, *A. schubertii* and *A. popoffii*. Unidentified *Aeromonas* spp. of ADH(+) and ADH(−) were found in 26 and 223 of total isolates respectively. *A. hydrophila* and *A. veronii* bv. sobria were both isolated from six of the eight points. Some species were more frequent in dams than in springs and wells. *A. jandaei*, *A. popoffii* and *A. hydrophila*-like bacteria were isolated only from the three dams, and *A. eucrenophila* was isolated only from the three wells. Although F well and H well had very high viable counts, the recovered strains of *Aeromonas* spp. were low (Table 1). The reason may be that because of the high viable count, concentrations of 10 times and original were used for measurement of *Aeromonas* species in both agar plates. Therefore, there were few isolated colonies, but the counts would likely be higher if these samples had been concentrated to 100 times like other sites.

Table 2 Isolated *Aeromonas* species from water samples of dams, springs and wells[a]

Isolated *Aeromonas*	A dam	B dam	C dam	D spring	E spring	F well	G well	H well	Total
A. hydrophila			6	2	11	12	2	7	40
A. hydrophila like[b]	3	1	1						5
A. caviae						19			19
A. caviae like[c]			3		6	1			10
A. eucrenophila						58	1	1	60
A. veronii biovar sobria	18	4	83		32	3		4	144
A. jandaei	6	13	7						26
A. schubertii	4								4
A. popoffii	4	3	1						8
Unidentified ADH(+)[d]	3	2	10	2		9			26
Unidentified ADH(−)[e]	28	3	34	7	147	3		1	223
Total	66	26	145	11	196	105	3	13	565

[a] *Aeromonas* colonies were harvested from plates at the sample concentration used for calculation of viable count, and from other plates which were lower concentrations than the plates used for calculation

[b] There are difficult isolates that distinguish *A. hydrophila* from *A. bestiarum*

[c] There are difficult isolates that distinguish *A. caviae* from *A. media*

[d] Unidentified ADH(+) is *Aeromonas* spp. of arginin dehydrolase (ADH) positive

[e] Unidentified ADH(−) is *Aeromonas* spp. of arginin dehydrolase (ADH) negative

Table 3 Detection of putative virulence factors from *Aeromonas* spp.

Isolated *Aeromonas*	Number of strains (%)	β-Hemolysis (sheep)	Protease (skim milk)	Gelatinase (gelatin)	Lipase (corn oil)	Esterase (Tween 80)	DNase
A. hydrophila	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)
A. hydrophila like[a]	5 (100)	3 (60.0)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
A. caviae	7 (100)	2 (28.6)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)
A. caviae like[b]	10 (100)	1 (10.0)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)
A. eucrenophila	6 (100)	1 (16.7)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)
A. veronii biovar sobria	30 (100)	29 (96.7)	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)
A. jandaei	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)
A. popoffii	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)
Unidentified ADH(+)[c]	19 (100)	14 (73.7)	19 (100)	19 (100)	19 (100)	19 (100)	19 (100)
Unidentified ADH(−)[d]	34 (100)	28 (82.4)	33 (97.1)	34 (100)	33 (97.1)	33 (97.1)	34 (100)
Total	152 (100)	119 (78.3)	151 (99.3)	152 (100)	151 (99.3)	151 (99.3)	152 (100)

[a] There are difficult isolates that distinguish *A. hydrophila* from *A. bestiarum*

[b] There are difficult isolates that distinguish *A. caviae* from *A. media*

[c] Arginin dehydrolase (ADH) positive

[d] Arginin dehydrolase (ADH) negative

Representative *Aeromonas* spp. isolates were investigated to determine if they possessed any kind of putative virulence factors (Table 3). Nearly all *Aeromonas* species produced protease, gelatinase, lipase, esterase and DNase, however *A. caviae*, *A. caviae*-like bacteria and *A. eucrenophila* belonging to *A. caviae* complex had low hemolytic activity. Since most *Aeromonas* hemolysins described to date are related to one of two toxins of the aerolysin [21] and AHH1 hemolysin [22], we investigated *aer* and *hly*A genes by the PCR method (Table 4). All *A. hydrophila* strains possessed *aer* and/or *hly*A genes, with 83.3 % having both *aer* and *hly*A genes. No *A. caviae* and *A.*

Table 4 Detection of *aer* and *hlyA* genes from *Aeromonas* spp.

Isolated *Aeromonas*	No. of strains (%)	*aer*(+)/hlyA(+)	*aer*(+)/*hlyA*(−)	*aer*(−)/*hlyA*(+)	*aer*(−)/*hlyA*(−)
A. hydrophila	24 (100)	20 (83.3)	1 (4.2)	3 (12.5)	0 (0)
A. hydrophila like[a]	5 (100)	0 (0)	2 (40.0)	0 (0)	3 (60.0)
A. caviae	7 (100)	0 (0)	0 (0)	0 (0)	7 (100)
A. caviae like[b]	10 (100)	0 (0)	5 (50.0)	0 (0)	5 (50.0)
A. eucrenophila	6 (100)	0 (0)	0 (0)	0 (0)	6 (100)
A. veronii biovar sobria	30 (100)	5 (16.7)	24 (80.0)	0 (0)	1 (3.3)
A. jandaei	9 (100)	0 (0)	2 (22.2)	0 (0)	7 (77.8)
A. popoffii	8 (100)	0 (0)	3 (37.5)	0 (0)	5 (62.5)
Unidentified ADH(+)[c]	19 (100)	7 (36.8)	6 (31.6)	0 (0)	6 (31.6)
Unidentified ADH(−)[d]	34 (100)	3 (8.8)	25 (73.5)	1 (2.9)	5 (14.7)
Total	152 (100)	35 (23.1)	68 (44.7)	4 (2.6)	45 (29.6)

[a] There are difficult isolates that distinguish *A. hydrophila* from *A. bestiarum*

[b] There are difficult isolates that distinguish *A. caviae* from *A. media*

[c] Arginin dehydrolase (ADH) positive

[d] Arginin dehydrolase (ADH) negative

eucrenophila strains possessed both genes, but 50 % of *A. caviae*-like isolates had the *aer* gene only, and the *hly*A gene was not present in any. Most (80 %) of the *A. veronii* bv. sobria isolates possessed only the *aer* gene, with most of the rest having both *aer* and *hly*A genes. Isolates of both *A. jandaei* and *A. popoffii* were 100 % positive for β-hemolysin, but 77.8 and 62.5 % had the *aer* (−)/*hly*A(−) pattern, respectively. Most of the unidentified *Aeromonas* spp. of ADH(+) and ADH(−) possessed *aer* and/or *hly*A genes.

Discussion

The genus *Aeromonas* has been isolated by Holmes et al. from aquatic environments such as rivers, lakes, ponds, seawater (estuaries), drinking water, groundwater, wastewater, and sewage in various stages of treatment [23]. In our study, *Aeromonas* spp. were found to exist at over 1000 CFU/100 ml in one spring and two wells, far higher than the <1 CFU/ml in ground water that has been reported by Holmes et al. [23]. *Aeromonas* in the E spring water accounted for 21.8 % of the total viable count. This water is used to make tea consumed at a traditional community event. Although *Aeromonas* numbers in dams were similar to what Holmes et al. reported, and also in lakes ($1–10^2$ CFU/ml) [23], various *Aeromonas* species were isolated from these sites. The water from dams in Okinawa Prefecture is filtered and disinfected for distribution as public water supplies. The D and E springs receive many sightseers since they were designated among "Selected 100 Exquisite and Well-Conserved Water Sites'' by the Japanese Ministry of Environment. Visitors often consume the raw water because it appears to be clear and pure. The water from wells is used mainly for watering garden plants, washing cars, and washing harvested vegetables, so household members can be exposed to *Aeromonas* spp. in these ways. In brief, raw water without chlorination from these sampling points, except for dams, is frequently consumed or contacted by people.

Several studies have reported *Aeromonas* isolation from aquatic environments [4, 24, 25] but these contained little data on the proportion of various component species, and no other studies done in Japan with such data were found. Investigation of *Aeromonas* species in the environment revealed the proportion of the bacteria in environmental water, as shown in Fig. 2 of this study. Although our sampling points were geographically distant, the isolation of *A. hydrophila* and *A. veronii* bv. sobria from six of eight points, respectively (Fig. 1 and Table 2), suggests that both these species exist widely in aquatic environments in Okinawa. *A. jandaei*, *A. popoffii* and *A. hydrophila*-like bacteria were isolated from three dams, but these species were not isolated from springs and wells. These species may exist in environments where water has become eutrophic due to photosynthesis of phytoplankton, fertilizer runoff or other reasons, or because the water of dams is muddier than springs and wells. On the other hand, *A. eucrenophila* was isolated from three wells but not from dams and springs. Since well water appears clear, *A. eucrenophila* may prefer an environment where there is little organic matter and/or it not illuminated by sunlight. This is supported by *A. eucrenophila* being found in unpolluted surface and groundwater such as oligosaprobic

streams, and in clean well water [14]. There was no apparent influence of water temperature in the sampled range, as seen in Table 1.

Reports indicate that ADH-positive species of *Aeromonas* are prevalent in strains isolated from clinical samples, and ADH-negative species are more prevalent in environmental strains [13], but the unidentified *Aeromonas* spp. of ADH(+) and ADH(−) strains in our study showed almost identical levels of protease, gelatinase, lipase, esterase, and DNase. The positive rate for β-hemolysin was somewhat higher in the unidentified ADH(−) *Aeromonas* spp. than the ADH(+) strain. We investigated *aer* and *hly*A genes in isolated species to test the existence of two hemolytic toxins because these genes may contribute to the virulence of *A. hydrophila* [26]. Although the primer set of Pollard et al. could detect the *aer* gene from only *A. hydrophila* [27], we could detect it from various species using the primer set of Nam and Joh [19]. The positive rate found for the *hly*A gene in *A. caviae*, *A. caviae*-like bacteria, and *A. eucrenophila* was 0 %, compared to 35 % found by of Heuzenroeder et al. [26] in *A. caviae*. The reason may lie in the difference of the sample source, because most positive samples of Heuzenroeder et al. were from fish rather than from water. On the other hand, the results for β-hemolysin and *hly*A gene of the *A. jandaei* and *A. popoffii* do not agree with each other in Tables 3 and 4, suggesting that there may be a hemolytic gene besides *aer* and *hly*A genes in the both species.

At least seven species of already-known *Aeromonas* isolates and the unidentified *Aeromonas* spp. with/without ADH exist in water of dams, springs and private wells that are in regular use in Okinawa. Six of the seven (excepting *A. eucrenophila)* already-known species have been reported in clinical *Aeromonas* isolates [28]. *A. hydrophila* and *A. veronii* bv. sobria were widely isolated at most of the sampling points. *A. hydrophila, A. caviae* and *A. veronii* bv. sobria are among the species mainly responsible for causing disease in humans [3, 29]. Infection caused by aeromonads in Okinawa is, therefore, also a possibility because species are widespread in freshwater environments.

Recently Khajanchi et al. found suggestive evidence of successful colonization and infection by particular strains of certain *Aeromonas* species after transmission to humans from water [30]. Voss et al. found that 13 of 28 *Aeromonas* wound and soft tissue infections during a 4-year period from 1985 to 1989 were associated with a water-related injury, and 43 % of the total could be directly related to water from a lake or river [7]. In Okinawa, 38 cases of extraintestinal *Aeromonas* infections were reported in a 5-year period from April 2008 to March 2013 in Urasoe General Hospital in Urasoe city (population about 114,000) adjacent to the prefectural capital Naha city [31]. Further, an afebrile 15-year-old girl who had been healthy until the onset of the illness died from *A. veronii* bv. sobria infection. The infection route was unknown [32]. Since these reports suggest the likelihood of transmission of *Aeromonas* species to humans from environmental freshwater, we believe that they support the significance of our study.

Through this study, we have quantified the prevalence, proportion, and the presence of various putative virulence factors of *Aeromonas* species in aquatic environments in Okinawa Prefecture. We clarified the trend of distribution of *Aeromonas* species in the environment. Our findings may help direct public health efforts to reduce *Aeromonas* infection transmitted from water to humans by pinpointing such risk factors as leisure activities in shallow waters at dams, children playing in springs, and using water from private wells for gardening or washing vegetables or cars. *Aeromonas* species are also potential pathogens when traumatic injury is exposed to environmental water, for immunocompetent as well as immunocompromised hosts. A survey of environmental water for *Aeromonas* spp. is, therefore, suggested.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest.

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