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Aeromonas Isolates from Fish and Patients in Tainan City, Taiwan: Genotypic and Phenotypic Characteristics

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ABSTRACT The present study aimed to isolate *Aeromonas* from fish sold in the markets as well as in sushi and seafood shops and compare their virulence factors and antimicrobial characteristics with those of clinical isolates. Among the 128 fish isolates and 47 clinical isolates, *Aeromonas caviae*, *A. dhakensis*, and *A. veronii* were the principal species. *A. dhakensis* isolates carried at least 5 virulence genes, more than other *Aeromonas* species. The predominant genotype of virulence genes was *hlyA lip alt col ela* in both *A. dhakensis* and *A. hydrophila* isolates, *alt col ela* in *A. caviae* isolates, and *act* in *A. veronii* isolates. *A. dhakensis*, *A. hydrophila*, and *A. veronii* isolates more often exhibited hemolytic and proteolytic activity and showed greater virulence than *A. caviae* isolates in *Caenorhabditis elegans* and the C2C12 cell line. However, the link between the genotypes and phenotypes of the studied virulence genes in *Aeromonas* species was not evident. Among the four major clinical *Aeromonas* species, nearly all (99.0%) *A. dhakensis*, *A. hydrophila*, and *A. veronii* isolates harbored bla_{CphA}, which encodes a carbapenemase, but only a minority (6.7%, 7/104) were nonsusceptible to carbapenem. Regarding AmpC β-lactamase genes, bla_{AQU-1} was exclusively found in *A. dhakensis* isolates, and bla_{MOX3} was found only in *A. caviae* isolates, but only 7.6% ($n = 6$) of the 79 *Aeromonas* isolates carrying bla_{AQU-1} or bla_{MOX3} exhibited a cefotaxime resistance phenotype. In conclusion, fish *Aeromonas* isolates carry a variety of combinations of virulence and β-lactamase resistance genes and exhibit virulence phenotypes and antimicrobial resistance profiles similar to those of clinical isolates.

IMPORTANCE *Aeromonas* species can cause severe infections in immunocompromised individuals upon exposure to virulent pathogens in the environment, but the characteristics of environmental *Aeromonas* species remain unclear. Our study showed that several pathogenic *Aeromonas* species possessing virulence traits and antimicrobial resistance similar to those of *Aeromonas* isolates causing clinical diseases were present in fish intended for human consumption in Tainan City, Taiwan.

KEYWORDS *Aeromonas*, virulence factors, β-lactam, antimicrobial susceptibility

Aeromonas species are widespread in aquatic creatures (1–4) and have been isolated from a variety of seafood (5, 6), such as in frozen fish ready for human consumption and in market-sold sushi products containing raw fish (7, 8). Moreover, *Aeromonas*

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species have been isolated from stool samples obtained from persons with diarrhea (9). However, certain individuals, especially those with biliary diseases or cancers, are at high risk of extraintestinal *Aeromonas* infections after consumption of contaminated food or associated food products (10, 11).

Fish constitute one of the protein food sources in southern Taiwan, where the prevalence of chronic liver diseases and *Aeromonas* bacteremia is high (12). In an earlier study conducted in northern Taiwan, *Aeromonas* isolates were found in 88% of seafood from markets and supermarkets, and among these isolates, 98% of *Aeromonas hydrophila* isolates and 94% of *A. sobria* isolates produced β-hemolysin (13). In a survey of virulence markers in *A. veronii* and *A. hydrophila* isolates recovered from freshwater fish and human stool samples, this virulence trait was obvious even at 4°C (14), suggesting that the presence of *A. hydrophila* and *A. veronii* in ice-stored freshwater fish conferred potential health risks. These results suggest that susceptible individuals consuming fish contaminated by pathogenic *Aeromonas* species may develop invasive extraintestinal infections, such as bacteremia or biliary tract infections.

Molecular identification of *Aeromonas* species is regarded as a standard method due to the inaccuracy of conventional phenotypic tests. For example, several isolates originally phenotypically identified as *A. hydrophila* were reclassified as *A. dhakensis* using *rpoD* or *gyrB* sequencing (15, 16). Therefore, the clinical significance of some *Aeromonas* species should be reevaluated, especially in areas of endemicity with a high prevalence of *Aeromonas* infections. Therefore, microbiological surveillance for *Aeromonas* species by molecular methods is warranted in Taiwan.

Three classes of chromosomally mediated β-lactamases, i.e., AmpC β-lactamases, metallo-β-lactamases (MBLs), and penicillinases, are present in clinical *Aeromonas* isolates (10, 17). Another important class of β-lactamases, the class A extended-spectrum β-lactamases (ESBLs), has been increasingly reported in clinical and environmental aeromonads (18, 19). The presence of ESBLs among pathogenic aeromonads raises the concern of inappropriate cephalosporin monotherapy for severe *Aeromonas* infection if the expression of β-lactamase can be induced by cephalosporin. In addition, data on the phenotypes and genotypes of antimicrobial resistance among pathogenic *Aeromonas* isolates in the environment or in food sources are very limited in Taiwan. Therefore, the present study aimed to survey *Aeromonas* species in fish from markets and from sushi and seafood shops in Tainan City, Taiwan, and to compare their virulence genes and antimicrobial resistance profiles with those of clinical isolates from the same geographical area.

RESULTS

Prevalence of *Aeromonas* species in fish. A total of 235 samples were collected, and 78.7% (185) grew bacteria on *Aeromonas* selective medium (LabM 167). Of those isolates, 128 (69.2%) were identified as *Aeromonas* species and the others were identified as *Enterobacter cloacae* ($n = 1$), *Shewanella* species ($n = 1$), *Vibrio* species ($n = 2$), and *Pseudomonas* species ($n = 53$) by sequencing of the *rpoD* genes. *A. caviae* ($n = 43$, 33.6%) was the most common species, followed by *A. veronii* ($n = 33$, 25.8%), *A. bivalvium* ($n = 13$, 10.2%), *A. dhakensis* ($n = 11$, 8.6%), and *A. hydrophila* ($n = 9$, 7.0%). Other species included *A. enteropelogenes* ($n = 6$), *A. taiwanensis* ($n = 6$), *A. media* ($n = 4$), *A. salmonicida* ($n = 2$), and *A. jandaei* ($n = 1$).

Prevalence of *Aeromonas* species in humans. In 2011, a total of 47 clinical isolates were available for further analysis (Table 1). Blood was the most common source, with 61.7% of the clinical isolates being recovered from the blood of 29 patients, and of these isolates, 27.6% were from 8 patients with concurrent bacteremia caused by other bacterial species, including *Escherichia coli*, *Klebsiella pneumoniae*, *E. cloacae*, *Acinetobacter baumannii*, *Acinetobacter lwoffii*, *Pseudomonas aeruginosa*, and *Clostridium perfringens*. The patients had a mean age ± standard deviation of 70 ± 14 years, with a slight male predominance (male to female ratio, 1.1). Common underlying diseases were cancer ($n = 11$ patients), diabetes mellitus ($n = 8$), biliary or gallbladder stones ($n = 7$), and liver cirrhosis ($n = 5$). The infection sources of *Aeromonas* bacteremia were

TABLE 1 Clinical sources of the 47 clinical *Aeromonas* isolates

Source	No. of isolates						
	A. dhakensis (*n* = 14)	*A. hydrophila* (*n* = 6)	*A. veronii* (*n* = 11)	*A. caviae* (*n* = 14)	*A. schubertii* (*n* = 1)	*A. taiwanensis* (*n* = 1)	Total (*n* = 47)
Blood	9	3	5	10	1	1	29
Ascites	1	0	3	0	0	0	4
Bile	1	0	2	1	0	0	4
Wound	1	1	0	2	0	0	4
Pus	1	1	1	0	0	0	3
Urine	1	0	0	0	0	0	1
Other	0	1	0	1	0	0	2

identified in 15 patients and included biliary tract infection (*n* = 9 patients), skin and soft tissue infection (*n* = 3), vascular catheter-associated infection (*n* = 2), and intra-abdominal infection (*n* = 1). All patients were hospitalized and received antimicrobial therapy, and four died in the hospital, resulting in an in-hospital mortality rate of 13.8%. Overall, the major *Aeromonas* species noted in humans and fish were *A. dhakensis*, *A. caviae*, and *A. veronii*; *A. bivalvium*, *A. media*, *A. salmonicida*, and *A. jandaei* were not present in any of the clinical samples (Fig. 1).

β-Hemolysis and exoprotease assays. A total of 145 (82.9%) isolates showed beta-hemolysis, and 88% expressed proteolytic activity, as shown in Table 2. All four *A. media* isolates lacked hemolytic activity on agar plates. Notably, the hemolytic phenotype and exoprotease activity were less common in *A. caviae* isolates (77.2% and 68.4%, respectively) than in *A. veronii* isolates (97.7% and 95.5%, respectively), *A. dhakensis* isolates (100% and 100%, respectively), or *A. hydrophila* isolates (100% and 100%, respectively) (*P* values were <0.05 for all comparisons). No significant difference in beta-hemolytic or proteolytic activity was found between fish and clinical isolates of *A. caviae*, *A. veronii*, *A. dhakensis*, and *A. hydrophila*.

Distribution of virulence genes. The distribution of virulence genes in *Aeromonas* species is shown in Table 2. The collagenase gene (*col*) was the most commonly found gene and was found in 74.3% of the 175 isolates, followed by the gene for elastase (*ela*) (*n* = 129, 73.7%). The genes encoding the ADP-ribosyltransferase toxin (*aexT*), aerolysin (*aerA*), components of the type III secretion system (*ascF-ascG* and *ascV*), and heat-stable cytotoxin (*ast*) were identified in less than 10% of the studied isolates. Although

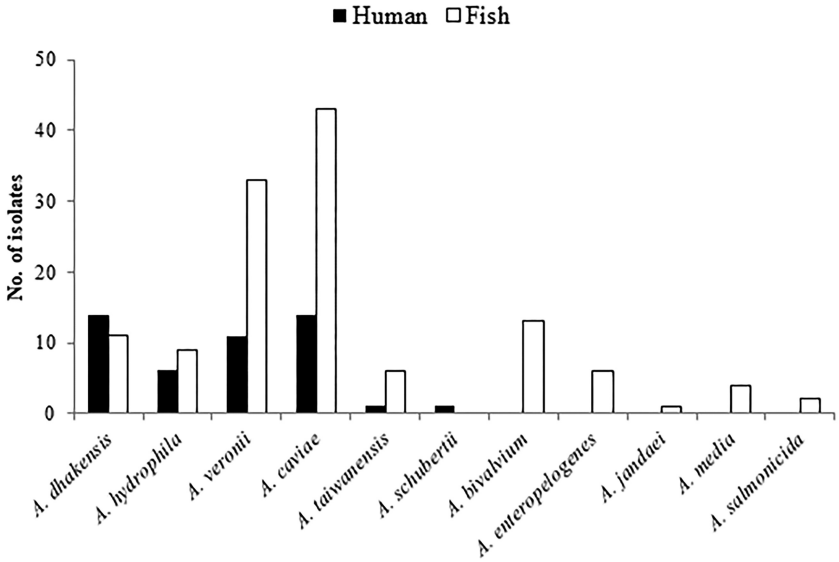

FIG 1 Distribution of *Aeromonas* species in clinical samples and in fish obtained from markets and from sushi and seafood shops.

TABLE 2 Prevalence of hemolytic and proteolytic activity and virulence genes in fish and clinical *Aeromonas* isolates

Species and source	No. (%) of isolates[a]												
	Total	Hemolysis	Proteolysis	*act*	*aerA*	*alt*	*ascF-ascG*	*aexT*	*ascV*	*ast*	*col*	*ela*	*hlyA*	*lip*
A. caviae	57													
Fish	43	31 (72.1)	30 (69.8)	0	0	21 (48.8)**	0	0	0	0	30 (69.8)	43 (100)	0	11 (25.6)
Human	14	13 (92.9)	9 (64.3)	1 (7.1)	0	0	0	0	0	0	14 (100)*	14 (100)	0	14 (100)***
A. veronii	44													
Fish	33	32 (97.0)	31 (93.9)	26 (78.8)	0	12 (36.4)	0	4 (12.1)	2 (6.1)	5 (15.2)	30 (90.9)***	5 (15.2)	0	2 (6.1)
Human	11	11 (100)	11 (100)	11 (100)	0	2 (18.2)	1 (9.1)	2 (18.2)	7 (63.6)***	0	2 (18.2)	0	0	3 (27.3)
A. dhakensis	24													
Fish	10	10 (100)	10 (100)	4 (40)	3 (30)	10 (100)	2 (20)	0	1 (10)	1 (10)	10 (100)	10 (100)	10 (100)	10 (100)
Human	14	14 (100)	14 (100)	4 (28.6)	4 (28.6)	14 (100)	2 (14.3)	0	2 (14.3)	0	14 (100)	14 (100)	13 (92.9)	14 (100)
A. hydrophila	16													
Fish	10	10 (100)	10 (100)	1 (10)	1 (10)	10 (100)	3 (30)	1 (10)	0	1 (10)	10 (100)	10 (100)	10 (100)	10 (100)
Human	6	6 (100)	6 (100)	2 (33.3)	2 (33.3)	6 (100)	3 (50)	1 (16.7)	3 (50)	6 (100)***	6 (100)	6 (100)	6 (100)	6 (100)
A. taiwanensis	7													
Fish	6	3 (50)	6 (100)	0	0	2 (33.3)	0	6 (100)	0	0	0	6 (100)	0	0
Human	1	0	0	0	0	0	0	0	0	0	0	1 (100)	0	1 (100)
A. schubertii, human	1	1 (100)	1 (100)	0	0	0	0	0	0	0	0	1 (100)	0	0
A. bivalvium, fish	13	5 (38.5)	13 (100)	0	0	4 (30.8)	0	0	0	0	10 (76.9)	13 (100)	0	8 (61.5)
A. enteropelogenes, fish	6	6 (100)	6 (100)	0	0	3 (50)	0	0	0	0	0	0	0	3 (50)
A. media, fish	4	0	4 (100)	0	0	2 (50)	0	0	0	0	2 (50)	4 (100)	0	0
A. salmonicida, fish	2	2 (100)	2 (100)	2 (100)	0	1 (50)	0	0	0	0	2 (100)	2 (100)	2 (100)	2 (100)
A. jandaei, fish	1	1 (100)	1 (100)	0	0	0	0	0	1 (100)	0	0	0	1 (100)	0
Total	175	145 (82.9)	154 (88.0)	51 (29.1)	10 (5.7)	87 (49.7)	11 (6.3)	14 (8.0)	16 (9.1)	13 (7.4)	130 (74.3)	129 (73.7)	42 (24.0)	84 (48.0)

[a]*act*, gene encoding a heat-labile cytotoxin; *aerA*, gene encoding an aerolysin; *alt*, gene encoding a heat-labile cytotoxin; *ascF-ascG*, *aexT*, and *ascV*, components of the type III secretion system; *ela*, gene encoding elastase; *col*, gene encoding collagenase; *hlyA*, gene encoding hemolysin; *lip*, gene encoding lipase; *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

TABLE 3 Common genotypes in the fish and clinical isolates of different *Aeromonas* species

Species and genotype[a]	No. (%) of isolates	
	Fish isolates	Clinical isolates
Aeromonas dhakensis		
Total	10	14
hlyA lip alt col ela	3 (30)	5 (35.7)
Aeromonas hydrophila		
Total	10	6
hlyA lip ascF-ascG alt col ela	3 (30)	0
hlyA lip alt col ela	3 (30)	0
act hlyA aerA lip ascF-ascG alt ast col ela	0	2 (33.3)
hlyA lip ascF-ascG alt ast col ela	0	2 (33.3)
Aeromonas caviae		
Total	33	14
lip col ela	5 (15.2)	9 (64.3)[b]
Aeromonas veronii		
Total	43	14
act	8 (18.6)	3 (21.4)

[a]*act*, gene encoding heat-labile cytotoxin; *aerA*, gene encoding aerolysin; *alt*, gene encoding heat-labile cytotoxin; *ascF-ascG*, *aexT*, and *ascV*, gene encoding components of the type III secretion system; *ela*, gene encoding elastase; *col*, gene encoding collagenase; *hlyA*, gene encoding hemolysin; *lip*, gene encoding lipase.
[b]$P < 0.005$.

the number of isolates was low, few of the virulence genes studied were detected in one clinical isolate of *A. schubertii* (only *ela*), one fish isolate of *A. jandaei* (*ascV* and *hlyA*), and all six fish isolates of *A. enteropelogenes* (*alt* and *lip*).

The distribution of some virulence genes was heterogeneous in the fish and clinical isolates of *Aeromonas* species. Notably, five virulence genes were noted in >90% of fish and clinical isolates of *A. dhakensis* and *A. hydrophila*, and the distribution of virulence genes was similar regardless of the origin of the isolates, except that *ast* was predominant in clinical isolates of *A. hydrophila* (100% in clinical isolates versus 10% of fish isolates; $P < 0.0001$). More clinical *A. caviae* isolates than fish *A. caviae* isolates carried *col* (100% versus 69.8%, $P = 0.025$) and *lip* (100% versus 25.6%, $P < 0.0001$). Similarly, more clinical *A. veronii* isolates than fish isolates had *ascV* (63.6% versus 6.1%, $P < 0.0001$), but *col* was more commonly found in the fish *A. veronii* isolates than in the clinical counterparts (90.9% versus 18.2%, $P < 0.0001$).

The common genotypes, as indicated by the combination of putative virulence factors in the fish and clinical isolates, are summarized in Table 3. Among the *A. dhakensis* isolates, the most common genotype was *hlyA lip alt col ela*, and the *act* genotype was the most common among *A. veronii* isolates. The major genotype in *A. caviae* isolates was *lip col ela*, which was more common in clinical isolates than in fish isolates (64.3% versus 15.2%, $P = 0.0015$).

***Caenorhabditis elegans* LT assays for the major genotypes.** Twenty isolates of the major genotypes belonging to four *Aeromonas* species were selected for the liquid toxicity (LT) assay. Almost all *C. elegans* worms fed *A. dhakensis* isolates died within 24 h (336/338, 99.4%; Fig. 2). In contrast, the survival rates of *C. elegans* worms infected with *A. hydrophila*, *A. veronii*, and *A. caviae* were 9.1% (57/623), 24.3% (76/313), and 90.7% (263/290), respectively. Overall, *A. caviae* isolates were less lethal than the isolates of the other species to *C. elegans* ($P < 0.0001$ for all comparisons).

Cytotoxicity of the major genotypes. The cytotoxicity of 20 selected isolates of the major genotypes belonging to four *Aeromonas* species was assessed in the C2C12 mouse myoblast cell line (Fig. 3). The mean level of lactate dehydrogenase (LDH) release induced by *A. dhakensis* isolates was 90.7% ± 26.1%, that induced by *A. hydrophila* was 92.1% ± 20.8%, that induced by *A. veronii* was 79.3% ± 41.3%, and that

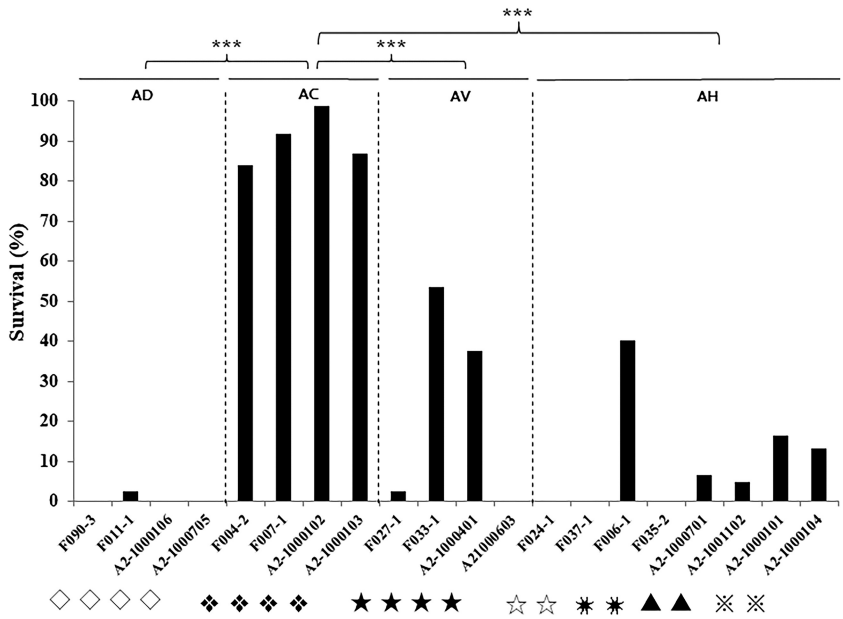

FIG 2 Twenty-four-hour survival rates of *Caenorhabditis elegans* worms infected by the major genotypes of fish and human isolates of four *Aeromonas* species, *Aeromonas dhakensis* (AD), *Aeromonas hydrophila* (AH), *Aeromonas veronii* (AV), and *Aeromonas caviae* (AC), in the liquid toxicity assay. ***, $P < 0.0001$ compared with *A. caviae*. F, fish isolates; A2, clinical isolates. Symbols for genotypes: ◇, *hlyA lip alt col ela*; ❖, *lip col ela*; ★, *act*; ☆, *hlyA lip ascF-ascG alt col ela*; ✷, *hlyA lip alt col ela*; ▲, *act hlyA aerA lip ascF-ascG alt ast col ela*; ※, *hlyA lip ascF-ascG alt ast col ela*.

induced by *A. caviae* was 24.7% ± 32.8% (one-way analysis of variance [ANOVA], $P < 0.0001$). The *post hoc* Tukey's honest significant difference (HSD) test demonstrated that *A. caviae* isolates were less cytotoxic to the C2C12 cell line than *A. dhakensis* ($P = 0.024$), *A. hydrophila* ($P = 0.008$), or *A. veronii* ($P = 0.08$) isolates.

Antimicrobial susceptibility. The antimicrobial susceptibility rates of *Aeromonas* isolates to 18 antimicrobial agents are shown in Table 4. Two species (*A. bivalvium* and *A. enteropelogenes*) found only in fish were susceptible to the tested antibiotics, with the exception of ampicillin, cefazolin, co-trimoxazole, and ampicillin-sulbactam. In general, clinical isolates of *A. dhakensis*, *A. hydrophila*, and *A. caviae* were less susceptible than their counterpart fish isolates to the commonly prescribed β-lactams, such as penicillins (piperacillin and piperacillin-tazobactam), broad-spectrum cephalosporins (cefuroxime, cefotaxime, and ceftazidime), and carbapenems (ertapenem and imipenem). Most (95.4%) *Aeromonas* isolates were susceptible to ertapenem and imipenem, but five *A. veronii* isolates, two *A. dhakensis* isolates, and one *A. caviae* isolate were nonsusceptible to a carbapenem. Although 88% ($n = 154$) of the 175 *Aeromonas* isolates were resistant to cefazolin, 51.5% ($n = 17$) of 33 *A. veronii* fish isolates were cefazolin susceptible, but none of the clinical *A. veronii* isolates were susceptible to cefazolin (51.5% versus 0%, $P = 0.003$). All fish *A. hydrophila* isolates were susceptible to cefuroxime, cefotaxime, and ceftazidime, but only half of the clinical *A. hydrophila* isolates were susceptible (100% versus 50%, $P = 0.04$). However, the co-trimoxazole susceptibility rate was significantly higher for clinical isolates than for their fish counterparts for *A. dhakensis* (92.9% versus 0%, $P < 0.00001$), *A. caviae* (57.1% versus 7%, $P = 0.002$), and *A. veronii* (100% versus 15.1%, $P < 0.00001$).

β-Lactamase genes. The targeted β-lactamase genes were found in only four species: *A. dhakensis*, *A. hydrophila*, *A. veronii*, and *A. caviae* (Table 5). The AmpC β-lactamase-encoding gene, bla_{AQU-1}, was present in all 14 clinical *A. dhakensis* isolates and 60% of 10 fish isolates. Almost all *A. dhakensis* (24/24), *A. hydrophila* (16/16), and *A. veronii* (43/44) isolates but none of the 57 *A. caviae* isolates carried bla_{CphA}. However, bla_{MOX3} was present in only two *A. caviae* isolates, and bla_{PER3} was present in one *A.*

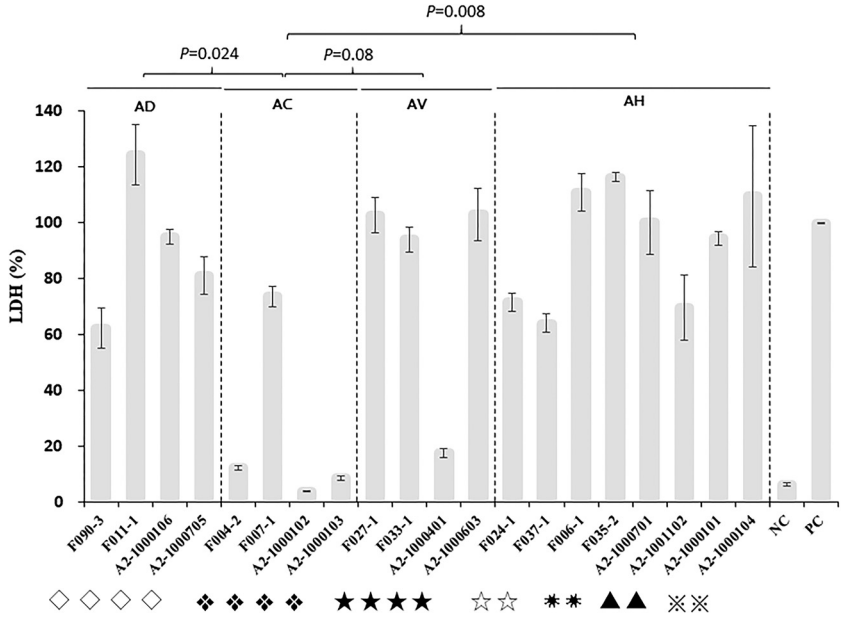

FIG 3 Cytotoxicity of *Aeromonas dhakensis* (AD), *Aeromonas hydrophila* (AH), *Aeromonas veronii* (AV), and *Aeromonas caviae* (AC) isolates to the C2C12 mouse fibroblast cell line, expressed as the proportion of the level of LDH release induced by the *Aeromonas* isolates compared with the level of LDH release induced by lysis solution (the value for the positive control was 100%). F, fish isolates; A2, clinical isolates; PC, positive control; NC, negative control. Symbols for genotypes: ◇, *hlyA lip alt col ela*; ❖, *lip col ela*; ★, *act*; ☆, *hlyA lip ascF-ascG alt col ela*; ✷, *hlyA lip alt col ela*; ▲, *act hlyA aerA lip ascF-ascG alt ast col ela*; ※, *hlyA lip ascF-ascG alt ast col ela*.

caviae isolate. ESBL genes, such as bla_{SHV12}, bla_{CTX1}, bla_{CTX2}, bla_{CTX3}, bla_{CTX9}, bla_{CTX13}, bla_{CTX14}, and bla_{CTX151}, were not detected in the studied *Aeromonas* isolates. The association of the MBL gene (bla_{CphA}) and AmpC β-lactamase genes (bla_{AQU-1} and bla_{MOX3}) with antimicrobial resistance is shown in Table 6. The presence of the bla_{MOX3} gene was not associated with cefotaxime nonsusceptibility (6.8% versus 3.7%, $P = 0.40$), and the bla_{CphA} gene was not associated with ertapenem resistance (4.8% versus 1.7%, $P = 0.65$). The proportion of isolates nonsusceptible to cefotaxime was higher among bla_{AQU-1}-positive isolates than among bla_{AQU-1}-negative isolates (10% versus 0%, $P = 0.019$).

DISCUSSION

Aeromonas is an important pathogen of community-acquired infections in southern Taiwan (12). Our data show that *Aeromonas* species causing clinical diseases, i.e., *A. dhakensis*, *A. hydrophila*, *A. veronii*, and *A. caviae*, can be isolated from fish and share virulence properties and antimicrobial resistance profiles similar to those of isolates of human origin. In a study conducted in Mexico City, Mexico, 82 *Aeromonas* isolates were discovered in 250 frozen fish ready for human consumption (7), and another study in Norway identified multiple *Aeromonas* species in fresh retail sushi (8). In these two studies, *A. veronii*, *A. hydrophila*, and *A. media* were the major species. A substantial proportion of our patients with bacteremia had underlying cancer, diabetes mellitus, biliary or gallbladder stones, or liver cirrhosis. Despite a lack of direct evidence supporting the foodborne nature of *Aeromonas* infection in case clusters or outbreaks, the above-described susceptible hosts carry the risk of invasive *Aeromonas* infection after the consumption of seafood contaminated with pathogenic *Aeromonas* species (20–22).

In the present study, the virulence traits of fish or clinical *Aeromonas* isolates were compared by hemolytic and proteolytic activity assays, *C. elegans* LT assays, and cytotoxicity assays. According to a previous report, the virulence manifested in the LT and cytotoxicity assays was correlated with the virulence traits demonstrated in a

TABLE 4 Antimicrobial susceptibility of *Aeromonas* isolates from humans and fish[a]

Drug	No. (%) of susceptible isolates or susceptibility															
	A. dhakensis		*A. hydrophila*		*A. caviae*		*A. veronii*		*A. bivalvium*	*A. enteropelogenes*	*A. taiwanensis*		*A. media*	*A. jandaei*	*A. salmonicida*	*A. schubertii*
	Fish ($n = 10$)	Human ($n = 14$)	Fish ($n = 10$)	Human ($n = 6$)	Fish ($n = 43$)	Human ($n = 14$)	Fish ($n = 33$)	Human ($n = 11$)	Fish ($n = 13$)	Fish ($n = 6$)	Fish ($n = 6$)	Human ($n = 1$)	Fish ($n = 4$)	Fish ($n = 1$)	Fish ($n = 2$)	Human ($n = 1$)
AM	2 (20)	0	0	0	0	0	1 (3.0)	0	0	4 (66.7)	0	R	0	R	0	R
SAM	2 (20)	0	0	0	4 (9.3)	0	1 (3.0)	0	0	5 (83.3)	0	R	0	R	0	R
PIP	9 (90)	12 (85.7)	9 (90)	3 (50)	37 (86.0)	11 (78.6)	28 (84.8)	10 (90.9)	10 (76.9)	6 (100)	5 (83.3)	S	3 (75.0)	S	2 (100)	S
TZP	10 (100)	12 (85.7)	10 (100)	6 (100)	42 (97.7)	12 (85.7)	32 (97.0)	11 (100)	13 (100)	6 (100)	5 (83.3)	S	3 (75.0)	R	2 (100)	S
CZ	0	0	0	0	2 (4.7)	0	17 (51.5)	0	0	2 (33.3)	0	R	0	I	0	I
CXM	10 (100)	12 (85.7)	10 (100)	3 (50)	43 (100)	10 (71.4)	33 (100)	11 (100)	13 (100)	6 (100)	6 (100)	I	4 (100)	S	2 (100)	S
CTX	10 (100)	12 (85.7)	10 (100)	3 (50)	43 (100)	10 (71.4)	33 (100)	11 (100)	13 (100)	6 (100)	6 (100)	I	4 (100)	S	2 (100)	S
CAZ	10 (100)	12 (85.7)	10 (100)	3 (50)	43 (100)	11 (78.6)	33 (100)	11 (100)	13 (100)	6 (100)	6 (100)	S	4 (100)	S	2 (100)	S
FEP	10 (100)	14 (100)	10 (100)	6 (100)	43 (100)	13 (92.9)	33 (100)	11 (100)	13 (100)	6 (100)	6 (100)	S	4 (100)	S	2 (100)	S
ATM	10 (100)	14 (100)	10 (100)	5 (83.3)	43 (100)	12 (85.7)	33 (100)	11 (100)	13 (100)	6 (100)	6 (100)	S	4 (100)	S	2 (100)	S
ETP	10 (100)	12 (85.7)	10 (100)	6 (100)	43 (100)	13 (92.9)	30 (90.9)	9 (81.8)	13 (100)	6 (100)	6 (100)	S	4 (100)	S	2 (100)	S
IMP	10 (100)	12 (85.7)	10 (100)	6 (100)	43 (100)	14 (100)	33 (100)	10 (90.9)	13 (100)	6 (100)	6 (100)	S	4 (100)	S	2 (100)	S
LVX	10 (100)	14 (100)	10 (100)	5 (83.3)	43 (100)	13 (92.9)	33 (100)	11 (100)	13 (100)	6 (100)	6 (100)	S	4 (100)	S	2 (100)	S
GM	10 (100)	14 (100)	9 (100)	6 (100)	43 (100)	13 (92.9)	33 (100)	11 (100)	13 (100)	6 (100)	6 (100)	S	4 (100)	S	2 (100)	S
AN	10 (100)	14 (100)	10 (100)	5 (83.3)	43 (100)	14 (100)	33 (100)	11 (100)	13 (100)	6 (100)	6 (100)	S	4 (100)	S	2 (100)	S
SXT	0	13 (92.9)	1 (10)	6 (100)	3 (7.0)	8 (57.1)	5 (15.1)	11 (100)	1 (7.7)	2 (33.3)	3 (50)	S	0	I	1 (50)	S
DXY	10 (100)	14 (100)	9 (90)	6 (100)	40 (93.0)	13 (92.9)	31 (93.9)	11 (100)	13 (100)	6 (100)	6 (100)	S	4 (100)	S	2 (100)	S
TET	9 (90)	7 (50)	7 (70)	5 (83.3)	36 (83.7)	11 (78.6)	17 (51.5)	8 (72.7)	13 (100)	6 (100)	5 (83.3)	S	4 (100)	S	2 (100)	S

[a]AM, ampicillin; SAM, ampicillin-sulbactam; PIP, piperacillin; TZP, piperacillin-tazobactam; CZ, cefazolin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; ETP, ertapenem; IMP, imipenem; LVX, levofloxacin; GM, gentamicin; AN, amikacin; SXT, co-trimoxazole; DXY, doxycycline; TET, tetracycline; S, susceptible; I, intermediate; R, resistant.

TABLE 5 Prevalence of four β-lactamase genes in *Aeromonas* isolates from humans and fish

Species and source	No. (%) of isolates carrying[a]:				
	Total	bla_{PER3}	bla_{AQU-1}	bla_{MOX3}	bla_{CphA}
Aeromonas dhakensis					
Fish	10	0	6 (60)	0	10 (100)
Human	14	0	14 (100)	0	14 (100)
Aeromonas hydrophila					
Fish	10	0	0	0	10 (100)
Human	6	0	0	0	6 (100)
Aeromonas veronii					
Fish	33	0	0	2 (6.1)	33 (100)
Human	11	0	0	0	10 (90.9)
Aeromonas caviae					
Fish	43	0	0	43 (100)	0
Human	14	1 (7.1)	0	14 (100)	0

[a]These genes encode metallo-β-lactamase (CphA), AmpC β-lactamase (AQU-1 or MOX3), or an extended spectrum β-lactamase (PER3).

mouse infection model (23). Our results indicated that the *A. dhakensis*, *A. hydrophila*, and *A. veronii* isolates were more virulent than the *A. caviae* isolates, as evidenced by the hemolytic and proteolytic phenotypes and as seen in the two infection models with *C. elegans* and C2C12 cells. Furthermore, the fish isolates of *A. dhakensis*, *A. hydrophila*, and *A. veronii* were as virulent as their human counterparts.

Although a variety of combinations of virulence genes among clinical and fish *Aeromonas* isolates were found in the present work, there was no clear link between the presence of a specific gene and the virulence phenotype, suggesting the need for more research to identify the virulence factors or regulatory mechanisms of *Aeromonas* species. Consistent with previous findings in *A. hydrophila* isolates, the virulence phenotype is the result of a molecular symphony from the cumulative effect of multiple contributing virulence factors (24). However, some virulence traits have been identified in specific *Aeromonas* species. *A. hydrophila* often carries *aerA*, *hlyA*, and *alt* (25, 26). Since previous studies did not distinguish *A. dhakensis* from *A. hydrophila* by the molecular typing method, it is possible that *A. dhakensis* may be misidentified as *A. hydrophila*. Our work showed that *aerA* and *hlyA* were distributed mostly in *A. hydrophila* and *A. dhakensis* isolates. *A. caviae* infrequently carries these genes (7, 25, 27). Aerolysin has been considered a potential virulence factor in *A. caviae* (28) but was not detected in our *A. caviae* isolates. *A. salmonicida*, an *Aeromonas* species prevalent in fish and seafood samples (1, 7, 8) but rare in clinical samples, has been reported to carry pathogenic genes, such as *act*, *alt*, and *hlyA* (8). A significant proportion of *A. veronii* isolates (84%, 37/44) carried *act*, and the *act*-positive isolates exhibited virulence phenotypes in the LT and cytotoxicity assays. Taken together, our findings indicate that the absence of *aerA*, *hly*, or *act* may be associated with the low virulence of *A. caviae* compared with that of other pathogenic *Aeromonas* species.

TABLE 6 Association between susceptibility to ertapenem or the 3rd-generation cephalosporin cefotaxime and the presence of bla_{CphA}, bla_{AQU-1}, or bla_{MOX3} in *Aeromonas dhakensis*, *Aeromonas hydrophila*, *Aeromonas veronii*, and *Aeromonas caviae* isolates

Susceptibility	Ertapenem			Cefotaxime					
	bla_{CphA}			bla_{AQU-1}			bla_{MOX3}		
	No. (%) of isolates			No. (%) of isolates			No. (%) of isolates		
	Positive ($n = 83$)	Negative ($n = 58$)	*P* value	Positive ($n = 20$)	Negative ($n = 141$)	*P* value	Positive ($n = 59$)	Negative ($n = 82$)	*P* value
Nonsusceptible	4 (4.8)	1 (1.7)	0.65	2 (10)	0	0.019	4 (6.8)	3 (3.7)	0.40
Susceptible	79 (95.2)	57 (98.3)		18 (90)	121 (85.8)		55 (93.2)	79 (96.3)	

Variations in antimicrobial susceptibility among clinical and fish *Aeromonas* isolates suggest the presence of different clones or the existence of selective pressure from antibiotics in the aquatic environment or clinical practice. Previous studies have shown that the genes encoding MBLs and AmpC β-lactamases are widely distributed among clinical *Aeromonas* species (29, 30) and that their corresponding resistance phenotypes did not manifest unless their expression was induced under specific circumstances (31–33). Genetic modifications, such as an extra insertion sequence in bla_{CphA}, may alter the expression of CphA (33). The emergence of resistance to cefotaxime was reported in a patient with *A. hydrophila* infection treated with cefotaxime (34), and imipenem therapy can induce carbapenem resistance in patients with severe *A. hydrophila* and *A. veronii* infections (35, 36). These observations emphasize that carbapenems should be used with caution for *Aeromonas* infections unless severe infections due to non-bla_{CphA}-carrying *A. caviae* are verified. Similarly, cefepime, a 4th-generation cephalosporin, should be considered for invasive infections caused by *Aeromonas* species expressing the AmpC β-lactamase, e.g., *A. hydrophila* and *A. caviae* (17).

In conclusion, multiple *Aeromonas* species were isolated from fish intended for human consumption in Tainan City. Fish isolates carry certain combinations of genes encoding putative virulence factors and β-lactam resistance that are also present in clinical isolates.

MATERIALS AND METHODS

Fish and clinical sample collection, storage, and preparation. Apparently healthy fish were purchased from traditional markets, supermarkets, and sushi and seafood shops in Tainan City between 1 and 30 June 2011. Each fish sample was individually packed in a clean polyethylene bag and transferred in a cooler to the laboratory for bacterial culture. Culture samples were collected from fish gills or surfaces using sterile swabs; plated on a selective *Aeromonas* selective medium, LabM 167 (Lab M Ltd., Lancashire, UK), as described previously (37); and cultivated at 37°C for 24 h.

Clinical *Aeromonas* isolates were obtained between January and December 2011 from the microbiological laboratory at National Cheng Kung University Hospital, a medical center in southern Taiwan, and stored at −70°C. *Aeromonas* isolates were identified by a positive oxidase test, D-glucose fermentation, a motility test, the absence of growth in 6.5% sodium chloride, resistance to the vibriostatic agent O/129 (150 μg), and identification by the Vitek GNI Plus system (bioMérieux, Marcy l'Etoile, France). The final species identification was determined based on the partial sequence of *rpoD* (38).

Antimicrobial susceptibility tests. The antimicrobial susceptibility of each *Aeromonas* isolate was measured on Mueller-Hinton agar (CMP, Creative Media Products, Ltd., New Taipei City, Taiwan) using the disc diffusion method (Becton, Dickinson Microbiology Systems, Sparks, MD, USA), and the interpretative criteria followed the Clinical and Laboratory Standards Institute (CLSI) recommendations for *Aeromonas* species (CLSI M45-A2, 2010) (39). The antimicrobial agents tested included ampicillin, ampicillin-sulbactam, piperacillin, piperacillin-tazobactam, cefazolin, cefuroxime, cefotaxime, ceftazidime, cefepime, aztreonam, ertapenem, imipenem, levofloxacin, gentamicin, amikacin, co-trimoxazole, doxycycline, and tetracycline.

PCR of virulence and drug resistance genes. The studied isolates were screened for the drug resistance genes reported in *Aeromonas* species, including those encoding ESBLs (CTX-M and PER-3), AmpC β-lactamases (AQU-1 and MOX-3), and MBL (CphA), by PCR. It has been reported that *Aeromonas* species can secrete a broad range of exotoxins and exoenzymes, which are responsible for clinical infections in humans (40). In the present study, we studied the genes encoding exotoxins or secretion system components and extracellular enzymes, including the genes for aerolysin (*aerA*), hemolysin (*hlyA*), type III secretion system components (*ascV* and *ascF-ascG*), ADP-ribosyltransferase toxin (*aexT*), heat-labile enterotoxin (*act*), heat-stable cytotoxin (*ast*), heat-labile cytotoxin (*alt*), lipase (*lip*), elastase (*ela*), and collagenase (*col*). All primers used for the detection of virulence and antimicrobial resistance genes and the associated references are summarized in Table S1 in the supplemental material (20, 41, 42).

Hemolysis and exoprotease assays. The degree of beta-hemolysis was assessed on Luria-Bertani (LB) agar containing 5% (vol/vol) sheep blood agar (Difco Laboratories, Detroit, MI, USA). Qualitative assays of exoprotease activity were performed on LB agar containing 2% (wt/vol) skim milk (Difco Laboratories, Detroit, MI, USA). The presence of clear zones surrounding the streaks indicated positive reactions in exoprotease and hemolytic tests (23).

LT assay in *Caenorhabditis elegans*. To compare the virulence of the fish isolates with the virulence of the clinical isolates, the isolates of different species with major genotypes according to the presence of virulence genes, including *hlyA lip alt col ela* (2 fish and 2 clinical *A. dhakensis* isolates), *lip col ela* (2 fish and 2 clinical *A. caviae* isolates), *act* (2 fish and 2 clinical *A. veronii* isolates), *hlyA lip ascF-ascG alt col ela* (2 fish *A. hydrophila* isolates), *hlyA lip alt col ela* (2 fish *A. hydrophila* isolates), *act hlyA aerA lip ascF-ascG alt ast col ela* (2 clinical *A. hydrophila* isolates), and *hlyA lip ascF-ascG alt ast col ela* (2 clinical *A. hydrophila* isolates), were selected for the *C. elegans* liquid toxicity (LT) assay. The detailed procedures for the LT assay were described previously (43). The survival rates of the worms were determined by dividing the number of live worms by the total number of worms after infection for 24 h.

Cytotoxicity assay. Cytotoxicity assays were conducted in a mouse C2C12 fibroblast cell line (American Type Culture Collection no. CRL-1772; BCRC no. 60083) obtained from the Bioresource Collection and Research Center, Hsinchu, Taiwan, and the levels of lactate dehydrogenase (LDH) release were measured (23). Cells were cultured in complete medium, consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), and incubated at 37°C in the presence of 5% CO_2. *Aeromonas* isolates were grown in 2 ml of LB medium for 16 h, and 50 μl of the bacterial solution was transferred to 5 ml of LB medium and cultivated for another 3 h at 37°C. C2C12 myoblast cells were separated by centrifugation and seeded into 12-well plates (5×10^4 cells/well). The cells were incubated with the bacterial cultures at a multiplicity of infection (MOI) of 20. After incubation at 37°C for 2 h, the culture medium was examined for LDH levels by use of a CytoTox 96 kit (Promega, Madison, WI). A group treated with lysis solution (Promega, Madison, WI) was used as the positive control, and an untreated group was used as the negative control. The cytotoxic activity was expressed as the percentage of the mean of triplicate measurements of the released LDH level induced by *Aeromonas* isolates compared with that induced by lysis solution (defined as 100% cytotoxicity).

Statistical analysis. Statistical analysis was performed to compare the differences in the variables between different *Aeromonas* isolates with the Statistical Package for the Social Sciences (version 21.0; SPSS, Chicago, IL, USA). Categorical variables were compared by the chi-square test or Fisher's exact test. Cytotoxicity was compared by one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) *post hoc* test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01360-19.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

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