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ORIGINAL ARTICLE

Aeromonas stool isolates from individuals with or without diarrhea in southern Taiwan: Predominance of *Aeromonas veronii*

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Background: Although aeromonads are important pathogens causing invasive infections in southern Taiwan, *Aeromonas*-associated intestinal infections have been rarely mentioned.
Purpose: The aim of this study was to understand the frequency of isolation and clinical significance of aeromonads recovered from adult stool samples in southern Taiwan.
Methods: During a 15-month study period, 514 adults with diarrhea and 167 asymptomatic controls were prospectively screened for the presence of aeromonads in stools. The identity of *Aeromonas* species was determined by the *rpoD* sequencing. Clinical information was retrieved from medical records, and *in vitro* cytotoxicity assay and polymerase chain reaction detection of putative virulent genes were performed.

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Results: Thirteen (2.5 %) of 514 diarrheal patients and six (3.6%) of 167 asymptomatic controls had *Aeromonas* isolates in their stools. Of 11 diarrheal patients with available clinical information, *Aeromonas veronii*, the predominant species, was noted in six patients, and another potential enteropathogen was present in four patients. The cytotoxicity of *A. veronii* isolates to the HT-29 cell line was more potent in the isolates from diarrheal patients than those from asymptomatic controls ($p = 0.015$). The cytotoxicity of *A. veronii* isolates was more potent than that of *A. caviae* from symptomatic patients ($p = 0.001$). Putative virulence markers, including AHCYTONE, ascV, ascF-ascG, and aexT, were detected exclusively in *A. veronii*. The presence of the *ascV* gene was associated with cytotoxicity in *A. veronii* isolates. All *Aeromonas* isolates were susceptible to varied antimicrobial agents, except ampicillin/sulbactam.
Conclusion: A. veronii is the predominant species in stools from individuals with or without diarrhea in southern Taiwan.

Introduction

Aeromonas species are important endemic pathogens in southern Taiwan, in which a variety of human *Aeromonas* infections, such as bacteremia, biliary tract infection, soft tissue infection, and pneumonia, have been reported.[1–4] In contrast, *Aeromonas*-associated intestinal infections in this area were rarely mentioned. Although *Aeromonas*-associated intestinal infections have been reported and discussed in the literature,[5–8] there are still several controversies about its role as an enteropathogen. The arguments about aeromonads as a true enteropathogen, summarized in a review by von Graevenitz,[9] come from several lines of evidence: (1) failure to identify a single clonally-related outbreak of diarrhea caused by these pathogens, even though they are ubiquitous in environments; (2) the lack of proven experimental pathogenicity for humans; and (3) frequently a self-limited clinical course of *Aeromonas*-associated diarrhea. However, with the advance of molecular techniques, more *Aeromonas* species are considered the causes of diarrhea, and the number of enterotoxins responsible for enteritis has been identified increasingly.[10] At least infraspecific subsets of *Aeromonas* strains with a particular array of enterotoxin genes are suggested to be potential enteropathogens.[9]

Several virulence factors have been considered to play an important role in causing gastrointestinal infections, such as type IV pili,[11] heat-labile enterotoxins (Act and Alt), heat-stable enterotoxin (Ast), a variety of hemolysins (AerA, HlyA, Ahh1, and Asa1), and type III secretion system (TTSS).[10–14] Some clinical studies have found that the cytotoxin was more frequently present in *Aeromonas* isolates from diarrheal patients than in the carriers.[15] However, controversy remains because some reports showed contradictory results.[16]

In Taiwan, diarrheal patients are often screened by clinical microbiology laboratories for several bacterial pathogens, such as *Campylobacter*, *Shigella*, or *Salmonella* species, but not for *Aeromonas* species. Therefore, our study aim was to study the frequency of isolation and clinical significance of aeromonads in fecal specimens, as well as their antimicrobial susceptibility, cytotoxicity, and virulence factors among fecal *Aeromonas* isolates obtained from diarrheal and asymptomatic patients.

Materials and methods

Cultivation and identification of *Aeromonas* isolates

This prospective study was conducted at the National Cheng Kung University Hospital, a medical center in southern Taiwan between September 2010 and December 2011. The stool samples from symptomatic patients for microbiological cultures of *Salmonella*, *Shigella*, or *Vibrio* species, were screened for *Aeromonas* species using the *Aeromonas* Selective Medium LabM 167 (Lab M; Lab M Ltd, Lancashire, UK) in the microbiological laboratory. Patients undergoing health examinations in the study hospital were recruited for participation (as the control group), and their stool samples were screened for aeromonads using the method described above.

The genus *Aeromonas* was identified as previously described.[17] *Aeromonas* isolates were stored at −70°C until use. Final species identification was made through the partial sequences of *rpoD*.[18] The reference strains for *rpoD* sequencing (GenBank accession no.) included *Aeromonas dhakensis* (*Aeromonas aquariorum* MDC47, FJ936132.1), *Aeromonas veronii* ATCC 9071^T (FN773340.1), *Aeromonas caviae* ATCC 13136^T (FN773319.1), and *Aeromonas sanarellii* A2-67^T (FJ472929.1).

Antimicrobial susceptibility

The performance procedures for antimicrobial susceptibility by the disk diffusion method and the interpretative criteria were interpreted following the Clinical and Laboratory Standards Institute recommendations for *Aeromonas* species.[19] The antimicrobial agents tested included amoxicillin/sulbactam, cefuroxime, ceftriaxone, cefepime, levofloxacin, and imipenem.

Detection of putative virulence factors

All isolates were studied by polymerase chain reaction to identify the genes encoding putative virulence factors: cytolytic enterotoxin (AHCYTOEN), aerolysin (aerA), hemolysin (hlyA), heat-labile enterotoxin (alt), heat-stable enterotoxin (ast), and three components of the TTSS—ascV, aexT, and ascF-ascG—as described elsewhere.[20]

Cytotoxicity of *Aeromonas* isolates

Fecal *Aeromonas* isolates were tested for cytotoxicity to the human colon carcinoma cell line, HT-29, according to a previously described method.[10] A 0.1% Triton X-100 solution was used as a positive control and serum-free RPMI (Roswell Park Memorial Institute) medium (GIBCO, Grand Island, NY, USA) as a negative control. The cytotoxic activity was expressed as the mean of triplicate measurements of released leukocyte lactate dehydrogenase levels, compared with that of Triton X-100 solution (defined as 100% of cytotoxicity).

Patient information

Symptomatic patients with *Aeromonas* species in their stools were investigated for their clinical presentations, if informed consent was obtained. The information reviewed included demographic data, food and occupation exposure history, and clinical presentations. The study was ethically approved by the Institutional Review Board of the study hospital (IRB no. ER-99-086).

Statistical analysis

Categorical variables were compared using the Chi-square test or Fisher's exact test, if the expected counts were less than 5. The median cytotoxicity was compared using the Mann—Whitney test and plotted using GraphPad Prism, version 5.01 (GraphPad Software Inc., La Jolla, CA, USA). The Cochran—Armitage trending statistic test was performed to assess the relationship between the incidence of *Aeromonas*-associated diarrhea and age.

Results

During the study period, stool samples were collected from 514 adults (≥18 years old) with diarrhea in the study hospital. In addition, 167 stool specimens were obtained from individuals undergoing health examinations during the study period. Of 514 patients with diarrhea, 13 (2.5%) had *Aeromonas* isolates in unformed stools. In contrast, 6 (3.6%) of 167 asymptomatic persons had *Aeromonas* in their stools. A total of 19 *Aeromonas* stool isolates were identified as *A. veronii* (10, 52.6%), *A. caviae* (7, 36.8%), *A. sanarelli* (1, 5.3%), and *A. dhakensis* (1, 5.3%), based on the matched *rpoD* sequences. The distribution of *Aeromonas* species in patients with and without diarrhea was similar ($p = 0.72$). The *Aeromonas* isolation rates among stool samples in different age stratification are given in Fig. 1. Our data showed a linear trend of the proportion of *Aeromonas* species recovered from individuals with diarrhea increasing with age ($p = 0.07$). Ten of 13 diarrheal episodes occurred during the summer season (April—September) in southern Taiwan.

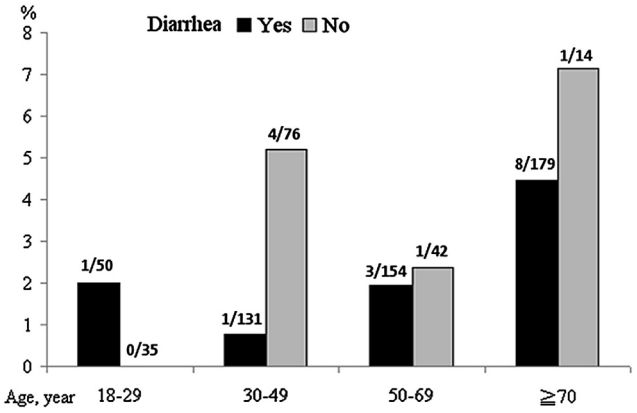

Figure 1. Isolation rates of *Aeromonas* species in the stools from individuals with and without diarrhea in different age groups. The *p* value of the trend test for diarrhea (■) and no diarrhea (□) is 0.07 and 0.41, respectively.

Informed consent was obtained from 11 of 13 symptomatic patients, and their clinical features are summarized in Table 1. In addition to diarrhea, their clinical presentations included abdominal cramping pain (3 patients), nausea/vomiting (1 patient), dysentery (1 patient), and fever (1 patient). Copathogens, including *Clostridium difficile* [2 patients with fecal growth of *C. difficile* and 1patient with fecal *C. difficile* toxin as revealed by: PREMIER ™ TOXINS A&B (Meridian Bioscience, Inc., Cincinnati, Ohio, USA), and *Vibrio parahaemolyticus* (1 patient), were noted. Eleven *Aeromonas* isolates were identified as *A. veronii* (6 patients), *A. caviae* (3 patients), *A. dhakensis* (1 patient), and *A. sanarellii* (1 patient). Their median age was 73 (range, 35—87) years. The underlying diseases of 11 patients included liver cirrhosis (2 patients), malignancy (2 patients), diabetes mellitus (2 patients), and Crohn's disease (1 patient). One patient had a history of eating lettuce with salad prior to the illness. Nine of 11 patients were admitted for medical care, and eight were treated by antibiotics. Although diarrhea lasting for more than 1 month was noted in two patients, enteritis symptoms resolved within 2 weeks in eight patients. Of note, a patient from whose stool *A. veronii* and *C. difficile* toxins were detected, developed severe colitis and hypotension requiring vasopressor support.

The identified virulence genes, including—*AHCYTOEN*, *ascF-ascG*, *ascV*, *aexT*, and *hlyA*—in *Aeromonas* stool isolates from patients with diarrhea are summarized in Table 1. Three genes, *aerA*, *ast*, and *alt*, were not detected. Of note, in five clinical *A. veronii* isolates, some putative virulence markers, including *AHCYTOEN* (4 isolates), *ascF-ascG* (3 isolates), *ascV* (3 isolates), *aexT* (1 isolate), and *hlyA* (1 isolate), were identified. All 19 isolates were susceptible to cefuroxime, ceftriaxone, cefepime, levofloxacin, and imipenem (Table 2). In contrast, the majority (94.7%) of isolates were not susceptible to ampicillin/sulbactam.

The cytotoxicity of *Aeromonas* isolates from the individuals with and without diarrhea, as assessed in the HT-

Table 1 Clinical features of 11 patients with *Aeromonas*-associated diarrhea

Characteristics	No. of patients (%)
Age (median, range), y	73, 35–87
Sex, male	8 (72.7)
Underlying disease	
Diabetes mellitus	2 (18.2)
Malignancy	2 (18.2)
Liver cirrhosis	2 (18.2)
HIV/AIDS	1 (9.1)
Crohn's disease	1 (9.1)
Treatment for diarrhea	
Admission	9 (81.8)
Antibiotic treatment	8 (72.7)
Clinical presentations	
Leukocytosis	5 (45.5)
Fever	3 (27.3)
Diarrhea >2 wk	2 (18.2)
Aeromonas species	
A. veronii	6 (54.6)
A. caviae	3 (27.3)
A. sanarellii	1 (9.1)
A. dhakensis	1 (9.1)
Virulence genes	
AHCYTOEN	4 (36.4)
ascF-ascG	4 (36.4)
ascV	3 (27.3)
aexT	1 (9.1)
hlyA	1 (9.1)
Virulent strains[a]	5 (45.5)
Concurrent enteropathogens	
Clostridium difficile[b]	3 (27.3)
Vibrio parahaemolyticus	1 (9.1)

[a] Defined as cytotoxicity > 50%, based on the released leukocyte lactate dehydrogenase level, as compared with that of Triton X-100 solution, which is defined as 100% of cytotoxicity in the HT-29 cell line.
[b] *Clostridium difficile* toxin A/B or toxigenic *C. difficile* was detected in stool.

29 cell line, is shown in Fig. 2. Five of 11 patients with diarrhea were infected with *Aeromonas* isolates with a cytotoxicity level of >50%. The median values of cytotoxicity levels for fecal *A. veronii* isolates from diarrheal patients were higher than those from asymptomatic controls (63.1% vs. 21.9%, $p = 0.015$). Nonetheless, the cytotoxicity of *A. caviae* isolates obtained from individuals with and without diarrhea was similar (10.7% vs. 7.30%, $p = 0.46$). However, cytotoxicity was more evident in fecal *A. veronii* than in *A. caviae* isolates from symptomatic patients (63.1% vs. 10.7%, $p = 0.001$). With regard to the correlation and virulence factors, the cytotoxicity was more evident in *ascV*$^+$ *A. veronii* isolates (73.0% vs. 26.2%, $p = 0.008$).

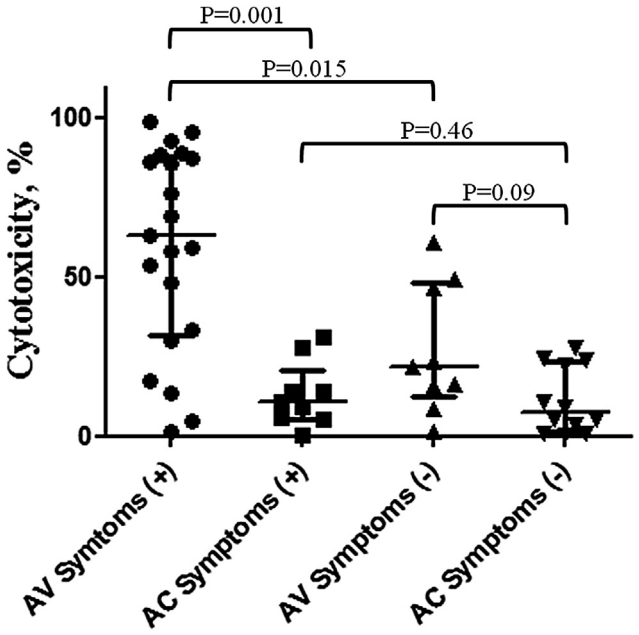

Figure 2. The medians with interquartiles of cytotoxicity for fecal *Aeromonas veronii* and *Aeromonas caviae* isolates from individuals with and without diarrhea.

Two *A. veronii* isolates from the patients with concomitant *V. parahaemolyticus* or *C. difficile* toxin in feces expressed high cytotoxicity levels (92.4% and 91.5%, respectively). In contrast, low cytotoxicity levels (15.9% and 21.7%, respectively) were present in an *A. veronii* isolate from a patient with healthcare-associated diarrhea, and an *A. caviae* isolate from a patient with AIDS and diarrhea. Although *C. difficile* was isolated from the stool samples of both patients, the toxigenic status of *C. difficile* isolates was not determined.

Table 2 *In vitro* susceptibility of *Aeromonas* stool isolates to six antimicrobial agents

Drugs	*A. veronii* ($n = 10$)			*A. caviae* ($n = 7$)			*Aeromonas* isolates ($n = 19$)
	S	I	R	S	I	R	S
Ampicillin/sulbactam	0	1 (10)	9 (90)	0	1 (14.3)	6 (85.7)	1 (5.3)
Cefuroxime	10 (100)	0	0	7 (100)	0	0	19 (100)
Ceftriaxone	10 (100)	0	0	7 (100)	0	0	19 (100)
Cefepime	10 (100)	0	0	7 (100)	0	0	19 (100)
Levofloxacin	10 (100)	0	0	7 (100)	0	0	19 (100)
Imipenem	10 (100)	0	0	7 (100)	0	0	19 (100)

I = intermediate; R = resistant; S = susceptible.

Discussion

In Taiwan, invasive *Aeromonas* infections were more often reported than *Aeromonas*-associated diarrhea. In the present study, the isolation rate in clinical stool samples from diarrheal patients was 2.5%, which is comparable with the isolation rates reported in Spain (2%), Sweden (2%), Israel (2%), Switzerland (4.8%), and Japan (5.6%),[6,21–24] and is similar to that in our asymptomatic controls (4.2%) and healthy individuals in Tokyo (3.8%, 74/1958 patients).[24] However, the isolation rates varied widely in different countries. For example, up to 52.4% of diarrheal infants and 8.7% in controls were reported in Peru.[25]

Similar to our previous observation of seasonal preference of *Aeromonas* bacteremia,[1,26] the predominance of our cases of *Aeromonas*-associated diarrhea was evident in warm seasons, which is probably related to the proliferation of aeromonads in water systems in higher ambient temperatures.[27] Although the trend test was not significant, the elderly have an increased risk of acquiring *Aeromonas*-associated diarrhea in this study. Similar findings of *Aeromonas* bacteremia have been reported.[28] Greater susceptibility to *Aeromonas* infections in the elderly may be related to concurrent chronic underlying diseases and waning host immunity.[28,29] Therefore, physicians should consider *Aeromonas* spp. as one of the possible enteropathogens causing gastrointestinal infections in the susceptible population.

The result that *A. veronii* was the predominant species in adults was not in accordance with other reports. In northern Taiwan, *Aeromonas hydrophila* has been discovered in 2.5% of 2150 diarrheal stool samples from children.[30] In a review article, *A. caviae* was referred to be the most common species, followed by *A. hydrophila* and *Aeromonas veronii* biovar *sobria*.[9] Among the etiologies of travelers' diarrhea among Finnish tourists traveling to Morocco, *A. veronii* biovar. *sobria* was the major species.[31] These varied results may be related to heterogeneous hosts, geographic locations, season of collections, and different culture media used.[5] Of note, *A. sanarellii* was isolated from a female presenting to the emergency department with diarrhea as well as urinary tract infection. She recovered after taking oral cephalexin (for 3 days), which was not *in vitro* active against *A. sanarellii*, for urinary tract infection. *A. sanarellii* has been first identified in Taiwan and associated with clinical wounds in humans.[3,32] Although further clinical evidence is required to confirm the enteropathogenicity of *A. sanarellii* in humans, this is the first report to describe the isolation of *A. sanarellii* from human stools.

Although many cases of diarrheal disease due to *Aeromonas* were mild, 82% (9/11 patients) of our cases were admitted and 73% (8/11 patients) received antibiotics, indicating severe illness in certain cases. Despite the fact that the role of antimicrobial therapy remains controversial for *Aeromonas*-associated diarrhea, most *Aeromonas* isolates were susceptible to broad-spectrum beta-lactams and fluoroquinolone. However, the clinical use of cephalothin, ampicillin, or ampicillin/sulbactam for *Aeromonas*-associated diarrhea will be discouraged because of antimicrobial resistance.[20,33]

Our study indicated that cytotoxicity levels were significantly higher in *A. veronii* isolates from diarrheal patients, in accordance with several published studies in which a significant association was found between cytotoxicity in cell lines and clinical diarrheal disease.[34,35] For example, in an Iranian study, cytotoxicity was present in 67.9% of *A. hydrophila* isolates from diarrheal patients, in contrast to 22.7% in asymptomatic persons ($p < 0.05$).[34] Moreover, the *ascV* gene encoding the TTSS has been considered an indicator of virulence in *Aeromonas*,[36,37] which was further supported by our finding that cytotoxicity was more evident in $ascV^+$ isolates than in $ascV^-$ isolates.

Although there were similar *Aeromonas* isolation rates from the stools of individuals with and without diarrhea, the microbiological characteristic suggests that *A. veronii* is a potent enteropathogen. Cytotoxicity was present in clinical *A. veronii* isolates, which carry an array of genes encoding virulence factors. Of note, a significant proportion of *A. veronii* isolates carried genes encoding a cytotoxin, AHCYTONE, and the components of TTSS (*ascV* and *ascF-ascG*). Moreover, the variable cytotoxicity among *A. veronii* isolates indicates that the colonization of nonpathogenic or low-level pathogenic *Aeromonas* isolates in the intestinal tracts of humans is possible. In contrast, the pathologic role of *A. caviae* in enteritis, at least in our study, is equivocal owing to the absence of known virulence factors and cytotoxicity in clinical isolates.

Gastroenteritis due to coinfections with *Aeromonas* and other enteropathogens have been rarely reported. A Spanish study of the role of *Aeromonas* species in travelers' diarrhea found that three (16.7%) of 18 patients with *Aeromonas* infections had other enteropathogens, such as *Shigella sonnei*, *Giardia lamblia*, or *Salmonella typhimurium*.[6] Moreover, four (36.4%) of our 11 patients with *Aeromonas*-associated diarrhea had another enteropathogen, either *C. difficile* or *V. parahaemolyticus*, in their stools. However, the enteropathogenicity or toxigenic status of the two *C. difficile* isolates was not confirmed. These results of the presence of concurrent enteropathogens and similar isolation rates of *Aeromonas* species from stool samples of individuals with and without diarrhea, not surprisingly, raise some concern regarding the enteropathogenicity of *Aeromonas* species in humans, a point of controversy raised by von Graevenitz.[9] However, many experts still include *Aeromonas* species in the list of enteropathogens of travelers' diarrhea,[38] acute dysentery,[39] or chronic diarrhea.[40]

In conclusion, *A. veronii* is the predominant species among fecal *Aeromonas* isolates, and its cytotoxicity was more evident in the isolates from diarrheal patients than from asymptomatic controls.

Conflicts of interests

The authors report no conflicts of interests.

Acknowledgments

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