Virulence Diversity among Bacteremic *Aeromonas* Isolates: *Ex Vivo*, Animal, and Clinical Evidences

Po-Lin Chen1,3, Chi-Jung Wu3,7, Pei-Jane Tsai4,8, Hung-Jen Tang9,12, Yin-Ching Chuang9,10,11, Nan-Yao Lee1, Ching-Chi Lee1, Chia-Wen Li1, Ming-Chi Li1, Chun-Chun Ou1, Chang-Shi Chen5, Wen-Chien Ko1,6.

1Department of Internal Medicine, National Cheng Kung University Hospital, Tainan, Taiwan, 2Department of Pathology, National Cheng Kung University Hospital, Tainan, Taiwan, 3Graduate Institute of Clinical Medicine, National Cheng Kung University College of Medicine, Tainan, Taiwan, 4Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University College of Medicine, Tainan, Taiwan, 5Department of Biochemistry and Molecular Biology, National Cheng Kung University College of Medicine, Tainan, Taiwan, 6Department of Medicine, National Cheng Kung University College of Medicine, Tainan, Taiwan, 7National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Taipei, Taiwan, 8Research Center of Infectious Disease and Signaling, National Cheng Kung University, Tainan, Taiwan, 9Department of Medicine, Chi Mei Medical Center, Tainan, Taiwan, 10Department of Medical Research, Chi Mei Medical Center, Tainan, Taiwan, 11Department of Clinical Pathology, Chi Mei Medical Center, Tainan, Taiwan, 12Department of Health and Nutrition, Chia Nan University of Pharmacy and Science, Tainan, Taiwan

Abstract

**Background:** The objective of this study was to compare virulence among different *Aeromonas* species causing bloodstream infections.

**Methodology/Principal Findings:** Nine of four species of *Aeromonas* blood isolates, including *A. dhakensis*, *A. hydrophila*, *A. veronii* and *A. caviae* were randomly selected for analysis. The species was identified by the DNA sequence matching of *rpoD*. Clinically, the patients with *A. dhakensis* bacteremia had a higher sepsis-related mortality rate than those with other species (37.5% vs. 0%, *P* = 0.028). Virulence of different *Aeromonas* species were tested in *C. elegans*, mouse fibroblast C2C12 cell line and BALB/c mice models. *C. elegans* fed with *A. dhakensis* and *A. caviae* had the lowest and highest survival rates compared with other species, respectively (all *P* values <0.0001). *A. dhakensis* isolates also exhibited more cytotoxicity in C2C12 cell line (all *P* values <0.0001). Fourteen-day survival rate of mice intramuscularly inoculated with *A. dhakensis* was lower than that of other species (all *P* values <0.0001). Hemolytic activity and several virulence factor genes were rarely detected in the *A. caviae* isolates.

**Conclusions/Significance:** Clinical data, *ex vivo* experiments, and animal studies suggest there is virulence variation among clinically important *Aeromonas* species.

Introduction

*Aeromonads*, belonging to the genus *Aeromonas*, are gram-negative rods, which can proliferate in aquatic environments and soils. They are important endemic pathogens in southern Taiwan as well as other areas worldwide [1,2], and have been implicated in a variety of human infectious diseases, including gastroenteritis, wound infections, septicemia, respiratory infections, hepatobiliary infections, and urinary tract infections [3]. Most human diseases were reported to be associated with three species *A. hydrophila*, *A. veronii*, and *A. caviae* [4–7]. The reported mortality rate among patients with *Aeromonas* bacteremia varies from 24% to 63% [5]. Of note, higher case fatality rates were noted in patients with *A. hydrophila* and *A. veronii* bacteremia in the literature, ranging from 33% to 56% [5,8,9]. Nevertheless, clinical infections due to *A. dhakensis* were rarely described in the literature for several reasons. First, *A. dhakensis*, previously named *A. aquariorum* or *A. hydrophila sub. dhakensis*, was often recognized as *A. hydrophila* by the current phenotype-based identification system. Second, correct identification of *A. dhakensis* needs specific molecular methods, such as *rpoD* or *gyrB* sequencing [10–12]. Therefore, the importance attributed to *A. dhakensis* in human infections should be re-evaluated due to the changing taxonomy. Morinaga et al. had reported that *A. dhakensis* could carry an array of virulence factors and exhibit the most potent toxicity to...
human blood cell lines among the tested *Aeromonas* species [11]. Our previous study also demonstrated that *A. dhakensis* isolates are more toxic to human normal skin cell lines than *A. hydrophila* isolates [12]. However, comparative studies of clinical presentations among *Aeromonas* species, including *A. dhakensis*, are not reported. Thus, our aim was to analyze the clinical presentations of bloodstream infections due to common *Aeromonas* species as well as their virulence in animal models of *Caenorhabditis elegans* and mice.

**Materials and Methods**

**Bacterial isolates**

The study isolates were selected from stored *Aeromonas* blood isolates between January of 2004 and April of 2011 at National Cheng Kung University Hospital, a medical center in southern Taiwan. The phenotype of species was determined by the Vitek 2 GN (bioMérieux, Inc., Durham, NC, USA) and/or API 20E (BioMérieux Marcy-l’Etoile, France) identification cards and biochemical tests. Species identification of each *Aeromonas* isolate was determined based on the partial sequences of *rpoD* as described before [13]. The GenBank accession numbers of the *rpoD* sequences for *Aeromonas* isolates are listed in the Table S1 in File S1. All *Aeromonas* isolates were stored at −70°C until use.

Nine isolates of each common *Aeromonas* species, including *A. dhakensis*, *A. hydrophila*, *A. veronii*, and *A. caviae*, were randomly selected. The reference strains for *rpoD* sequencing (GenBank accession no.) included *A. hydrophila* subsp. *dhakensis* CECT 5744 (EF465510.1), *A. hydrophila* ATCC 7966 (AY127856.1), *A. veronii* CECT 4246 (AY987685.1), and *A. caviae* CECT 838 (AY169337). Clinical details of these 36 patients were obtained from medical charts. The study was ethically approved by The Institutional Review Board of National Cheng Kung University Hospital (IRB no. B-ER-101-031) and the requirement for informed consent was waived.

**Definitions**

The medical records of the selected patients were reviewed retrospectively. The sites of infection were determined on the basis of clinical findings or bacterial culture results [9]. Acute cholangitis was diagnosed by the presence of clinical signs of right upper quadrant pain, fever, and jaundice, in addition to *Aeromonas* growth in the bile, which was collected by percutaneous transhepatic cholangiodrainage [14]. Catheter-related bloodstream infection was defined as a positive semi-quantitative tip culture (≥15 colony-forming units), bacteremia, and/or high clinical suspicion [14]. Diagnosis of spontaneous bacterial peritonitis was based on the presence of a polymorphonuclear leukocyte count of ≥250/mm³ in ascitic fluid, which was collected by diagnostic paracentesis, and the exclusion of secondary peritonitis [15]. Those without apparent infection sites were defined as the cases of primary bacteremia.

Sepsis-related mortality was the death of a patient with a clinical course suggestive of persistently active infection without an obvious explanation [16] and death due to any cause during hospitalization was referred to as in-hospital mortality. The severity of bacteremia when first presented at our hospital was graded by the Pittsburgh bacteremia score, which was based on the evaluation of mental status, body temperature, blood pressure, need for mechanical ventilation and presence or absence of cardiac arrest, and critical illness was defined as a score of at least 4 points [17]. Empirical antimicrobial therapy was considered to be appropriate, if the etiological pathogen was susceptible in vitro to at least one of the drugs administered within 3 days after the onset of bacteremia [18].

**Liquid-toxic (LT) assay of *C. elegans* infected by aeromonads**

The virulence of 36 blood isolates of four *Aeromonas* species were tested by the LT assay of *C. elegans*. The detailed procedures for LT assays were described elsewhere [12]. In brief, the survival rate of worms in LT assay was determined by counting the number of live worms out of the total number of worms under a dissecting scope. The mean survival rates of *C. elegans* from day 1 to day 3 were determined for four *Aeromonas* species. LT assay procedures are detailed in the File S1.

**Cytotoxicity assay**

Cytotoxicity assays were performed 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. The cells were cultured in a complete medium consisting of Dulbecco’s Modified Eagle’s medium (DMEM, Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). All cells were incubated in 10-cm tissue culture dishes (BD Falcon, San Jose, CA, USA) at 37°C and 5% CO2. They were ready for use after cultivation for at least 2–3 days. The *Aeromonas* isolates were grown in 1 ml of LB medium for 3 hours, and 0.5 ml of the bacterial solution was transferred to 15 ml disposable tubes and cultivated for another 17 hours at 37°C. The C2C12 fibroblast cells were separated by centrifugation and seeded into 96-well plates (1×10⁵ cells/well). The cells were incubated with bacterial cultures at a multiplicity of infection (MOI) of 25. After incubation at 37°C for 2 hours, the culture medium was examined for the release of lactate dehydrogenase (LDH) by a CytoTox 96 kit (Promega, Madison, WI). A 0.1% Triton X-100 solution was used as a positive control, and serum-free Roswell Park Memorial Institute (RPMI) medium (GIBCO, Grand Island, N.Y., USA) was used as a negative control. The cytotoxicity activity was expressed as the mean of triplicate measurements of released LDH levels compared with Triton X-100 exposure (defined as 100% cytotoxicity).

**Life span and pathology of BALB/c mice with *Aeromonas* intramuscular infection**

Three clinical blood isolates of each species, i.e. *A. dhakensis*: A2-061, A2-094, A2-107; *A. hydrophila*: A2-011, A2-066, A2-078; *A. caviae*: A2-9307121, A2-961204, A2-9310251; *A. veronii*: A2-007, A2-029, A2-041, were randomly selected for the mouse study. All the isolates of the same species were genetically different as evidenced by the gel profiles of arbitrarily primed polymerase chain reaction (AP-PCR) methods [19] (data not shown). Six to ten week-old female BALB/c mice weighing 18–22 grams were obtained from National Laboratory Animal Center. Animals were housed in a pathogen-free environment using 12 h alternating periods of light and dark until the initiation of experiments. Each mouse was injected intramuscularly at the right thigh with 100 IL containing 2.5×10⁶ colony forming units (CFU) of *Aeromonas* isolates. Seven mice were tested for each isolate. At 24 h after injection, one mouse was sacrificed for pathological examination. The infected soft tissues were dissected and fixed in 10% v/v neutral-buffered formalin, and then stained with hematoxylin and eosin for light microscopy.

The severity of soft tissue damage was evaluated according to the extent of inflammatory cell infiltration, edema, or myonecrosis.
based on a semi-quantitative score designed for evaluating peripheral compartment syndrome [20]. In brief, five high-powered (100x) representative fields were scored by a blinded pathologist based on the following criteria, including items of inflammatory cell infiltrate (1, inflammatory cell penetration into <10% of muscle parenchyma; 2, 11%–50% of parenchyma; 3, >50% of parenchyma; edema (1, edema visible in <10% of muscle parenchyma; 2, 11%–50% of parenchyma; 3, >50% of muscle parenchyma; and myonecrosis (1, abnormal muscle fibers in <10%; 2, 11%–50%; 3, >50% of views), with a sum score range of 3 to 9. The survival of six mice was monitored daily for 14 days.

All the animal experiments in this study were carried out in strict accordance with the recommendations in the Guidelines for Committee of Laboratory Care and Use, developed by the National Cheng Kung University. The protocol was ethically approved by the Institutional Animal Care and Use Committees and the National Cheng Kung University (Permit No, 101050). Experiments were planned and conducted with environmental enrichment, veterinary oversight and the use of appropriate analgesics and anesthesia when needed. All animals were monitored daily by trained personnel. The frequency of monitoring was increased when animals developed or if they were anticipated to develop clinical signs of severe sepsis. In the study, the animals were humanely sacrificed when they met either the criteria of hypothermia <34 °C or >20% body weight loss. Monitoring body temperature was accomplished using laser directed infrared temperature scanners. Intramuscular inoculation with pathogens was performed under anesthesia by inhalation of 2% isoflurane with 1.5L/min oxygen, and all efforts were made to minimize suffering. Euthanasia at the completion of experiments was carried out by exsanguination, under deep anesthesia with inhalation of 3% isoflurane with 1.5L/min oxygen.

Exoenzyme assay

Qualitative assays of exoprotease activity were performed on LB agar containing 2% (wt/vol) skimmed milk (Difco Laboratories, Detroit, MI, USA). Hemolytic activity was assayed on LB agar containing 5% (vol/vol) sheep blood, amylase activity on starch agar (Difco Laboratories, Detroit, MI, USA), and nuclease activity on DNase agar with methyl green (Difco Laboratories, Detroit, MI, USA). A single streak of undiluted organisms were inoculated on blood agar plates and incubated at 37°C for 24 hours, and on starch and DNase agar plates for 48 hours. Positive reactions for exoprotease and hemolytic tests were the presence of clear zones surrounding the streaks. Amylase activity was examined by removing growth from each streak to expose the agar plates to Gram iodine. Starch hydrolysis was indicated by a colorless zone surrounding colonies. Bacillus subtilis ATCC 6633 and Escherichia coli ATCC 25922 were positive and negative control strains for the amylase test, respectively. For the DNase test agar with methyl green, positive reactions were identified as decolorizing around the streaks. Positive and negative control strains for the DNase test were Staphylococcus aureus ATCC 29253 and Staphylococcus epidermidis ATCC 12228, respectively.

PCR detection of the genes encoding putative virulence factors

Polymerase chain reactions (PCRs) using previously described primers and conditions were conducted to detect the genes encoding heat-stable enterotoxin (ast), hemolysin (ahh1), acrosin (aerA), components of the type III secretion system (TTSS) (astV) or ADP-ribosylating toxin (aexT) [21]. A. hydrophila ATCC 7966 was used as a positive control for ahh1, aerA and ast [21,22] and A. veronii ATCC 9071 was a positive control for astV and aexT [12].

Results

Clinical features of patients with Aeromonas bacteremia were summarized in Table 1. Polymicrobial infection was more common in patients with A. veronii bacteremia (P = 0.032). Patients with A. dhakensis bacteremia tended to have liver cirrhosis (P = 0.029). The sources of Aeromonas bacteremia were identified in 36.1% (13 of 36 patients, including vascular catheter-related infections (4), spontaneous bacterial peritonitis (3), necrotizing fasciitis (2), biliary tract infections (2), pleural empyema (1), and appendicitis (1). Four (44.4%) of 9 patients with A. dhakensis bacteremia empirically received in vitro active antimicrobial agents, in contrast to 20 (74.1%) of 27 patients with non-dhakensis Aeromonas bacteremia (P = 0.12, Fisher’s exact test). The proportion of critical illness, i.e. Pittsburgh bacteremia score ≥4, was similar among the patients with bacteremia due to four Aeromonas species.

The sepsis-related and in-hospital mortality rates of patients with A. dhakensis bacteremia were significantly higher than those of bacteremia caused by non-dhakensis Aeromonas species (P = 0.024 and 0.004, respectively). Even taking monomicrobial Aeromonas bacteremia (i.e. 8 episodes of A. dhakensis bacteremia, 7 A. hydrophila, 3 A. veronii, and 6 A. caviae) into consideration, the sepsis-related (37.5% vs. 0%, P = 0.029) or in-hospital mortality rate (50% vs. 0%, P = 0.007) of A. dhakensis bacteremia remained higher than that of monomicrobial bacteremia due to non-dhakensis Aeromonas species. Furthermore, the patients with A. dhakensis bacteremia and appropriate empirical therapy fared worse than those with non-dhakensis Aeromonas bacteremia and appropriate empirical therapy (14-day sepsis-related mortality rate: 2/4, 50% vs. 0/20, 0%; P = 0.02). Among three fatal patients with A. dhakensis bacteremia, each had severe underlying disease (i.e. liver cirrhosis in two patients and end-stage renal disease 1). Two had received appropriate empirical antibiotic therapy, but expired within 7 days after the onset of bacteremia.

Cytotoxicity of a total of 36 Aeromonas isolates from 4 species was assessed in C2C12 mouse fibroblast cell line. The mean values ± standard errors of the released LDH levels induced by Aeromonas isolates as compared with the LDH level by 0.1% Triton X-100 (a positive control: 100%), were 38.8±8.4% by A. dhakensis; 20.5±7.1% by A. hydrophila; 26.1±7.6% by A. veronii, and, −0.39±1.5% by A. caviae (Figure 2) (one-way ANOVA test,

Statistical analysis

Statistical analyses were performed to compare the variables among the adults infected by different Aeromonas isolates. Categorical variables were compared by the Chi-square test or Fisher’s exact test, if the expected counts were less than 5. Cytotoxicity was compared by one-way analysis of variance (ANOVA) with Turkey’s HSD (Honestly Significantly Difference) post hoc test. The scores for muscle damage in BALB/c mice were compared by the Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn’s post hoc test. Mouse survivals were analyzed by the log-rank test. Data were analyzed by the software of GraphPad Prism, version 5.01 (GraphPad Software Inc. California, USA).

Statistical analyses were performed to compare the variables among the adults infected by different Aeromonas isolates. Categorical variables were compared by the Chi-square test or Fisher’s exact test, if the expected counts were less than 5. Cytotoxicity was compared by one-way analysis of variance (ANOVA) with Turkey’s HSD (Honestly Significantly Difference) post hoc test. The scores for muscle damage in BALB/c mice were compared by the Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn’s post hoc test. Mouse survivals were analyzed by the log-rank test. Data were analyzed by the software of GraphPad Prism, version 5.01 (GraphPad Software Inc. California, USA).
Post-Hoc Turkey’s HSD test demonstrated that A. dhakensis isolates exhibited more potent cytotoxicity than other species (all P values <0.05), and A. veronii isolates higher cytotoxicity than A. caviae (P, 0.05) to the C2C12 cell line. The life spans of the mice infected by different Aeromonas species intramuscularly was shown in Figure 3. After two weeks, 18 mice infected by A. dhakensis, only four (22.2%) survived. Of note, 14 mice expired within 48 hours. In contrast, 16 (88.9%) of 18 mice infected by A. hydrophila and all by A. veronii or A. caviae survived for 14 days (all P values <0.0001). Kruskal–Wallis one-way ANOVA with Dunn’s post hoc test revealed that a similar severity of muscle damage at the inoculated sites after 24 hours of infection was discerned in the mice infected by A. dhakensis, A. hydrophila, and A. veronii (see Figure S1). The severity of muscle damage induced by A. caviae infection was significantly milder than that by A. hydrophila (P<0.05). Pathological characteristics

The characteristics of the patients with septicemia caused by different Aeromonas species are shown in Table 1. There were no significant differences in age and gender among the four groups. However, the incidence of monomicrobial bacteremia was significantly higher in the A. dhakensis group than in the A. hydrophila and A. veronii groups (P=0.032). The Pittsburgh bacteremia score was significantly higher in the A. hydrophila group than in the A. dhakensis group (P=0.029). The mortality was significantly higher in the A. hydrophila group than in the A. dhakensis group (P=0.024). The mortality was significantly higher in the A. hydrophila group than in the A. dhakensis group (P=0.004).

Table 1. Clinical features of patients with septicemia caused by different Aeromonas species.

| Characteristics                     | No. (%) of patients | P values |
|-------------------------------------|---------------------|----------|
|                                     | A. dhakensis n=9    | A. hydrophila n=9 | A. veronii n=9 | A. caviae n=9 |
| Age ≥60 year-old                    | 3 (33.3)            | 7 (77.8)     | 5 (55.6)       | 5 (55.6)       | 0.308 |
| Male gender                         | 8 (88.9)            | 6 (66.7)     | 5 (55.6)       | 4 (44.4)       | 0.239 |
| Monomicrobial bacteremia            | 8 (88.9)            | 8 (88.9)     | 3 (33.3)       | 6 (66.7)       | 0.032 |
| Source of infection                 |                     |             |                |                |      |
| Primary bacteremia                  | 6 (66.7)            | 6 (66.7)     | 6 (66.7)       | 5 (55.6)       | 0.948 |
| Secondary bacteremia                | 3 (33.3)            | 3 (33.3)     | 3 (33.3)       | 4 (44.4)       |      |
| Spontaneous bacterial peritonitis   | 2                   | 1            | -              | -              |      |
| Vascular-catheter related infection | -                   | 1            | -              | 3              |      |
| Necrotizing fascitis                | 1                   | 1            | -              | -              |      |
| Others                              | -                   | -            | 3              | 1              |      |
| Underlying diseases                 |                     |             |                |                |      |
| Liver cirrhosis                     | 6 (66.7)            | 3 (33.3)     | 3 (33.3)       | 0              | 0.029 |
| Active malignant diseases           | 1 (11.1)            | 4 (44.4)     | 2 (22.2)       | 4 (44.4)       | 0.316 |
| Pittsburgh bacteremia score ≥4     | 2 (22.2)            | 2 (22.2)     | 0              | 1 (11.1)       | 0.465 |
| Appropriate empirical antibiotics   | 4 (44.4)            | 6 (66.7)     | 9 (100)        | 5 (55.6)       | 0.06  |
| Mortality                           |                     |             |                |                |      |
| Sepsis-related                      | 3 (33.3)            | 0/8*         | 0              | 0              | 0.024 |
| In-hospital                         | 5 (55.6)            | 0/8*         | 1 (11.1)       | 0              | 0.004 |

*One patient with necrotizing fascitis was transferred to another hospital.
*Biliary tract infection, pleural empyema, appendicitis.
**Biliary tract infection.
doi:10.1371/journal.pone.0111213.t001

Figure 1. Three-day survivals of Caenorhabditis elegans co-cultivated with Aeromonas isolates of different species: Aeromonas dhakensis (AD), Aeromonas hydrophila (AH), Aeromonas veronii (AV), and Aeromonas caviae (AC) in the liquid-toxic assay. ***P<0.0001, as compared with AD.

doi:10.1371/journal.pone.0111213.g001

Figure 2. Cytotoxicity of Aeromonas dhakensis (AD, n=9), A. hydrophila (AH, n=9), A. veronii (AV, n=9) and A. caviae (AC, n=9) isolates to C2C12 mouse fibroblast cell lines, which is expressed as the proportions of the released LDH levels induced by Aeromonas isolates, as compared with the LDH level by 0.1% Triton X-100 (a positive control: 100%). *P<0.05.

doi:10.1371/journal.pone.0111213.g002
of muscle tissue damage, such as fragmentation of muscle fibers, edema of myocytes, and infiltration of inflammatory cells, were rarely seen in mice with \(A.\ caviae\) infection (see Figure S2).

The results of agar plate assays for phenotypic activity of exoenzymes, including exoprotease, amylase, and DNase, were demonstrated in Table 2. The proportion of hemolytic phenotype in the \(A.\ caviae\) isolates was 44.4%, which was significantly lower than the other species (\(P=0.001\)). Of four \(Aeromonas\) species, the activity of amylase, DNase, and exoprotease was present in most of the isolates.

The genetic distribution of virulence factors among \(Aeromonas\) blood isolates was summarized in Figure 4. In all \(A.\ dhakensis\) and \(A.\ hydrophila\) isolates, \(ahh1\) was detected, and \(aerA\) in 33.3% of both \(A.\ dhakensis\) and \(A.\ hydrophila\) isolates, respectively. However, \(ahh1\) and \(aerA\) were not found in \(A.\ veronii\) and \(A.\ caviae\). Of note, \(aexT\) was only identified in \(A.\ veronii\) isolates and none of \(A.\ caviae\) isolates possessed any of five tested genes. Among bacteremic isolates of \(A.\ hydrophila\) and \(A.\ dhakensis\), \(ast\) (100% vs. 11.1%, \(P<0.0001\)) was primarily present in \(A.\ hydrophila\) isolates.

**Discussions**

In the present study, the mortality rate of the patients with \(A.\ dhakensis\) bacteremia was higher than that of bacteremia due to non-\(dhakensis\) \(Aeromonas\) species (33.3%, 3/9 vs. 0%, 0/26; \(P=0.001\)), and the difference remained significant, if only those with appropriate empirical therapy were taken into consideration (2/4, 50% vs. 0/20, 0%; \(P=0.02\)). Therefore, our results reminded the clinicians that \(A.\ dhakensis\) infections can be life-threatening in susceptible hosts, despite of early appropriate antimicrobial therapy. In addition, our work provided more \textit{in vivo}\ and \textit{in vitro} evidences of the potent virulence of \(A.\ dhakensis\), and such a finding was in accordance with the clinical findings. \(A.\ dhakensis\) has been reported to be more toxic than other species to human blood cell lines [11], and wound isolates of \(A.\ dhakensis\) had been demonstrated to be more virulent in the \textit{C. elegans} model and human normal skin fibroblast cells than wound isolates of \(A.\ hydrophila\) [12]. However, the prevalence of human infections caused by \(A.\ dhakensis\) is often underestimated due to the misidentification as \(A.\ hydrophila\) by current phenotype-based identification schemes. Several reports indicated that the isolates phenotypically identified as \(A.\ hydrophila\) were \(A.\ dhakensis\), if \(rpoD\) or \(gyrB\) was sequenced [10,11]. Thus, with the potent virulence of \(A.\ dhakensis\), it is justified to precisely differentiate \(A.\ dhakensis\) from other \(Aeromonas\) species.

In the sepsis-related mortality rate of 9 cases of \(A.\ dhakensis\) bacteremia, 33.3%, were comparable with that of \(A.\ hydrophila\) bacteremia in the literature (33–36%) [5,8,9]. However, none of the 9 cases of bacteremia in our study died of sepsis due to \(A.\ hydrophila\). As mentioned before, phenotypically identified \(A.\ hydrophila\) was actually \(A.\ dhakensis\) by molecular methods.

**Table 2.** The results of agar plate assays for exoprotease, amylase, DNase, and hemolytic activity of isolates of four \(Aeromonas\) species: \(A.\ dhakensis\), \(A.\ hydrophila\), \(A.\ veronii\) and \(A.\ caviae\).

| Isolate number (%) | A. dhakensis, n=9 | A. hydrophila, n=9 | A. veronii, n=9 | A. caviae, n=9 | \(P\) value |
|--------------------|------------------|------------------|----------------|----------------|------------|
| Exoprotease        | 9 (100)          | 9 (100)          | 9 (100)        | 7 (77.8)       | 0.096      |
| Amylase            | 9 (100)          | 9 (100)          | 8 (88.9)       | 8 (88.9)       | 0.548      |
| DNase              | 9 (100)          | 9 (100)          | 8 (88.9)       | 9 (100)        | 0.379      |
| Hemolysis*         | 9 (100)          | 9 (100)          | 9 (100)        | 4 (44.4)       | 0.001      |

*All positive isolates showed \(\beta\)-hemolysis.

doi:10.1371/journal.pone.0111213.t002
Therefore, it is not surprising that the clinical outcome of published cases of *A. hydrophila* is similar to that of *A. dhakensis*. In addition to the significant virulence of *A. dhakensis*, other clinically relevant information from the present work is the low virulence of *A. caviae*. The heterogeneous distribution of virulence genes in bacteremic *Aeromonas* isolates of four species may partially account for the virulence comparative results in animals or patients. The so-called “virulent species” in the present study, *i.e.*, *A. dhakensis*, *A. hydrophila*, or *A. veronii*, harbored an array of virulence factors, such as hemolysin (*ahh1*), aerolysin (*aexA*), cytotoxin (*ast*), type III secretion system (*ascV* and *ascF-G*) [11,23,24].

*A. veronii* and *A. caviae* bacteremia have been associated with a high mortality, for example 42% in *A. veronii* bacteremia in Taiwan [5], and 20% and 17% in *A. veronii* and *A. caviae* bacteremia, respectively, in Japan [25]. The difference of mortality rates between studies may be related to the heterogeneous study population. Of our 36 cases, only 33.3% had liver cirrhosis. In contrast, of 154 cases in another Taiwanese study, 64.3% had liver cirrhosis [5], and in a Japanese study 36.1% of 36 cases had chronic hepatic disease [25]. Moreover, in the two published studies, the accuracy of species identification based on traditional biochemical tests was questionable. Therefore, generalization of studies, the accuracy of species identification based on traditional biochemical tests was questionable. Therefore, generalization of our conclusions to other hospitals or areas should be cautious.

A correlation between the virulence and hemolytic activity of aeromonads has been proposed [22]. The production of hemolysin or aerolysin in aeromonads has been related to their pathogenic potential in hosts [26-29] and inactivation of aerolysin and hemolysin genes in *A. hydrophila* attenuates the pathogenicity in wound and systemic infection models of mice [26,27]. Hemolysin (*ahh1*) or aerolysin genes (*aexA*) were not found in *A. caviae* isolates, as reported by Osman et al. in their *Aeromonas* isolates from retail meats in Egypt [30]. In addition, the genes encoding other important virulence factors, such as cytotoxin (*ast* and *alt*) [31] or TTSS genes (*ascV* and *ascF*) [21], were rarely found in *A. caviae* isolates. These results are in accordance with the impression that *A. caviae* is less invasive in humans and animal models. Comparisons of genetic information from whole genome sequences of clinical *Aeromonas* strains may identify potential genetic traits responsible for virulence [32].

*Aeromonas* skin and soft-tissue infections often were polymicrobial infections after exposure to aquatic environments [12], in immunocompromised subjects with liver cirrhosis [33], chronic renal failure, or malignancy [6,7,34]. The precise contribution of *Aeromonas* species or host factors to the severity of skin and soft-tissue infection is difficult to estimate in the real world. Animal models with controlled environmental and host variables may be used to compare the pathogenicity between species. Several animal models have been proposed for studying *Aeromonas* infections. These models, including leech, blue gourami, zebrafish, amoebae, nematode, or mice, had distinct advantages to link the pathogenicity in human [2]. We had demonstrated that the *Caenorhabditis elegans* LT assay is a plausible model to study the virulence of aeromonads, with several experimental advantages, such as a short round time, rapid generation time, large progeny, and ease of observation [12,35].

In the mice with intramuscular infection, though *A. dhakensis*, *A. hydrophila* and *A. veronii* isolates can all cause extensive tissue damage at the initial 24 hours, *A. dhakensis* infections lead to more fatality in mice at 2 weeks. These results suggest efficient adaption of *A. dhakensis* to the host immune or more pathogenicity to mice. Such a mouse model with intramuscular infection has been used by Grim et al. to evaluate the pathogenicity of different genotypes of *A. hydrophila* [36]. Therefore, it is possible that the mouse model of intramuscular infection could be a research platform to investigate the virulence signatures of *Aeromonas* species in human infections.

Moreover, the toxicity difference among varied *Aeromonas* species, *i.e.*, invasive species like *A. dhakensis* and less invasive species as *A. caviae* in *Caenorhabditis elegans* LT assay, was in accordance to those findings in the BALB/c mouse model, which is a feasible mammalian model to investigate the pathogenicity of *Aeromonas* species in soft-tissue infections. The majority (78%) of mice intramuscularly infected by *A. dhakensis* died within 48 hours and the degree of inflammatory response in mouse muscles was less severe in *A. caviae* than other species on pathological examination. The survival outcomes in BALB/c mice with intramuscular infections are compatible to the poor prognosis in necrotizing fasciitis, myonecrosis, or severe soft-tissue infectious due to *A. dhakensis*, *A. hydrophila*, or *A. veronii* in clinical reports [6,12,34,37-39]. These findings suggest virulence variation among *Aeromonas* species.

There are several limitations in the present study. Firstly, all the isolates were collected from a medical center, and therefore the caveat is that interpretations from our results may not be generalized to other areas. For example, the prevalence of virulence genes among *A. caviae* isolates here is low. In contrast, clinical *A. caviae* stool isolates from Spain and Mexico may carry aerolysin and hemolysin genes, with a prevalence of 96.0% and 84.2%, respectively [24]. Nevertheless, all reports together suggested the geographical genetic variation not only in environmental but also in clinical aeromonads. Secondly, our case number of *Aeromonas* bacteremia is too limited to represent the clinical outcome of *Aeromonas* bacteremia due to different species. A clinical study including more cases of *Aeromonas* bacteremia is ongoing to disclose the virulence variation of *Aeromonas* species. Nevertheless, our study highlights that correct identification of *A. dhakensis* among *Aeromonas* isolates is of clinical value due to its potential virulence. Third, the possibility of underestimation of infection sources should be considered, because clinical data in this study was obtained from the retrospective review of medical charts. However, the identification rate of foci of *Aeromonas* bacteremia was 36.1%, which was comparable to those reported in two published reports in Taiwan (43.3% and 48.5%) [9,40].

In conclusion, clinical data, *ex vivo* experiments, and animal studies suggest there is virulence variation among clinically important *Aeromonas* species. More clinical investigations and laboratory work are warranted to compare the pathogenicity of *Aeromonas* species in human infections.

**Supporting Information**

**Figure S1** Pathological scores of soft-tissue damage at 24 hours following inoculation with 100 μL of Luria-Bertani solution containing 2.5×10⁶ colony forming units of four *Aeromonas* species, *i.e.*, *A. caviae*, *A. hydrophila*, *A. veronii*, and *A. dhakensis*, over right thigh of BALB/c mice. There are three isolates of each species for the test. The infected soft tissues of mice were dissected and fixed in 10%v/v neutral-buffered formalin, and then stained with haematoxylin and eosin for light microscopy. (TIF)

**Figure S2** Fragmentation (arrows) of myocytes, inflammatory cells infiltration (arrowheads), and edema (dashed arrows) of muscle parenchyma were observed in high-powered fields (100x) of hematoxylin and eosin staining of infected muscle of BALB/c mice with inoculation of 4 *Aeromonas* species for 24 hours (*A. caviae*, *B. A. hydrophila*, *C. A. veronii*, *D. A. dhakensis*). (TIF)
File SI (DOCX)

Acknowledgments

We thank Chi-I Lin for their technical assistance. We are grateful to Prof. Chung-Yi Li, Sheng-Hsiang Lin, and Jia-Lin Wu in the Research Center of Clinical Medicine, National Cheng Kung University Hospital, for providing statistical consulting services.

Author Contributions

Conceived and designed the experiments: PLC CJW. Performed the experiments: CJC. Analyzed the data: FJT HYL CCL CWL MCL. Contributed reagents/materials/analysis tools: YCC HWT CCO. Wrote the paper: PLC CSC WCK.

References

1. Wu CJ, Chen PL, Tang HJ, Chen HM, Tseng FC, et al. (2014) Incidence of Aeromonas bacteremia in southern Taiwan: Vibrio and Salinemona bacteremia as comparators. J Microbiol Immunol Infect 47: 145–148.
2. Janda JM, Abbott SL (2010) The genus Aeromonas: taxonomy, pathogenicity, and infection. Clin Microbiol Rev 23: 35–73.
3. Parker JL, Shaw JG (2011) Aeromonas infection. Clin Microbiol Rev 24: 283–304.
4. Janda JM, Abbott SL (2010) The genus Aeromonas: taxonomy, pathogenicity, and infection. Clin Microbiol Rev 23: 35–73.
5. Chao CM, Lai CC, Gau SJ, Hsueh PR (2013) Skin and soft tissue infection due to Aeromonas strains isolated from fish. J Microbiol Immunol Infect 47: 267–273.
6. Ko WC, Chuang YC (1995) Aeromonas bacteremia: review of 59 episodes. Clin Infect Dis 20: 1306–1308.
7. Ko WC, Lee HC, Chuang YC, Liu CC, Wu JJ (2000) Clinical features and therapeutic implications of 104 episodes of monomicrobial Aeromonas bacteremia. J Infect 40: 267–273.
8. Aravena-Roman M, Harnett GB, Riley TV, Inglis TJ, Chang BJ (2011) Aeromonas aquariorum is widely distributed in clinical and environmental specimens and can be misidentified as Aeromonas hydrophila. J Clin Microbiol 49: 3006–3008.
9. Morimasa Y, Yanagihara K, Esingen FL, Beaz-Hidalgo R, Kohno S, et al. (2013) Identification error of Aeromonas aquariorum: A causative agent of septicemia. Diagn Microb Infect Dis 76: 106–109.
10. Chen PL, Wu CJ, Chen CS, Tsai PJ, Tang HJ, et al. (2013) A comparative study of clinical Aeromonas hydrophila and Aeromonas hydrophila isolates in southern Taiwan: A. hydrophila is more predominant and virulent. Clin Microbiol Infect Dis: 20101111469012456. [Epub ahead of print].
11. Soder L, Yanez MA, Chacon MR, Aguilar-Arreola MG, Catalan V, et al. (2004) Phylogeny analysis of the genus Aeromonas based on two housekeeping genes. J Bacteriol 186: 1511–1519.
12. Tang HJ, Lai CC, Lin HL, Chao CM (2014) Clinical manifestations of Aeromonas strains on the basis of multilocus sequence typing, phenotype, and presence of putative virulence genes. J Microbiol Immunol Infect 47: 145–148.
13. Wang G, Clark CG, Liu P, Pucknell C, Munro CK, et al. (2003) Detection and characterization of the hemolysin genes in Aeromonas hydrophila and Aeromonas sobria by multiple PCR. J Clin Microbiol 41: 1048–1054.
14. Figueras MJ, Aperti A, Saavedra MJ, Ko WC, Gonzalez N, et al. (2009) Clinical relevance of the recently described species Aeromonas aquariorum. J Clin Microbiol 47: 3742–3746.
15. Emekdas G, Aslan G, Tezcan S, Serin MS, Yildiz C, et al. (2006) Detection of Aeromonas hydrophila toxin genes in clinical and environmental isolates of Aeromonas spp.: correlation with virulence in a sucking mouse model. FEMS Microbiol Lett 164: 195–201.
16. Heuzenroeder MW, Flowe RL (1998) Inactivation of two haemolytic toxin genes in Aeromonas hydrophila attenuates virulence in a sucking mouse model. Microbiology 144 (Pt 2): 291–296.
17. Heuzenroeder MW, Yong CJ, Flowe RL (1999) Distribution of two hemolytic toxin genes in clinical and environmental isolates of Aeromonas spp.: correlation with virulence in a sucking mouse model. FEMS Microbiol Lett 174: 131–136.
18. Santos JA, Gonzalez CJ, Otero A, Garcia-Lopez ML (1999) Hemolytic activity and siderophore production in different Aeromonas species isolated from fish. Appl Environ Microbiol 65: 5612–5614.
19. Osman K, Ayy M, Kheader A, Mahbouk K (2012) Molecular detection of the Aeromonas virulence aerolysin gene in retail meats from different animal sources in Egypt. World J Microbiol Biotechnol 28: 1063–1070.
20. Yi SW, You CJ, Cho HS, Lee CS, Kwon JK, et al. (2013) Molecular characterization of Aeromonas strains isolated from farmed eels (Anguilla japonica). Vet Microbiol 164: 195–200.
21. Wu CJ, Wang HC, Chen CS, Shu HY, Kao AW, et al. (2012) Genome sequence of a novel human pathogen, Aeromonas aquariorum. J Bacteriol 194: 4144–4153.
22. Lee CC, Chi CH, Lee NY, Lee HC, Chen CL, et al. (2008) Necrotizing fasciitis in patients with liver cirrhosis: predominance of monomicrobial Gram-negative bacillary infections. Diagn Microb Infect Dis 62: 219–225.
23. Papadakis V, Porinios N, Katsiari K, Chariotapegiou AE, Anastasopoulos J, et al. (2012) Fulminant Aeromonas hydrophila infection during acute lymphoblastic leukemia treatment. J Microbiol Immunol Infect 45: 134–137.
24. Bogaerts A, Temmerman I, Boerjan B, Husson SJ, Schoofs L, et al. (2010) A differential proteomics study of Caenorhabditis elegans infected with Aeromonas hydrophila. Dev Comp Immunol 34: 690–698.
25. Gray CJ, Koduwa BV, Poonnamay D, Fitts EC, Sha J, et al. (2014) Functional genomic characterization of virulence factors from necrotizing fasciitis-causing strains of Aeromonas hydrophila. Appl Environ Microbiol 80: 4162–4183.
26. Kelly KA, Koehler JM, Ashdown LR (1993) Spectrum of extraintestinal disease caused by Aeromonas caviae and Aeromonas sobria. J Infect 23: 74–80.
27. Lee CC, Chi CH, Lee NY, Lee HC, Chen CL, et al. (2008) Necrotizing fasciitis in patients with liver cirrhosis: predominance of monomicrobial Gram-negative bacillary infections. Diagn Microb Infect Dis 62: 219–225.
28. Papadakis V, Porinios N, Katsiari K, Chariotapegiou AE, Anastasopoulos J, et al. (2012) Fulminant Aeromonas hydrophila infection during acute lymphoblastic leukemia treatment. J Microbiol Immunol Infect 45: 134–137.
29. Bogaerts A, Temmerman I, Boerjan B, Husson SJ, Schoofs L, et al. (2010) A differential proteomics study of Caenorhabditis elegans infected with Aeromonas hydrophila. Dev Comp Immunol 34: 690–698.
30. Gray CJ, Koduwa BV, Poonnamay D, Fitts EC, Sha J, et al. (2014) Functional genomic characterization of virulence factors from necrotizing fasciitis-causing strains of Aeromonas hydrophila. Appl Environ Microbiol 80: 4162–4183.
31. Lee CC, Chi CH, Lee NY, Lee HC, Chen CL, et al. (2008) Necrotizing fasciitis in patients with liver cirrhosis: predominance of monomicrobial Gram-negative bacillary infections. Diagn Microb Infect Dis 62: 219–225.
32. Papadakis V, Porinios N, Katsiari K, Chariotapegiou AE, Anastasopoulos J, et al. (2012) Fulminant Aeromonas hydrophila infection during acute lymphoblastic leukemia treatment. J Microbiol Immunol Infect 45: 134–137.
33. Bogaerts A, Temmerman I, Boerjan B, Husson SJ, Schoofs L, et al. (2010) A differential proteomics study of Caenorhabditis elegans infected with Aeromonas hydrophila. Dev Comp Immunol 34: 690–698.