Phenotypic and Genetic Diversity of *Aeromonas* Species Isolated from Fresh Water Lakes in Malaysia

Wei Ching Khor¹, Suat Moi Puah¹, Jin Ai Mary Anne Tan¹, SD Puthucheary², Kek Heng Chua¹*

¹ Department of Biomedical Science, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, ² Department of Medical Education, Research and Evaluation, Duke-NUS Medical School Singapore, Singapore

*khchua@um.edu.my*

Abstract

Gram-negative bacilli of the genus *Aeromonas* are primarily inhabitants of the aquatic environment. Humans acquire this organism from a wide range of food and water sources as well as during aquatic recreational activities. In the present study, the diversity and distribution of *Aeromonas* species from freshwater lakes in Malaysia was investigated using glycerophospholipid-cholesterol acyltransferase (GCAT) and RNA polymerase sigma-factor (*rpoD*) genes for speciation. A total of 122 possible *Aeromonas* strains were isolated and confirmed to genus level using the API20E system. The clonality of the isolates was investigated using ERIC-PCR and 20 duplicate isolates were excluded from the study. The specific GCAT-PCR identified all isolates as belonging to the genus *Aeromonas*, in agreement with the biochemical identification. A phylogenetic tree was constructed using the *rpoD* gene sequence and all 102 isolates were identified as: *A. veronii* 43%, *A. jandaei* 37%, *A. hydrophila* 6%, *A. caviae* 4%, *A. salmonicida* 2%, *A. media* 2%, *A. allosaccharophila* 1%, *A. dhakensis* 1% and *Aeromonas* spp. 4%. Twelve virulence genes were present in the following proportions—*exu* 96%, *ser* 93%, *aer* 87%, *fla* 83%, *enolase* 70%, *ela* 62%, *act* 54%, *aexT* 33%, *lip* 16%, *dam* 16%, *alt* 8% and *ast* 4%, and at least 2 of these genes were present in all 102 strains. The *ascV*, *aexU* and *hlyA* genes were not detected among the isolates. *A. hydrophila* was the main species containing virulence genes *alt* and *ast* either present alone or in combination. It is possible that different mechanisms may be used by each genospecies to demonstrate virulence. In summary, with the use of GCAT and *rpoD* genes, unambiguous identification of *Aeromonas* species is possible and provides valuable data on the phylogenetic diversity of the organism.

Introduction

*Aeromonas* organisms are oxidase-positive, polar flagellated, non-sporulating facultative anaerobic rods [1]. These Gram-negative aeromonads are essentially ubiquitous in the
microbial biosphere and found in almost every environmental niche, including aquatic habitats, fish, foods, domesticated pets, birds and soil. The aquatic environment is the natural habitat of aeromonads and they can be isolated from rivers, lakes, ponds, groundwater, surface water and chlorinated water. They are well known as causative agents of disease in fish, prawns, shrimps, oysters and other seafood [2]. *A. salmonicida* cause furunculosis and septicemia that result in huge economical loss in the fishing industry [3,4]. Disease may also be caused by the mesophilic *A. hydrophila* which has been linked to several epidemic outbreaks in the fishing industry [2,5]. In humans, aeromonads have been reported to be responsible for both gastrointestinal and extraintestinal infections particularly in immunocompromised patients [2,6,7]. Humans acquire aeromonads from a wide range of food and water [2]. Recreational activities such as boating, skiing, fishing and diving pose risks leading to infections [4,8].

The taxonomy of *Aeromonas* is in transition and presently this genus consists of 30 species [9]. Identification of *Aeromonas* to the species level can be difficult due to its complex phenotypic and genotypic heterogeneity [10]. The use of molecular approaches has led to a more refined identification that has revealed a number of discrepancies in the biochemical identification of this organism [11]. A molecular identification for *Aeromonas* species using *GCAT* and *rpoD* genes was reported by Puthucheary et al. [12]. *GCAT* is a highly conserved lipase gene present in practically all *Aeromonas* strains and a specific PCR probe was designed by Chacón et al. [13] that avoids confusion with other genera, such as *Vibrio* and *Plesiomonas*. The *rpoD* gene, a housekeeping gene, was reported to be an excellent tool for identification and for inferring the taxonomy of the genus *Aeromonas* [11]. Enterobacterial repetitive intergenic consensus (ERIC) sequences are short repetitive sequences in genomes of bacteria and the ERIC-PCR approach has been widely used for genomic fingerprinting of a broad range of bacterial species [14]. This method allows both phylogenetic inference and clonal differentiation of bacterial strains.

The production of virulence factors is essential for bacteria to establish infections and in *Aeromonas* a number of virulence genes have been described [15–18]. Pore-forming aerolysins *aer* and enterotoxins *act*, *alt* and *ast* are virulence determinants associated with gastroenteritis and diarrheal syndromes [19–21]. Other virulence factors described are extracellular lipases *lip*, *lipH3*, *pla* and *plc* that alter the host plasma membranes [22]. Type 3 secretion system (T3SS) effectors, *AexT* and *AexU* with the ability to cause host cell death have also been characterised [23,24]. Virulence in *Aeromonas* is a complex process and the detection of virulence factors is necessary in determining the potential pathogenicity and subsequent possible targets for vaccines. Hence the objectives of this study were (a) to isolate aeromonads from their natural aquatic habitat, (b) to investigate the clonality of the isolates using ERIC-PCR, (c) to identify and speciate these isolates using the *GCAT* and *rpoD* genes and (d) to screen for 15 virulence determinants.

**Materials and Methods**

**Sample collection and bacterial isolation**

Surface water samples were collected from 5 fresh water multi-purpose recreational lakes in Selangor, i.e., Tasik Aman (lake 1) (N 03.10261 °, E 101.62477 °), Tasik Taman Jaya (lake 2) (N 3.10418 °, E 101.64929 °), Tasik Varsiti Universiti Malaya (lake 3) (N 3.11941 °, E 101.65808 °) and two unnamed lakes in Rawang (lake 4 and lake 5) (N 03.36606 °, E 101.63717 ° and N 03.36752 °, E 101.63043 °). All samples were kept at 4°C and analysed within 30 hours of collection.
The samples were pre-filtered to remove residue and subsequently filtered through a 0.45 μm nitrocellulose membrane (Sartorius, Germany) using a vacuum system. The membranes were then suspended in broth and plated onto m-Aeromonas selective media (Biolife, Italia Srl) supplemented with ampicillin (10 mg/l) [25]. Yellow colonies on the agar plates due to dextrin fermentation, after 18–24 hours of incubation at 30°C were presumed to be *Aeromonas* species and tested with oxidase reagent (bioMérieux, France), checked for growth on MacConkey agar and 6.5% (w/v) NaCl-Luria Bertani (LB) broth. Oxidase-positive colonies growing on MacConkey agar but not in 6.5% NaCl-LB broth were further confirmed to genus level by the API 20E system (bioMérieux, France), then grown in LB broth, cryopreserved in 20% (v/v) glycerol at -80°C and maintained in LB agar and broth as working cultures.

**Bacterial DNA extraction**

Genomic DNA extraction was carried out using GeneAll® Exgene™ Cell SV DNA isolation kit (GeneAll Technology, Korea). Overnight cultures were pelleted, lysed using 20 mg/ml Proteinase K, passed through a spin column and washed with buffers and the purified DNA was subjected to concentration and standardisation for subsequent molecular analysis.

**Molecular identification**

**ERIC-PCR analysis.** All isolates were subjected to ERIC-PCR fingerprinting using primers and PCR conditions as described previously [26]. The amplification products were electrophoresed in 1.5% (W/V) agarose gels containing ethidium bromide at 56V for 6 hours in Tris-borate-EDTA buffer. Gene Ruler 100 bp DNA Ladder Plus (Fermentas) was used as a molecular size reference. The electrophoresed gels were visualised using a UV light transilluminator. The digitised profiles were analysed by BioNumerics software, version 7.5 (Applied Maths, Belgium). Similarity between the fingerprints was calculated with the band-matching Dice coefficient. Cluster analysis was performed using the unweighted pair-group method with average linkages (UPGMA). Representative samples from each cluster were amplified using the *rpoD* gene and sent for sequencing for verification.

**Genus identification.** The *GCAT* gene was amplified using primer pair as reported previously [13]. Presence of this gene (237 bp) was visualised on 1.5% agarose gel stained with ethidium bromide.

**Species identification.** Strains with the *GCAT* gene were further subjected to *rpoD* gene sequencing whereby the 816 bp gene region was amplified by a touch-down PCR using a degenerate primer pair as described by Yamamoto et al. [27]. The amplified product of the *rpoD* gene was resolved on a 2% agarose gel, excised and subjected to purification using QIAquick Gel Extraction kit (Qiagen, Germany). The gel was dissolved completely to release the PCR product which was then passed through a spin column and washed with buffers for purification, then sent for sequencing using specific primers as reported by Yamamoto et al. [27]. The resulting DNA sequences were then compared with the GenBank database using Basic Local Alignment Search Tool (BLAST).

**Phylogenetic analysis**

Phylogenetic data analysis using a partial nucleotide sequence (670 bp) of the *rpoD* gene was performed, the sequences (GenBank accession numbers: KT187565 to KT187686) were aligned using the ClustalW program and pairwise sequence identity matrices were calculated using the Bioedit software version 7.0.9 [28]. A phylogenetic tree was constructed based on neighbour-joining method via the MEGA 6 program with bootstrapping for 1000 replicates [29]. The genetic distances were also computed using Kimura’s two-parameter model. The *rpoD* gene
sequences of type strains representing all the known *Aeromonas* species were obtained from National Centre for Biotechnology Information (NCBI) database and used as reference gene sequences in the phylogenetic tree construction (Table 1). *Vibrio parahaemolyticus* ATCC 43996 (JQ015347.1) was included as an outgroup.

**Screening of virulence genes**

Isolates identified as *Aeromonas* species by *rpoD* gene analysis were subjected to direct PCR to detect the existence of the following 15 known virulence genes encoding for, DNAse (*exu*), heat-labile cytotoxic enterotoxin (*alt*), serine protease (*ser*), aerolysin/hemolysin (*aer*), cytotoxic enterotoxin (*act*), heat-stable cytotoxic enterotoxin (*ast*), lipase (*lip*), flagellin (*vla*), elastase (*ela*), ADP-ribosyltransferase toxins (*aexT* and *aexU*), DNA adenine methyltransferase (*dam*), enolase (*enolase*), T3SS membrane component (*ascV*) and hemolysin (*hlyA*). Screening was performed using the PCR primers reported previously (Table 2) at annealing temperatures from 55 to 68°C. Two-tailed Fisher’s exact test was carried out to determine the presence of combination of virulence genes. The purified PCR products of the genes, detected by QIAquick gel extraction kit (Qiagen, Germany) were sent for sequencing for validation.

**Table 1. Reference gene sequences used in the phylogenetic tree construction.**

| Species                      | Accession no. |
|------------------------------|---------------|
| 1   *A. allosaccharophila* CECT 4199 | HQ442825 |
| 2   *A. australiensis* strain 266      | FN773335 |
| 3   *A. bestiarum* CECT 4227           | HQ442854 |
| 4   *A. bivalvium* CECT 7113           | HQ442817 |
| 5   *A. carvenicola* CECT 7862         | HQ442864 |
| 6   *A. caviae* CECT 838              | HQ442790 |
| 7   *A. dhakensis* CECT 7289           | HQ442798 |
| 8   *A. diversa* CECT 4254            | HQ442805 |
| 9   *A. encheleia* CECT 4342           | HQ442778 |
| 10  *A. eucnephila* CECT 4224          | HQ442770 |
| 11  *A. fluvialis* strain 717          | FJ603453 |
| 12  *A. hydrophila* CECT 839           | HQ442791 |
| 13  *A. jandaei* CECT 4228             | HQ442840 |
| 14  *A. media* CECT 4232              | HQ442785 |
| 15  *A. molluscorum* CECT 5864         | HQ442812 |
| 16  *A. piscicola* CECT 7443           | HQ442859 |
| 17  *A. popoffii* CECT 5176            | HQ442853 |
| 18  *A. rivuli* DSM 22539              | FJ969433 |
| 19  *A. salmonicida* CECT 894          | HQ442843 |
| 20  *A. sanarellii* strain A2-67        | FJ807275 |
| 21  *A. schuberti* CECT 4240           | HQ442809 |
| 22  *A. simiae* CIP 107798             | HQ442811 |
| 23  *A. sobria* CECT 4245              | HQ442867 |
| 24  *A. taiwanensis* strain A2-50       | FJ807271 |
| 25  *A. tecta* CECT 7082               | HQ442762 |
| 26  *A. trota* CECT 4255               | HQ442822 |
| 27  *A. veronii* CECT 4257             | HQ442833 |

doi:10.1371/journal.pone.0145933.t001
Results and Discussion

A total of 122 probable aeromonad strains were isolated from the 5 lakes in Selangor and identified as *Aeromonas* spp. using the API20E system, i.e. 27 from lake 1, 21 from lake 2, 15 from lake 3, 18 from lake 4 and 21 from lake 5.

ERIC fingerprinting revealed 10 clusters consisting of 30 isolates at the 100% similarity level (Fig 1). The strains within each cluster appeared to have identical fingerprints and were considered as belonging to the same clone. The verification test demonstrated identical *rpoD* gene sequences for the representative isolates of the same cluster thus confirming the clonality. Replicate isolates were excluded from the study and 102 samples were subjected to further analyses.

The genus-specific *GCAT* gene present in the 102 isolates, confirmed that all the environmental isolates belonged to the genus *Aeromonas*. Hence, the biochemical identification system API20E is in agreement with the *GCAT*-PCR in identifying *Aeromonas* to the genus level. But the API20E has limitations in accurately identifying *Aeromonas* species without additional biochemical, morphological and physiological tests, thus the necessity of molecular methods for a higher resolution identification of *Aeromonas* species.

Based on the constructed phylogenetic tree using the partial *rpoD* sequence, 98 isolates showed clustering with the reference strains of respective species and were identified as:

| Gene | Primer sequence (5’ to 3’), F/R | Size (bp) | Reference |
|------|---------------------------------|-----------|-----------|
| *exu* | (A/G) GACATGCAACACTTCCC/GCTTGGTATTGCC (C/T) TGCAA (C/G) | 323 | [30] |
| *ser* | ACCGAGTGCTGTCCTCCACTCCAG/CCGTTCATCCACCCGCTTG | 211 | [31] |
| *aer* | CCTATGGCCTGACGAGGAG/CCAGTTCCAGTCTCCACT | 431 | [15] |
| *fla* | TCCAAAACGTTGACCT/GMYTGGTGCCCTTCTT | 608 | [18] |
| *act* | AGAGAGTGCAACACTCTAC/GAATTCCACCCGCTTG | 232 | [32] |
| *ela* | ACACGCTCAAGGATGAC/CCCTGGTGCTCGAGCAAGG | 513 | [18] |
| *aexT* | CGTGCCACTCAAGAGAAG/GCAGCTTGGCTGTCCT | 425 | [33] |
| *lil* | ATCTTCCTCCGATCGTGTG/CCGCCCAGACTGTCCTT | 382 | [18] |
| *alt* | AAAGGTCTGACAGCGGAG/AGGACATAGCGCTTCTT | 320 | [34] |
| *ast* | ATCAGTCAGCGAGCAGAGT/CTCTCCCTTGGTCTGT | 504 | [34] |
| *dam* | ATGAAAAACACGCCTTTTTTTTTAAAAATGG/TCCACCGCTGGTGTGCTGG | 873 | [16] |
| *enolase* | ATGCCACATCGTTAAGATGAT/TTAAGCTGTCATTCCGCTT | 1302 | [35] |
| *ascV* | ATCAGACCGGCTGCTCCAC/GTACGCAGACCTTCCAGC | 2166 | [36] |
| *aexU* | ATGCAGATTCAACATACCCAG/ATGACGATGACGCTGTTGTCG | 1539 | [23] |
| *hlyA* | ATGAGTGTGTGCCCCGATGGTTTTATGGCTGTT/GCTGCTTGGCCCGGCTTG | 1320 | [37] |

PLOS ONE | DOI:10.1371/journal.pone.0145933 December 28, 2015
Fig 1. Dendrogram showing ERIC fingerprints of the 122 strains of Aeromonas isolates using Dice similarity coefficient and UPGMA cluster method.

doi:10.1371/journal.pone.0145933.g001
Phenotypic and Genetic Diversity of Aeromonas Isolates

A. veronii—44, A. jandaei—38, A. hydrophila—6, A. caviae—4, A. salmonicida—2, A. media—2, A. allosaccharophila—1 and A. dhakensis—1 (Fig 2). Four isolates were not grouped into any cluster with known species strain. They formed clusters branching close to A. allosaccharophila and A. veronii, suggesting that these isolates need further investigation using more than one target gene or other approaches. The 4 isolates were considered as "Aeromonas spp." in this study. The pairwise sequence identity matrix using the rpoD gene revealed that intraspecies similarities for the isolates were 96.2–100% for A. veronii, 95.2–100% for A. jandaei, 97.3–98.6% for A. hydrophila, 97.1–99.4% for A. caviae, 98.0% for A. salmonicida and 100% for A. media and 96.7–100% for Aeromonas spp.

A. veronii was the most common species—43% followed by A. jandaei 37%, A. hydrophila 6%, A. caviae 4%, A. salmonicida 2%, A. media 2%, A. allosaccharophila 1% and A. dhakensis 1% and Aeromonas spp. 4%. We found dominance of A. veronii in environmental samples [38,39]. A. jandaei—the second common species in this study, was reported to be associated with epizootic occurrence in farmed eels [40] and possible implications for clinical infections [41]. A. salmonicida is a predominant species related to fish and fresh water samples [2]. However, only 2 strains of A. salmonicida were detected in our study and this may be due to several factors such as geographical location, type of aquatic environment selected or the temperature and pH of the water as A. salmonicida is not able to grow at temperatures around 37°C [42,43]. This variance can also be related to different regions or different areas of the same gene being targeted for the bacterial identification.

Biochemically, all Aeromonas isolates were oxidase-positive and fermented glucose and mannose, but showed negative reactions for ornithine decarboxylase, hydrogen sulfide, urease, tryptophan deaminase and rhamnose fermentation. Most A. jandaei were citrate-positive whereas only half of A. veronii were able to use citrate as the sole carbon source. Thirty-four percent of A. jandaei fermented melibiose while none of the A. veronii strains showed fermentation. The findings were in agreement with Abbott et al., [44]. However, 7 (18%) A. jandaei strains were observed to be atypically sucrose-positive.

Multiple virulence genes were present in all the 102 Aeromonas strains and all contained at least 2 of the virulence genes but none possessed only 1 virulence gene (Table 3). A single A. hydrophila isolate from the lake 4 carried a complement of 12 of the 15 virulence genes. The exu gene was the most prevalent, being present in 96% of the isolates and widely distributed in all species, except of A. media, similar to the study by Chacón et al. [15]. Other virulence genes detected were ser 93%, aer 87%, fla 83%, enolase 70%, ela 62, act 54%, aexT 33%, lip 16%, dam 16%, alt 8% and ast 4%. The sequencing results were compared to the Genbank database using BLAST. The nucleotide BLAST homology search revealed high homologies (>90%) of the virulence gene PCR products with the deposited sequences in the database.

Two-tailed Fisher’s exact test based on each virulence gene revealed statistically significant associations in A. hydrophila with alt, ast, lip, aexT and dam; A. caviae with lip; A. veronii with act and aexT and A. salmonicida with dam (Table 4). The serine protease gene was detected in all species (≥95%) with the exception of A. caviae (25%). The presence of aer gene was observed in A. veronii 94% and A. hydrophila 83%, but less frequently among A. caviae 25%, in agreement with Yousr et al. [45]. Inverse associations were observed in A. jandaei with the lip, fla and aexT genes and A. veronii with the lip, ela and enolase genes. None of the isolates demonstrated the presence of the ascV, aexU and hlyA genes. Khajanchi et al [46] reported that 30–47% of their isolates obtained from water samples were positive for the ascV, aexU and hlyA genes. This discordance can be explained by the fact that pathogenetic mechanisms of Aeromonas species may be different in different geographical locations. The discrepancies can also be related to the different dominant species of isolates in different studies. In their study, the
Fig 2. Phylogenetic tree of 102 *Aeromonas* and reference strains based on the *rpoD* gene sequences using neighbour-joining method with bootstrap replication of 1000.

doi:10.1371/journal.pone.0145933.g002
predominant species was *A. hydrophila* (59.5%)—this species harboured the 3 genes. In contrast, there were only 6 strains of *A. hydrophila* (6%) in our study.

Four virulence genes: *exu*, *ser*, *aer* and *fla* were the most frequently detected (>80%) and based on these 4 genes, 8 different subsets were observed and a statistically significant association was observed in *A. allosaccharophila* with *exu*/*ser*/*fla*; *A. caviae* with *exu*/*fla* and *A. media* with *ser*/*fla* (Table 4). The cytotoxic enterotoxin genes *alt* and *ast* were less frequently detected (*alt*-7%) and (*ast*-3%) and however mainly present in *A. hydrophila*. A significant association (*p*<0.0001) was found between these 2 genes and *A. hydrophila* either singly or in combination (*alt*/*ast*), and this implies that *A. hydrophila* probably possess a distinct set of virulence genes vis-à-vis other *Aeromonas* species. The *alt* and *ast* virulence determinants are associated with diarrhea, and found to be more prevalent in children with diarrhea compared to healthy controls, probably due to the presence of enterotoxins in *Aeromonas* [19]. The different proportions of virulence genes present suggest that different mechanisms may be used by each subpopulation of *Aeromonas* to colonise and induce infections.

**Conclusions**

Phenotypic and genotypic diversity of *Aeromonas* organisms from aquatic environments were investigated in this study. The use of both the approaches are useful for the characterisation of *Aeromonas*, however phenotypic studies have limitations emphasising the need for molecular identification methods. Ninety-six percent of the aeromonad isolates from aquatic environments were confirmed as *Aeromonas* using the *GCAT* and *rpoD* genes. The results confirm that by the use of these two genes, the definitive identification of environmental *Aeromonas* species is possible. *A. veronii* was the predominant species 43%, occurring in the freshwater lakes. Multiple virulence genes were present and different subsets of these genes existed

### Table 3. Presence of multiple virulence genes in 102 *Aeromonas* isolates.

| Species              | Frequency of isolates harbouring the indicated number of virulence gene, % |
|----------------------|--------------------------------------------------------------------------|
|                      | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | Total |
| *A. veronii*         | -  | -  | 3  | 11 | 16 | 9  | 5  | -  | -  | -  | -  | -  | -  | -  | -  | 44  |
| (n = 44)            |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100  |
| *A. jandaei*        | -  | -  | 2  | 2  | 6  | 21 | 6  | 1  | -  | -  | -  | -  | -  | -  | -  | 38  |
| (n = 38)            |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100  |
| *A. hydrophila*     | -  | -  | -  | -  | -  | -  | -  | 1  | -  | 3  | 1  | 1  | -  | -  | -  | 6   |
| (n = 6)             |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100  |
| *A. caviae*         | -  | -  | -  | -  | -  | -  | 2  | 1  | -  | 1  | -  | -  | -  | -  | -  | 4   |
| (n = 4)             |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100  |
| *A. salmonicida*    | -  | -  | -  | -  | -  | -  | -  | -  | -  | 2  | -  | -  | -  | -  | -  | 2   |
| (n = 2)             |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100  |
| *A. media*          | -  | -  | -  | 1  | 1  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 2   |
| (n = 2)             |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100  |
| *A. allosaccharophila* | -  | -  | -  | 1  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 1   |
| (n = 1)             |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100  |
| *A. dhakensis*      | -  | -  | -  | -  | -  | -  | 1  | -  | -  | -  | -  | -  | -  | -  | -  | 1   |
| (n = 1)             |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100  |
| *Aeromonas* spp.    | -  | -  | -  | 2  | 1  | -  | 1  | -  | -  | -  | -  | -  | -  | -  | -  | 4   |
| (n = 4)             |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100  |

doi:10.1371/journal.pone.0145933.t003
in the various Aeromonas species, leading to the suggestion that each species has a distinct set of virulence genes.

**Author Contributions**

Conceived and designed the experiments: JAMAT SDP KHC. Performed the experiments: WCK SMP. Analyzed the data: WCK SMP KHC. Contributed reagents/materials/analysis tools: JAMAT KHC. Wrote the paper: WCK SMP JAMAT SDP KHC.

**References**

1. Nordmann P, Poirel L. Emerging carbapenemases in Gram-negative aerobes. Clin Microbiol Infect. 2002; 8: 321–331. PMID: 12084099

2. Janda JM, Abbott SL. The genus Aeromonas: taxonomy, pathogenicity, and infection. Clin Microbiol Rev. 2010; 23: 35–73. doi: 10.1128/CMR.00039-09 PMID: 20065325
3. Faisal M, Eissa AE, Elsayed EE. Isolation of Aeromonas salmonicida from sea lamprey (Petromyzon marinus) with furuncle-like lesions in Lake Ontario. J Wildl Dis. 2007; 43: 618–622. doi: 10.7589/0090-3558-43.4.618 PMID: 17984256

4. Janda JM, Abbott SL. Evolving concepts regarding the genus Aeromonas: an expanding Panorama of species, disease presentations, and unanswered questions. Clin Infect Dis. 1998; 27: 332–344. PMID: 9709884

5. Hossain MJ, Sun D, McGarey DJ, Wrenn S, Alexander LM, Martino ME, et al. An Asian origin of virulent Aeromonas hydrophila responsible for disease epidemics in United States-farmed catfish. MBio. 2014; 5: e00848–14. doi: 10.1128/mBio.00848-14 PMID: 24895303

6. Lee WS, Puthucheary SD. Retrospective study of Aeromonas infection in a Malaysian urban area: a 10-year experience. Singapore Med J. 2001; 42: 57–60.

7. Figueras MJ. Clinical relevance of Aeromonas sM503. Rev Med Microbiol. 2005; 16: 145–153.

8. Bossi-Kupfer M, Genini A, Peduzzi R, Demarta A. Tracheobronchitis caused by Aeromonas veronii biovar sobria after near-drowning. J Med Microbiol. Microbiology Society; 2007; 56: 1563–1564. doi: 10.1099/jmm.0.47202-0

9. Beaz-Hidalgo R, Hossain MJ, Liles MR, Figueras MJ. Strategies to Avoid Wrongly Labeled Genomes Using as Example the Detected Wrong Taxonomic Affiliation for Aeromonas Genomes in the GenBank Database. PLoS One. 2015; 10: e0115813. doi: 10.1371/journal.pone.0115813 PMID: 25607802

10. Lamy B, Laurent F, Decousser J-W, Lecaillon E, Marchandin H, et al. Accuracy of 6 commercial systems for identifying clinical Aeromonas. Diagn Microbiol Infect Dis. 2010; 67: 9–14. doi: 10.1016/j.diagmicrobio.2009.12.012 PMID: 20167449

11. Beaz-Hidalgo R, Alperi A, Buján N, Rimalde JL, Figueras MJ. Comparison of phenotypical and genetic identification of Aeromonas strains isolated from diseased fish. Syst Appl Microbiol. 2010; 33: 149–153. doi: 10.1016/j.syapm.2010.02.002 PMID: 2027844

12. Puthucheary SD, Puah SM, Chua KH. Molecular characterisation of clinical isolates of Aeromonas species from Malaysia. PLoS One. 2012; 7: e30205. doi: 10.1371/journal.pone.0030205 PMID: 22383958

13. Chacón MR, Castro-Escarpulli G, Soler L, Guaroo J, Figueras MJ. A DNA probe specific for Aeromonas colonies. Diagn Microbiol Infect Dis. 2002; 44: 221–225. PMID: 12493167

14. Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 1991; 19: 6823–6831. PMID: 1762913

15. Chacón MR, Figueras MJ, Castro-Escarpulli G, Soler L, Guaroo J. Distribution of virulence genes in clinical and environmental isolates of Aeromonas spp. António Van Leeuwenhoek. 2003; 84: 269–278. doi: 10.1023/A:1026042125243 PMID: 14574104

16. Erova TE, Pillai L, Fadi AA, Sha J, Wang S, Galindo CL, et al. DNA adenine methyltransferase influences the virulence of Aeromonas hydrophila. Infect Immun. 2006; 74: 410–424. doi: 10.1128/IAI.74.1.410-424.2006 PMID: 16368997

17. Pillai L, Sha J, Erova TE, Fadi AA, Khajanchi BK, Chopra AK. Molecular and functional characterisation of a ToxR-regulated lipoprotein from a clinical isolate of Aeromonas hydrophila. Infect Immun. 2006; 74: 3742–3755. doi: 10.1128/IAI.00402-06 PMID: 16790746

18. Sen K, Rodgers M. Distribution of six virulence factors in Aeromonas species isolated from US drinking water utilities: a PCR identification. J Appl Microbiol. 2004; 97: 1077–1086. doi: 10.1111/j.1365-2672.2004.02398.x PMID: 15479425

19. Albert MJ, Ansaruzzaman M, Talukder KA, Chopra AK, Kuhn I, Rahman M, et al. Prevalence of enterotoxin genes in Aeromonas spp. isolated from children with diarrhea, healthy controls, and the environment. J Clin Microbiol. 2000; 38: 3785–3790. PMID: 11015403

20. Chopra AK, Houston CW. Enterotoxins in Aeromonas-associated gastroenteritis. Microbes Infect. 1999; 1: 1129–1137. doi: 10.1016/S1286-4579(99)00202-6 PMID: 10572317

21. Sha J, Kozlova E V, Chopra AK. Role of various enterotoxins in Aeromonas hydrophila-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxic activity. Infect Immun. 2002; 70: 1924–1935. PMID: 11895956

22. Pemberton JM, Kidd SP, Schmidt R. Secreted enzymes of Aeromonas. FEMS Microbiol Lett. 1997; 152: 1–10. doi: 10.1111/j.1574-6968.1997.tb10041.x PMID: 9228763

23. Sha J, Wang SF, Suarez G, Sierra JC, Fadi AA, Erova TE, et al. Further characterisation of a type III secretion system (T3SS) and of a new effector protein from a clinical isolate of Aeromonas hydrophila—part I. Microb Pathog. 2007; 43: 127–146. doi: 10.1016/j.micpath.2007.05.002 PMID: 17644303
24. Grim CJ, Kozlova E V, Sha J, Fitts EC, van Lier CJ, Kirtley ML, et al. Characterisation of Aeromonas hydrophila wound pathotypes by comparative genomic and functional analyses of virulence genes. MBio. 2013; 4: e00064–13. doi: 10.1128/mBio.00064-13 PMID: 23611906

25. Havelaar AH, Vonk M. The preparation of ampicillin dextrin agar for the enumeration of Aeromonas in water. Lett Appl Microbiol. 1988; 7: 169–171. doi: 10.1111/j.1472-765X.1988.tb01271.x

26. Szczuka E, Kaznowski A. Typing of clinical and environmental Aeromonas sp. strains by random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus sequence PCR. J Clin Microbiol. 2004; 42: 220–228. PMID: 14715756

27. Yamamoto S, Kasai H, Arnold DL, Jackson RW, Vivian A, Harayama S. Phylogeny of the genus Pseudomonas: intragenic structure reconstructed from the nucleotide sequences of gyrB and rpoD genes. Microbiology. 2000; 146: 2385–2394. doi: 10.1099/00221287-146-10-2385 PMID: 11021915

28. Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999; 41: 95–98.

29. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013; 30: 2725–2729. doi: 10.1093/molbev/mst197 PMID: 24132122

30. Nawaz M, Khan SA, Khan AA, Sung K, Tran Q, Kerdahi K, et al. Detection and characterisation of virulence genes and integrons in Aeromonas veronii isolated from catfish. Food Microbiol. 2010; 27: 327–331. doi: 10.1016/j.fm.2009.11.007 PMID: 20227596

31. Nam IY, Joh K. Rapid detection of virulence factors of Aeromonas isolated from a trout farm by hexaplex-PCR. J Microbiol. 2007; 45: 297–304. PMID: 17846582

32. Kingcombe CI, Huys G, Tonolla M, Albert MJ, Swings J, Peduzzi R, et al. PCR detection, characterisation, and distribution of virulence genes in Aeromonas spp. Appl Environ Microbiol. 1999; 65: 5293–5302. PMID: 10583979

33. Vilches S, Wilhelms M, Yu HB, Leung KY, Tomás JM, Merino S. Aeromonas hydrophila AH-3 AdET is an ADP-ribosylating toxin secreted through the type III secretion system. Microb Pathog. 2008; 44: 1–12. doi: 10.1016/j.micpath.2007.06.004 PMID: 17689917

34. Aguilera-Arreola MG, Hernández-Rodríguez C, Zúñiga G, Figueras MJ, Castro-Escarpulli G. Aeromonas hydrophila clinical and environmental ecotypes as revealed by genetic diversity and virulence genes. FEMS Microbiol Lett. 2005; 242: 231–240. doi: 10.1016/j.femsle.2004.11.011 PMID: 15621443

35. Sha J, Galindo CL, Pancholi V, Popov VL, Zhao Y, Houston CW, et al. Differential expression of the enolase gene under in vivo versus in vitro growth conditions of Aeromonas hydrophila. Microb Pathog. 2003; 34: 195–204. doi: 10.1016/S0882-4010(03)00028-7 PMID: 12668143

36. Sha J, Pillai L, Fadl AA, Galindo CL, Erova TE, Chopra AK. The Type III Secretion System and Cytotoxic Enterotoxin Alter the Virulence of Aeromonas hydrophila. Infect Immun. 2005; 73: 6446–6457. doi: 10.1128/IAI.73.10.6446-6457.2005 PMID: 16177316

37. Erova TE, Sha J, Hornerman AJ, Borchardt MA, Khajanchi BK, Fadl AA, et al. Identification of a new hemolysin from diarrheal isolate SSU of Aeromonas hydrophila. FEMS Microbiol Lett. 2007; 275: 301–311. doi: 10.1111/j.1574-6968.2007.00895.x PMID: 17725618

38. Hu M, Wang N, Pan ZH, Lu CP, Liu Y.J. Identity and virulence properties of Aeromonas isolates from diseased fish, healthy controls and water environment in China. Lett Appl Microbiol. 2012; 55: 224–233. doi: 10.1111/j.1472-765X.2012.03281.x PMID: 22725694

39. Sreedharan K, Philip R, Singh ISB. Characterisation and virulence potential of phenotypically diverse Aeromonas veronii isolates recovered from moribund freshwater ornamental fishes of Kerala, India. Antonie Van Leeuwenhoek. 2012; 103: 53–67. doi: 10.1007/s10482-012-9786-z PMID: 22843161

40. Esteve C, Valera L, Gutiérrez C, Ventosa A. Taxonomic study of sucrose-positive Aeromonas jandaei-like isolates from faeces, water and eels: emendation of A. jandaei Carnahan et al. 1992. Int J Syst Evol Microbiol. 2003; 53: 1411–1419. doi: 10.1099/ijss.0.05204-0

41. Corry JEL, Curtis GDW, Baird RM. Handbook of culture media for food microbiology. Amsterdam: Elsevier; 2003.

42. Altwegg M, Steigenwald AG, Altwegg-Bissig R, Lüthy-Hottenstein, Brenner DJ. Biochemical identification of Aeromonas genospecies isolated from humans. J Clin Microbiol. 1990; 28(2): 258–264

43. Rhodes MW, Kator H. Seasonal occurrence of mesophilic Aeromonas spp. as a function of biotype and water quality in temperate freshwater lakes. Water Res. 1994; 28: 2241–2251. doi: 10.1016/0043-1354(94)90039-6

44. Abbott SL, Cheung WKW, Janda JM. The genus Aeromonas: biochemical characteristics, atypical reactions, and phenotypic identification schemes. J Clin Microbiol. 2003; 41: 2348–57. PMID: 12791848

45. Yousr AH, Napis S, Rahmat AGR, Radu S. Detection of aerolysin and hemolysin genes in Aeromonas spp. isolated from environmental and shellfish sources by polymerase chain reaction. ASEAN food J. 2007; 14: 115–122.
46. Khajanchi BK, Fadl AA, Borchardt MA, Berg RL, Horneman AJ, Stemper ME, et al. Distribution of virulence factors and molecular fingerprinting of Aeromonas species isolates from water and clinical samples: suggestive evidence of water-to-human transmission. Appl Environ Microbiol. 2010; 76:2313–2325. doi:10.1128/AEM.02535-09 PMID: 20154106