Characterization and antimicrobial susceptibility of motile aeromonads isolated from freshwater ornamental fish showing signs of septicaemia

S. S. de S. Jagoda1,2, T. G. Wijewardana2,4, A. Arulkanthan2, Y. Igarashi1, E. Tan1, S. Kinoshita1, S. Watabe3, S. Asakawa1,*

1Laboratory of Aquatic Molecular Biology and Biotechnology, Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
2Center for Aquatic Animal Disease Diagnosis and Research, Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya 20400, Sri Lanka
3School of Marine Biosciences, Kitasato University, Minami Ku, Sagamihara, Kanagawa 252-0373, Japan
4Present address: No. 408/35, Mount Pleasant Garden II, Bowellawatte, Kandy 20000, Sri Lanka

ABSTRACT: A total of 74 phenotypically identified presumptive motile Aeromonas isolates recovered from septicaemic freshwater ornamental fish in Sri Lanka were genetically characterized by sequencing of rpoD and gyrB genes. rpoD/gyrB phylogeny confirmed only 53 isolates as Aeromonas, among which A. veronii was the predominant species (79.2%), followed by A. hydrophila (7.5%), A. caviae (5.7%), A. jandaei (1.9%), A. dhakensis (3.8%) and A. entero pelogenes (1.9%). The aeromonads confirmed by sequencing were further subjected to 16S rDNA PCR-RFLP which substantiated sequencing results for 83% of isolates. Fingerprinting of A. enteropelogenes (n = 42) using ERIC-PCR revealed no dominant clones, and the majority were genetically distinct. All isolates were screened by PCR for 7 virulence determinant genes (aer, act, ast, alt, fla, ser, exu) and 2 integrase encoding genes (intI1, intI2). Each isolate contained ≥3 of the virulence genes tested for, with a heterogeneous distribution. Of the isolates, 77% harboured the intI1 gene, while none had intI2. In vitro antimicrobial susceptibility testing showed highest resistances towards tetracycline (58.5%) and erythromycin (54.7%). Our results indicate the diverse range of aeromonads that could potentially be associated with motile aeromonad septicaemia in ornamental fish. This is the first isolation of A. dhakensis from a septicaemic ornamental fish since its original description from the same host.

KEY WORDS: Aeromonas veronii · Antimicrobial resistance · Motile aeromonad septicaemia · Ornamental fish · Phylogenetic identification · Virulence genes

INTRODUCTION

The ornamental fish industry provides numerous economic and social benefits. Export of live aquarium fish has emerged as a valuable foreign exchange earner, particularly in developing countries. Sri Lanka supplies about 2.7% of the world’s demand for ornamental fish, generating an annual income of ~10 million US$ (as of 2011; Sri Lanka EDB 2014). The current trend towards intensification and commercialization of ornamental fish culture increases the risk of fish diseases. Aeromonads are Gram-negative rods ubiquitous in aquatic environments (Janda & Abbott 2010, Beaz-Hidalgo & Figueras 2012). Motile aeromonad septicaemia (MAS), with mesophilic, motile Aeromonas species implicated in its development, is probably the most common bacterial disease in freshwater aquarium fish (Lewbart 2001). It is often an opportunistic infection, characterized by non-specific signs.

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such as fin rot, ulceration, haemorrhages, exophthalmia and dropsy. Historically, *Aeromonas hydrophila* has gained much attention as the most common fish pathogenic motile *Aeromonas* species, but other *Aeromonas* species may also play an important role in fish pathology that needs to be explored (Beaz-Hidalgo & Figueras 2012). Well-documented discrepancies in phenotypic identification of *Aeromonas* at the species level (Kozińska 2007, Beaz-Hidalgo et al. 2010) may have impacted the species designations of the fish pathogenic aeromonads identified to date (Austin 2011), but the use of molecular approaches, such as the sequencing of *rpoD* and *gyrB* genes (Yáñez et al. 2003, Soler et al. 2004, Martínez-Murcia et al. 2011), has led to increased accuracy of *Aeromonas* species identification.

In this context, the present study aimed to characterize a collection of *Aeromonas* isolates, from ornamental fish with clinical signs of MAS, at the species level using a polyphasic approach; our focus was to determine the relative occurrence of different mesophilic *Aeromonas* species as ornamental fish pathogens. This is an area that remains largely unexplored despite the common occurrence of MAS in ornamental fish. We also investigated the antimicrobial susceptibility patterns and the frequency of occurrence of some virulence genes in the study isolates. Molecular fingerprinting of *A. veronii*, which was the predominant species isolated in the present study, was also performed using enterobacterial repetitive intergenic consensus (ERIC-)PCR to determine the presence of any dominant clones associated with septicaemia in fish.

**MATERIALS AND METHODS**

**Sampling of freshwater ornamental fish**

Moribund freshwater ornamental fish presenting at least one or more of the clinical signs of septicaemia (focal ulcerations on skin, haemorrhages on skin and fins, fin rot, exophthalmia, abdominal distension and scale protrusion) were collected from commercial aquaria and breeding farms located in the northwestern, central, and north central provinces of Sri Lanka during the period from May 2007 to June 2008. Fish were transported to the laboratory in well-aerated water. Selection of aquaria from which to collect fish was based on the availability of diseased fish at the time of sampling, accessibility and convenience. In addition, septicaemic fish samples submitted for disease investigation to the Center for Aquatic Animal Disease Diagnosis and Research (University of Peradeniya) were also included.

**Bacterial isolation and phenotypic characterization**

All the diseased fish (Table 1) were subjected to a detailed preliminary laboratory examination and humanely euthanized using an overdose of MS 222. The kidneys and/or liver of affected fish were cultured aseptically on trypticase soy agar (TSA; Oxoid) for bacterial isolation and on *Aeromonas* starch DNA agar (Himedia) and Rimler Shotts agar (Himedia) for the preferential selection of aeromonads, and cultures were incubated in duplicate at 28°C and 35°C for 24 h. On few occasions where the fish were not sacrificed for sample collection, swabs from external lesions (ulcers) were used. Cultures in all plates were examined for colony morphology and Gram-staining reactions. Colonies were picked in order to represent all types of colonies in pure cultures and mixed cultures, and were subcultured on TSA. All Gram-negative isolates were subjected to a series of classical phenotypic tests including a cytochrome oxidase test, a motility test, an oxidation fermentation test and a catalase test. Furthermore, the ability to grow at 0% NaCl and the susceptibility to novobiocin were tested. Accordingly, the isolates that were Gram negative, cytochrome oxidase positive, motile, fermentative, catalase positive, able to grow at 0% NaCl and resistant to novobiocin were presumed to be motile *Aeromonas* and were used in further analysis. In instances where more than 1 fish was sampled from the same disease incidence, only 1 presumptive aeromonad isolate was analysed. Stock cultures were maintained for short periods at room temperature on TSA slants, and, for long-term storage, they were maintained at −20°C in tryptic soy broth medium supplemented with 10% glycerol (v/v). Details of the *Aeromonas* cultures used in this study are included in Table 1.

**DNA extraction and molecular identification**

Genomic bacterial DNA was extracted from cultures using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer’s instructions and stored at −20°C for further use. All presumptive *Aeromonas* isolates were first screened using *Aeromonas*-genus-specific primers (Chacón et al. 2002) targeting the glycerophospholipid cholesterol
Acyltransferase gene (gcac) using the reaction and cycling conditions described by Soler et al. (2002); they were then identified at the species level based on the sequencing analysis of 2 housekeeping genes, rpoD and gyrB. An approximately 1100 bp fragment of gyrB and a 820 bp fragment of rpoD were amplified from the template DNA, purified using the FastGene Gel/PCR extraction kit (Nippon Genetics) and sequenced using the BigDye Terminator V3.1 Cycle Sequencing Kit on the 3130xl Genetic Analyzer (Applied Biosystems). The primers and conditions used for PCR amplifications and DNA sequencing were those described by Soler et al. (2004) and Yáñez et al. (2003). Resulting rpoD and gyrB contigs were assembled separately (DNA baser V.3.5.3, Hercul BioSoft), and a BLAST search was carried out to compare the sequences with those held in the National Centre for Biotechnology Information (NCBI, Bethesda, MD).

The aeromonads confirmed by nucleotide sequencing were re-identified using RFLP (restriction fragment length polymorphism) analysis of the PCR-amplified 16S rRNA gene. The objective of this step was to evaluate the concordance between rpoD/gyrB gene sequencing and 16S rDNA-RFLP, since the latter can easily be adopted in ordinary fish disease diagnostic laboratories with no access to gene sequencing facilities. Primers, amplification conditions and the endonuclease digestion conditions for 16S rDNA-RFLP were those described by Borrell et al. (1997) and Figueras et al. (2000). The 16S rDNA digestion products were electrophoresed in 18% v/v polyacrylamide gels at 155 V for 4 h.

**Phylogenetic analysis**

A 667 bp sequence of rpoD and a 926 bp sequence of gyrB were used in the final analysis (Martínez-Murcia et al. 2011). Each nucleotide sequence was determined at least twice to resolve ambiguous areas. These partial gene sequences were aligned (both independently and as a concatenated sequence of 1593 bp) using Clustal W in MEGA Version 5 (Tamura et al. 2011) with those from Aeromonas reference strains representing all published species to date. Genetic distances and clustering were determined using Kimura’s 2-parameter model, and evolutionary trees were constructed by the neighbour-joining method with MEGA 5, with bootstrapping determined for 1000 replicates. The nucleotide sequences determined in this study have been

<p>| Table 1. Origin, laboratory reference numbers and the phylogenetic identification through rpoD/gyrB sequencing of Aeromonas isolates (n = 53) recovered from diseased freshwater ornamental fish |
|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Host</th>
<th>Sample source</th>
<th>Tissue</th>
<th>n</th>
<th>Isolates recovered</th>
<th>Phylogenetic identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poecilia reticulata</td>
<td>Kidney</td>
<td>15</td>
<td>Ae 1, Ae 3, Ae 13, Ae 15, Ae 20, Ae 22, Ae 31, Ae 39, Ae 44, Ae 46, Ae 47, Ae 49−Ae 51, Ae 53</td>
<td>15 A. veronii</td>
<td></td>
</tr>
<tr>
<td>Carassius auratus</td>
<td>Kidney</td>
<td>9</td>
<td>Ae 4, Ae 6, Ae 10, Ae 11, Ae 21, Ae 32, Ae 35, Ae 45, Ae 52</td>
<td>3 A. hydrophila, 4 A. veronii, 1 A. caviae, 1 A. jandaei</td>
<td></td>
</tr>
<tr>
<td>Cyprinus carpio (koi)</td>
<td>Kidney</td>
<td>5</td>
<td>Ae 23, Ae 25, Ae 26, Ae 34, Ae 41</td>
<td>3 A. veronii, 1 A. hydrophila, 1 A. enteropelogenes</td>
<td></td>
</tr>
<tr>
<td>Betta splendens</td>
<td>Kidney</td>
<td>5</td>
<td>Ae 2, Ae 19, Ae 29, Ae 33, Ae 38</td>
<td>5 A. veronii</td>
<td></td>
</tr>
<tr>
<td>Pterophyllum spp.</td>
<td>Kidney</td>
<td>6</td>
<td>Ae 9, Ae 14, Ae 18, Ae 28, Ae 36, Ae 40</td>
<td>5 A. veronii, 1 A. caviae</td>
<td></td>
</tr>
<tr>
<td>Helostoma temminckii</td>
<td>Kidney</td>
<td>3</td>
<td>Ae 5, Ae 17, Ae 30</td>
<td>3 A. veronii</td>
<td></td>
</tr>
<tr>
<td>Poecilia sphenops</td>
<td>Kidney</td>
<td>3</td>
<td>Ae 12, Ae 37, Ae 48</td>
<td>3 A. veronii</td>
<td></td>
</tr>
<tr>
<td>Osphronemus goramy</td>
<td>Ulcer</td>
<td>1</td>
<td>Ae 24</td>
<td>1 A. dhakensis</td>
<td></td>
</tr>
<tr>
<td>Symphysodon spp.</td>
<td>Liver</td>
<td>1</td>
<td>Ae 7</td>
<td>1 A. veronii</td>
<td></td>
</tr>
<tr>
<td>Xiphophorus helleri</td>
<td>Liver</td>
<td>1</td>
<td>Ae 16</td>
<td>1 A. veronii</td>
<td></td>
</tr>
</tbody>
</table>
deposited in DDBJ/EMBL/GenBank databases with the following accession numbers: rpoD AB828727–AB828779 and gyrB AB829112–AB829164 (Table S1 in the Supplement at www.int-res.com/articles/suppl/d109p127_supp.pdf). The GenBank accession numbers for the rpoD and gyrB gene sequences of reference strains used in alignments are listed in Table S2 in the Supplement.

Molecular fingerprinting of Aeromonas veronii using ERIC-PCR

The genetic diversity of Aeromonas veronii isolates (n = 42) was assessed by ERIC-PCR using the primers ERIC 1 (5’-ATG TAA GCT CCT GGG GAT TCA C-3’) and ERIC 2 (5’-AAG TAA GTG ACT GGG GTG AGC G-3’) (Versalovic et al. 1991) and the amplification conditions described by Fontes et al. (2011). Reactions were carried out 2 times for each isolate to confirm reproducibility. The amplification products were electrophoresed in a 1.2% agarose gel in Tris-borate buffer, and the fragment sizes were analysed using PyElph (Pavel & Vasile 2012). Banding patterns of each isolate were converted into a binary matrix, based on the presence and absence of the DNA fragments. To construct the dendrogram, levels of similarity between the profiles were calculated using the Dice coefficient, and cluster analysis of similarity matrices was calculated with the unweighted pair group method with arithmetic averages (UPGMA) (Garcia-Vallvé et al. 1999, Garcia-Vallvé & Puigloo 2002).

Determination of the susceptibility of isolates to antimicrobial agents

Susceptibility of all isolates to 8 different antimicrobial agents was determined by the disk diffusion (Kirby-Bauer) method on Mueller Hinton agar (Oxoid) according to the protocols established by the Clinical and Laboratory Standard Institute (CLSI 2008). The antibiotics and concentration (per disc) tested were as follows: amoxicillin (10 µg), neomycin (10 µg), sulphonmethoxazole-trimethoprim (25 µg), chloramphenicol (30 µg), tetracycline (30 µg), enrofloxacin (0.5 µg), erythromycin (15 µg) and nitrofurantoin (50 µg) (Oxoid). The antimicrobial agents used for susceptibility testing were chosen to cover different antibiotic groups that are used in ornamental fish aquaculture. Plates were incubated at 28°C for 24 h. Based on the size of the inhibition zones, isolates were characterised as sensitive, intermediate, or resistant.

Detection of virulence-related genes by PCR

All the isolates were screened by PCR for the presence of 7 genomic markers potentially linked to virulence—aerolysin (aerA), cytotoxic enterotoxin (act), the heat-stable and heat-labile cytotoxins (ast and alt, respectively), serine protease (ser), DNase (exu) and flagellin (fla)—using the primers and conditions described by Nawaz et al. (2010) and Soler et al. (2002). The sequence of each primer used to amplify the target genes, the expected size of the PCR products and their references are listed in Table S3 in the Supplement.

PCR amplification of integrons

To determine whether the Aeromonas isolates carry integrons, we used PCR amplification to detect Class 1 and Class 2 integrase genes, intI1 and intI2, respectively (Mazel et al. 2000). The primers used for the amplification of these genes and the predicted sizes of the amplification products are listed in Table S3 in the Supplement.

RESULTS

Characteristics of diseased fish and phenotypic identification of isolates

A total of 173 moribund freshwater fish (guppy Poecilia reticulata, n = 42; goldfish Carassius auratus, n = 56; koi carp Cyprinus carpio, n = 19; fighter Betta splendens, n = 27; kissing gourami Helostoma temminckii, n = 9; giant gourami Osphronemus goramy, n = 1; platy Xiphophorus maculatus, n = 2; angel Pterophyllum spp., n = 12; swordtail Xiphophorus spp., n = 1; molly Poecilia sphenops, n = 3; and discus Symphysodon spp., n = 1) originating from 34 aquaria (commercial level, n = 29; small-scale, household-based, n = 5) and ornamental fish breeding farms (n = 7) were examined in this study. All the aquaria/farms from which the diseased fish were collected had many species of fish. Fin rot, haemorrhages, dermal ulceration, ascites, scale protrusion and exophthalmia were the most commonly observed gross signs associated with infections.

Of the bacterial isolates selected from cultures grown on TSA and Aeromonas-selective media, a total of 74 isolates were presumptive Aeromonas (Table 2), as identified by the phenotypic methods, while the rest of the isolates consisted of Citrobacter.
Out of 74 isolates presumptively identified as Aeromonas spp. by phenotypic tests, only 62 isolates (62/74 = 84%) showed amplification of the gcat gene, suggesting that 12 isolates do not belong to the genus Aeromonas (Table 2). However, when the identities of all 74 isolates were confirmed with housekeeping gene, correct identification at the genus level occurred in 98% (52/53) of the Aeromonas isolates characterized in the present study, corroborating the findings of Chacón et al. (2002, 2003) and Beaz-Hidalgo et al. (2010). The rest of the gcat-negative isolates were found by nucleotide sequencing to belong to other genera (data not shown). However, a false positive amplification was observed for 10 isolates which were subsequently identified as Vibrio spp.

Molecular identification

Amplification of gcat gene

Out of 74 isolates subjected to 16S rDNA RFLP, 44 isolates (83%) exhibited 'typical' restriction patterns enabling an accurate speciation comparable with identification obtained through housekeeping gene sequencing. The remaining 9 isolates (7 Aeromonas veronii and 2 A. dhakensis) (17%) could not be assigned to a known species by 16S rDNA RFLP. Those 7 A. veronii isolates exhibited atypical restriction patterns (i.e. different to the pattern published for this species; Borrell et al. 1997, Figueras et al. 2000) (Table 2, Fig. S1 in the Supplement). The 2 A. dhakensis isolates shared an atypical pattern that is closely related to A. caviae. Figueras et al. (2009) reported that A. dhakensis produce either the A. caviae RFLP pattern or a somewhat similar pattern with extra bands.

Identification by 16S rDNA PCR-RFLP

Out of 53 isolates subjected to 16S rDNA RFLP, 44 isolates (83%) exhibited 'typical' restriction patterns enabling an accurate speciation comparable with identification obtained through housekeeping gene sequencing. The remaining 9 isolates (7 Aeromonas veronii and 2 A. dhakensis) (17%) could not be assigned to a known species by 16S rDNA RFLP. Those 7 A. veronii isolates exhibited atypical restriction patterns (i.e. different to the pattern published for this species; Borrell et al. 1997, Figueras et al. 2000) (Table 2, Fig. S1 in the Supplement). The 2 A. dhakensis isolates shared an atypical pattern that is closely related to A. caviae. Figueras et al. (2009) reported that A. dhakensis produce either the A. caviae RFLP pattern or a somewhat similar pattern with extra bands.

Molecular fingerprinting of Aeromonas veronii by ERIC-PCR

ERIC-PCR fingerprints of Aeromonas isolates (Fig. S2 in the Supplement) consisted of 2 to 12 fragments ranging from 125 to 5015 bp. The dendrogram obtained from the ERIC-PCR analysis (Fig. 2) revealed 7 clusters at the 90% similarity level. Isolates within these clusters were considered to be genetically related. Six of these clusters were 2-isolate...
clusters and had 100% similarity. In the remaining cluster, 3 isolates grouped with a similarity level of 100% and grouped with a similarity of 90.9% with the fourth isolate. The ERIC patterns of all remaining isolates (n = 26) were diverse and hence considered as genetically distinct and unrelated, with similarities below 90%. Among these genetically unrelated members, the highest similarity between 2 isolates was 83.3%, as indicated by the Dice coefficient and observed for the isolates Ae 13 and Ae 46, which

Fig. 1. Unrooted neighbour-joining phylogenetic tree constructed from the concatenated partial rpoD and gyrB gene sequences showing the relationships of the 53 Aeromonas isolates isolated in this study with reference strains. Numbers shown next to each node indicate bootstrap values (percentages of 1000 replicates). Coloured circles indicate the presence of the virulence factor genes analysed in this study for each isolate; the coloured triangle indicates the presence of the integrase 1 gene (intI). aerA: aerolysin; ser: serine protease; fla: flagellin; exu: DNase; ast: heat-stable cytotoxic enterotoxin; alt: heat-labile cytotoxic enterotoxin; act: cytotoxic enterotoxin.
were both isolated from the same fish species (guppy).

It was of interest to see that isolates with identical ERIC-PCR patterns (100% similarity) were isolated from different species of fish, indicating that genetically related isolates could infect different species of fish.

**Antimicrobial susceptibility testing**

The susceptibility levels of the 53 *Aeromonas* isolates against 8 antimicrobial agents are shown in Table 3. Besides the classical resistance of aeromonads to amoxicillin (beta lactam antibiotics) (98.1%), the highest resistances encountered were 58.5% to tetracycline and 54.7% to erythromycin. In contrast, the majority of isolates were susceptible to enrofloxacin (84.9%), followed by chloramphenicol (81.1%), neomycin (77.3%) and sulphamethoxazole-trimethoprim (71.7%). Multi-resistance to the tested antibiotics was found in 26 isolates (49%).

**Detection of virulence-related genes and integrons**

The distribution of the 7 virulence genes among motile *Aeromonas* isolates is shown in Table 4. All the isolates harboured at least 3 of the virulence genes tested, while all the virulence genes were present in 6/53 (11%) of isolates that included 5 isolates of *A. veronii* and 1 isolate of *A. dhakensis*. Overall, the genomic marker for flagellin (*fla*, 100%) was the most prevalent, followed by that for DNase (*exu*, 98%), aerolysin/hemolysin (*aerA*, 94%), cytotoxic enterotoxin (*act*, 83%), serine protease (*ser*, 62%), heat-labile cytotoxic enterotoxin (*alt*, 49%) and heat-stable cytotoxic enterotoxin (*ast*, 38%) in the isolates analysed. The most common combination of putative virulence genes was *aer*+*ser*+*fla*+*exu*+*act*+*alt*−*ast*, which was present in 19% (10/53) of isolates. Results of the amplification of integrase genes revealed that *intI1*-carrying bacteria corresponded to 77% (41) of the isolates, whereas the *intI2* gene was not detected in any of the isolates.
found Nielsen et al. 2001, Austin 2011). In contrast, we freshwater fish (Hettiarachchi & Cheong 1994, species classically linked to MAS in Aeromonas from each infection incidence. aeromonads and characterized only a single isolate the current study, which focused preferentially on highly diverse microbial communities. However, uncommon in ornamental fish that live together with 2008). Moreover, mixed infections are probably not has been recognized (Lewbart 2001, Musa et al. since a certain role of these genera as fish pathogens gens with aeromonads, should not be overlooked rio spp., isolated either as sole pathogens or co-patho-
sis the most cited motile species which is in agreement with others who reported the dominance of A. veronii among fish pathogenic aeromonad isolates following molecular identification (Sreedharan et al. 2011, 2013, Hu et al. 2012, Yi et al. 2013). This difference could possibly be related to the host species selected and the geographical location, but it may have been due, at least in part, to the methods of bacterial identification employed. In a study done to investigate the causative organisms behind incidences of bacterial disease (n = 23) among a number of different species of ornamental fish in Sri Lanka, Hettiarachchi & Cheong (1994) found A. hydrophila to be the most dominant species, associated with 18 incidences (78.26%). In contrast, Sreedharan et al. (2013) investigated 3 incidences of disease (1 each in gourami, goldfish and oscar), in Kerala, India, and isolated A. veronii as the causative organism in all 3 cases. A comparatively higher occurrence of A. veronii over A. hydrophila has been reported among aeromonads isolated from diseased eels in the Republic of Korea (Yi et al. 2013) and diseased freshwater aquaculture fish from China (Hu et al. 2012).

The identification of aeromonads is fraught with numerous difficulties due to the phenotypic, serological and genotypic heterogeneity existing within the genus. Discrepancies in identification of Aeromonas resulting from poor correlation between phenotypic and genetic identification schemes have been well documented (Kozinka 2007, Beaz-Hidalgo et al. 2010). This problem also occurred in the present study, where identification using specific but a limited number of phenotypic properties resulted in the apparent misidentification of 21 isolates as aeromonads.

gcat, a gene that is used to identify Aeromonas at the genus level (Chacón et al. 2002), was useful in the majority (98%) of isolates, with slight deviations in the rest. gcat is often considered a virulence-related gene but aeromonads for which the gcat gene cannot be amplified have been reported (Nawaz et al. 2010), as we observed for 1 of our isolates. This could be the result of a possible mismatch of primers, as noted by Chacón et al. (2002). However, false positive amplification of this gene resulted in the misidentification of 10 isolates as belonging to the genus Aeromonas even though they actually belonged to the genus Vibrio. This could have been due to the low annealing temperature (56°C) during amplification. Weak amplification of the same size fragment at

<table>
<thead>
<tr>
<th>Aeromonas species</th>
<th>n</th>
<th>aerA</th>
<th>act</th>
<th>alt</th>
<th>virulence gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. veronii</td>
<td>42</td>
<td>42 (100)</td>
<td>36 (86)</td>
<td>22 (52)</td>
<td>alt = 14 (33); ser = 27 (64); exu = 41 (98); fla = 42 (100)</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td>4</td>
<td>2 (50)</td>
<td>3 (75)</td>
<td>2 (50)</td>
<td>aerA = 4 (100); alt = 3 (75); ser = 4 (100); exu = 4 (100)</td>
</tr>
<tr>
<td>A. caviae</td>
<td>3</td>
<td>2 (67)</td>
<td>2 (67)</td>
<td>1 (33)</td>
<td>alt = 1 (33); ser = 0 (0); exu = 3 (100)</td>
</tr>
<tr>
<td>A. dhakensis</td>
<td>2</td>
<td>2 (100)</td>
<td>2 (100)</td>
<td>1 (50)</td>
<td>ser = 1 (100); exu = 2 (100); fla = 2 (100)</td>
</tr>
<tr>
<td>A. jandaei</td>
<td>1</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>ser = 1 (100); exu = 1 (100)</td>
</tr>
<tr>
<td>A. enteropelogenes</td>
<td>1</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td>ser = 1 (100); exu = 1 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>50 (94)</td>
<td>44 (83)</td>
<td>26 (49)</td>
<td>20 (38); 33 (62); 52 (98); 53 (100)</td>
</tr>
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</table>

### DISCUSSION

Isolation of 6 different species of motile aeromonads from 10 diverse species of ornamental fish during the current study adds more evidence for the wide host range and geographical distribution of this bacterium. Motile aeromonads often act as secondary pathogens in fish; therefore, their isolation from septicaeamic fish is not so surprising in view of the intensive culture practices in ornamental fish farming that might favour opportunistic infections. The role of other bacteria such as Citrobacter spp., Pseudomonas spp., Flavobacterium spp., Enterobacter spp., or Vibrio spp., isolated either as sole pathogens or co-pathogens with aeromonads, should not be overlooked since a certain role of these genera as fish pathogens has been recognized (Lewbart 2001, Musa et al. 2008). Moreover, mixed infections are probably not uncommon in ornamental fish that live together with highly diverse microbial communities. However, incidences of mixed infections were not examined in the current study, which focused preferentially on aeromonads and characterized only a single isolate from each infection incidence.

Aeromonas hydrophila is the most cited motile Aeromonas species classically linked to MAS in freshwater fish (Hettiarachchi & Cheong 1994, Nielsen et al. 2001, Austin 2011). In contrast, we found A. veronii (79.2%) to be the predominant species which is in agreement with others who reported the dominance of A. veronii among fish pathogenic aeromonad isolates following molecular identification (Sreedharan et al. 2011, 2013, Hu et al. 2012, Yi et al. 2013). This difference could possibly be related to the host species selected and the geographical location, but it may have been due, at least in part, to the methods of bacterial identification employed. In a study done to investigate the causative organisms behind incidences of bacterial disease (n = 23) among

### Table 4. Numbers of putative virulence genes in a total of 53 Aeromonas isolates recovered from ornamental fish. Percent of isolates in parentheses. aerA: aerolysin; act: cytotoxic enterotoxin; alt: heat-labile cytotoxic enterotoxin; ser: serine protease; exu: DNase; fla: flagellin

<table>
<thead>
<tr>
<th>Aeromonas species</th>
<th>n</th>
<th>aerA</th>
<th>act</th>
<th>alt</th>
<th>Ser</th>
<th>Exu</th>
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<td>A. veronii</td>
<td>42</td>
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<td>27 (64)</td>
<td>41 (98)</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td>4</td>
<td>2 (50)</td>
<td>3 (75)</td>
<td>2 (50)</td>
<td>4 (100)</td>
<td>3 (75)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>A. caviae</td>
<td>3</td>
<td>2 (67)</td>
<td>2 (67)</td>
<td>1 (33)</td>
<td>1 (33)</td>
<td>0 (0)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>A. dhakensis</td>
<td>2</td>
<td>2 (100)</td>
<td>2 (100)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>2 (100)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>A. jandaei</td>
<td>1</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>A. enteropelogenes</td>
<td>1</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (100)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>50 (94)</td>
<td>44 (83)</td>
<td>26 (49)</td>
<td>20 (38)</td>
<td>33 (62)</td>
<td>52 (98)</td>
</tr>
</tbody>
</table>
low annealing temperatures was observed in some *Vibrio* isolates by Chacón et al. (2002); this problem was overcome by increasing the temperature to 65°C.

The *rpoD* and *gyrB* genes have already been used successfully as accurate, unequivocal molecular chronometers for identification of the genus *Aeromonas* (Yañez et al. 2003, Soler et al. 2004, Martínez-Murcia et al. 2011). Combined analysis of the *rpoD* and *gyrB* genes improved resolution and enabled unambiguous speciation of all isolates used in the present study. In comparison, 16S rRNA PCR-RFLP (Borrell et al. 1997, Figueras et al. 2000), while being discriminatory for the majority of isolates (83%), produced either atypical restriction patterns or patterns that were very similar to those of other species in the rest of the isolates. This could be due to the high sequence similarity and the occurrence of microheterogeneities in the 16S rRNA genes (Alperi et al. 2008). Closely related species (i.e. those that have an identical or almost identical 16S rRNA gene sequence) produce the same RFLP pattern, as in the case of *A. piscicola* which has the same pattern as *A. salmonicida* and *A. bestiarum* (Beaz-Hidalgo et al. 2010) and *A. dhakensis* which has the same pattern as *A. caviae* (Figueras et al. 2009). According to these observations, a need for the incorporation of polyphasic molecular approaches in precise species identification of aeromonads becomes clearly evident. While the exact taxonomic position of fish-pathogenic *Aeromonas* species might not be of interest to all fish pathologists, its use in epidemiological studies and in recognizing new pathogenic species and subspecies should not be overlooked. However, for ordinary fish disease diagnostic laboratories, with no access to gene sequencing, other techniques such as those mentioned above are still of use, even though the known limitations could result in misidentification/underestimation of different pathogenic species.

*A. jandaei* and *A. enteropelogenes* have rarely been isolated from ornamental fish (John & Hatha 2012), and little information is available regarding their association with clinical disease in ornamental fish. To the best of our knowledge, this is the first time that *A. dhakensis* has been recovered from clinically diseased ornamental fish since the original description of this species from the aquarium water and skin of ornamental fish (Martínez-Murcia et al. 2008, Beaz-Hidalgo et al. 2013). Indeed, it is the first time this species has been isolated in Sri Lanka. Isolation of this seemingly globally distributed species (Aravena-Román et al. 2011, Yi et al. 2013) from diseased ornamental fish provides additional evidence that a diverse range of motile aeromonads could potentially be associated with septicaemia in ornamental fish. The occurrence and pathogenesis of these rarely isolated motile aeromonads in tropical aquarium fish are not well understood and deserve further study.

ERIC-PCR, a low cost, rapid, reproducible strain typing method with high discriminatory power (Soler et al. 2003) revealed the intraspecific diversity that exists within the *A. veronii* isolated in the present study. The majority of isolates were genetically distinct (62% of the isolates had similarities below 90%), with no dominant clones of *A. veronii* associated with MAS in the fish population investigated. This kind of high genetic diversity had been observed among clinical and environmental isolates of aeromonads from different sources typed by the above technique (Davin-Regli et al. 1998, Szczuka & Kaznowski 2004).

High levels of antimicrobial resistance in bacteria isolated from ornamental fish and their environment is not a novel observation (Verner-Jeffreys et al. 2009, Cizek et al. 2010, Dias et al. 2012). In agreement, surprisingly numerous multi-antibiotic-resistant bacteria (49%) were observed among our isolates, apart from their classical resistance to beta lactam antibiotics (Janda & Abbott 2010). Tolerance to tetracycline and erythromycin was particularly widespread (>50%), a finding that was in common with other comparable investigations of motile aeromonads from ornamental fish (Dias et al. 2012, Sreedharan et al. 2012). While tolerance of these antibiotics has likely resulted from their use in the aquarium fish industry, resistance can also arise from gene mutations or by acquisition of transferable genetic elements such as integrons (Jacobs & Chenia 2007). The observed levels of multi-resistance could be attributed to the horizontal spread of resistance genes, which is further supported by the presence of Class 1 integrons in 77% of the isolates.

Screening for the presence of virulence genes as a method to evaluate the potential virulence of aeromonads could be speculative, since virulence is a complex process, and empirical testing with a disease challenge is often necessary for conclusive results. However, expression of the putative virulence-associated factors in *Aeromonas* appears to be affected by environmental conditions (Tso & Dooley 1995, Merino et al. 1998), making the detection of true virulent strains difficult. Nevertheless, screening for virulence genes has been used in many studies as a practical approach for evaluating the genetic potential of aeromonads to express virulence factors (Puthucheary et al. 2012). In agreement with previ-
ous studies (Nawaz et al. 2010, Hu et al. 2012, Yi et al. 2013), we found high heterogeneity in the distribution of toxin genes among the tested isolates, with a heterogeneous distribution forming 18 different virulence gene combinations. High prevalences of *gcat*, *exu*, *fia*, *act* and *aerA* genes are consistent with the results of Yi et al. (2013) and Nawas et al. (2010) from clinical isolates of fish.

In conclusion, the present study highlights the diversity of mesophilic *Aeromonas* species that could potentially be associated with MAS in ornamental fish. The ability of rare aeromonad species to act as fish pathogens needs to be explored further, in order to clarify their role in disease. The relatively high prevalence of antimicrobial-resistant bacteria harbouring multiple virulence genes raises concerns about possible treatment failures in fish disease outbreaks and the public health threats they may pose, given the importance of aeromonads as emerging human pathogens (Janda & Abbott 2010). Overall, the results obtained highlight the need to promote responsible ornamental fish ownership, good husbandry practices and prudent use of antimicrobials in the ornamental fish industry.

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