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Isolation and molecular identification of *Aeromonas* species from the tank water of ornamental fishes

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RESEARCH ARTICLE

ABSTRACT

Aeromonads are recognised as important pathogens of fishes. In this study, ten water samples were randomly collected from pet shops' fish tanks and home aquaria inhabited by several fish species (silver arowana, koi, goldfish, catfish, pictus fish, silver shark and silver dollar fish). Altogether 298 colonies were isolated using *Aeromonas* selective agar. A total of 154 isolates were then confirmed as belonging to the genus *Aeromonas* using the *GCAT* gene. Using ERIC-PCR, a total of 40 duplicate isolates were excluded from the study and 114 isolates were subjected to PCR-RFLP targeting the RNA polymerase sigma factor (*rpoD*) gene using lab-on-chip. A total of 13 different *Aeromonas* species were identified. The most prevalent species were *A. veronii* (27%, 31/114), followed by *A. dhakensis* (17%, 19/114), *A. finlandiensis* (9%, 10/114), *A. caviae* (8%, 9/114), *A. hydrophila* (4%, 4/114), *A. jandaei* (4%, 4/114), *A. rivuli* (3%, 3/114), *A. enteropelogens* (2%, 2/114), *A. tecta* (2%, 2/114), *A. allosaccharophila* (1%, 1/114), *A. eucrenophila* (1%, 1/114), *A. media* (1%, 1/114) and *A. diversa* (1%, 1/114). Twenty-six isolates (23%) were unidentifiable at species level. The present study demonstrates that *Aeromonas* species are highly diverse in freshwater fish tanks, and suggests the potential risks posed by the isolated bacteria to the health of ornamental fish species.

KEYWORDS

aeromonads, freshwater ornamental fish, micro-fluid chip, PCR-RFLP targeting the *rpoD* gene

INTRODUCTION

Aeromonas are oxidase-positive, facultative anaerobic Gram-negative rods. They are ubiquitous in nature, and they are present in a wide range of clinical and environmental habitats (Puah et al., 2013; Khor et al., 2015). The genus *Aeromonas* was formerly classified within the family *Vibrionaceae* and later a new family, known as *Aeromonadaceae*, was created for it. The taxonomical characterisation of the genus is difficult due to its phenotypic and genotypic heterogeneity. To date, a total of 36 species have been identified (Figueras et al., 2017).

Aeromonas infections are the most common bacterial diseases of freshwater fishes (Kadlec et al., 2011). Two major types of fish diseases caused by *Aeromonas* infections can be identified. Fish furunculosis caused by *A. salmonicida* is characterised by septicemia with haemorrhages. The other type of infection by mesophilic species (mainly by *A. hydrophila* and *A. veronii*) causes a similar assortment of illness in fishes (Dumen et al., 2018). *Aeromonas* bacteria are also capable of generating disease in humans. In recent years, increasing numbers of *Aeromonas* infection cases have been reported, producing diseases ranging from gastrointestinal to extraintestinal infections (Janda and Abbott, 2010; Puthucheary et al., 2012; Khor et al., 2018).

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Ornamental fish husbandry is an increasingly popular hobby with a steady expansion in trade in more than 125 countries (Dey, 2016). This hobby makes fishes one of the most common pets kept in Western households (Sullivan et al., 2016). In Malaysia, the ornamental fish industry is considered an important source of income with the major export to markets in the United States of America and Japan. However, intensified fish breeding and keeping has often resulted in poor broodstock quality and increased susceptibility to infections (Dey, 2016). Recently, Gauthier (2015) has raised concerns of the risk of bacterial zoonoses originating from ornamental fishes, especially among fish hobbyists. Of the many potential bacterial agents of fish-borne zoonoses, *Aeromonas* was reported as the most common zoonotic bacterium isolated from ornamental fishes (Weir et al., 2012).

Thus far, the study of ornamental fish infections in Malaysia, especially those caused by *Aeromonas* species, has received very little attention. However, investigations on the importance of *Aeromonas* infections in ornamental fishes and their water have been conducted in other countries. Dias et al. (2012) and Baron et al. (2017) showed that most aeromonads isolated from ornamental fishes are potential reservoirs of antibacterial resistance genes and that ornamental fishes could be a possible transmission route for aeromonads. This finding was also supported by Weir et al. (2012) in a systematic study conducted in Canada, which showed that the most common zoonotic bacterium isolated from ornamental fishes was *Aeromonas*. Hence, in the present study, our aim was to investigate the presence and diversity of *Aeromonas* in water samples collected from the fish tanks of randomly selected pet shops and home aquaria (or tanks) in Klang Valley, Malaysia.

MATERIALS AND METHODS

Sample collection

From October 11, 2016 to December 5, 2016, ten water samples were collected from different spots of the Klang

Valley, Malaysia. This included 5 samples from home aquaria ($n = 2$) and koi ponds ($n = 3$), and 5 samples from the fish tanks of randomly selected pet shops. The details of the fish species reared in home aquaria, fish ponds and tanks are described in Table 1. One litre of surface water was collected into sterilised plastic bottles with tight screw caps for each sample. All water samples were kept at 4 °C until analysis (up to 48 h after collection).

Bacterial isolation and biochemical tests

Bacterial isolation was conducted using the EPA Method 1605 (USEPA, 2001). Water samples were pre-filtered, and 500 mL of them were subjected to membrane filtration using 0.45-µm nitrocellulose membranes (Sartorius Stedim, Biotech). Filter membranes were incubated in pre-enriched Luria Bertani (LB) broth overnight at 30 °C, subjected to serial dilutions, and then plated onto *Aeromonas* selective agar (Biolife, Italiana S.r.l.) supplemented with 10 mg/L ampicillin (Gifco, Thermo Fisher Scientific). Yellow colonies ($n = 10$ –40) from each water sample were selected randomly, and purified by streaking on LB agar. Single colonies were subjected to three phenotypic tests for further characterisation: oxidase test, lactose fermentation test and 6.5% salt tolerance test. For the oxidase test and the lactose fermentation test, *A. hydrophila* ATCC 7966 and *Escherichia coli* ATCC 25922 were used as positive and negative controls, respectively. For the salt tolerance test, *Staphylococcus aureus* ATCC 25923 was used as positive control, whereas *A. hydrophila* ATCC 7966 as negative control. Oxidase-positive colonies with negative growth in the salt tolerance test and non-lactose fermenters were presumptively identified as *Aeromonas* species, and they were selected for molecular analysis.

DNA extraction

An in-house-adapted method for DNA extraction was performed (Puah et al., 2017). A total of 2 mL of the overnight bacterial culture was pelleted and washed vigorously with sterile, deionised water. The supernatant was discarded, and the pellet was resuspended in deionised water and incubated

Table 1. Main features of the source of water samples, including the inhabiting fish species

Source	Fish species	Source ID
Home aquarium	Home koi pond	Koi (<i>Cyprinus carpio</i>)
	Home aquarium	Goldfish (<i>Carassius auratus</i>)
	Home aquarium	Silver arowana (<i>Osteoglossum bicirrhosum</i>)
	Home koi pond	Koi (<i>Cyprinus carpio</i>)
	Second Residential College koi pond	Koi and catfish (<i>Cyprinus carpio</i> and <i>Clarias gariepinus</i>)
Pet shop	Irene's Aquarium	Goldfish (<i>Carassius auratus</i>)
	Akarikoi	Koi (<i>Cyprinus carpio</i>)
	The Pet Family	Pictus catfish (<i>Pimelodus pictus</i>)
	Loy's Aquarium	Koi (<i>Cyprinus carpio</i>)
	Fish Depot	Silver shark and silver dollar fish (<i>Balantiocheilos melanopterus</i> and <i>Metynnis argenteus</i>)

Table 2. Isolation, characterisation and species identification of aeromonads isolated from the tank water of ornamental fishes: Numbers in cells indicate the number of isolates that passed the analysis (i.e. gave a positive result)

Water sampling site	Microbiological analysis				Molecular analysis		
	Phenotype on <i>Aeromonas</i> selective agar	Oxidase test	6.5% salt tolerance test	Lactose fermentation test	GCAT screening	Number of isolates after clonal exclusion (ERIC-PCR)	<i>rpoD</i> -RFLP
Home aquarium (including koi pond)	149	141	140	139	74	54	<i>A. veronii</i> (n = 16) <i>A. dhakensis</i> (n = 13) <i>A. caviae</i> (n = 4) <i>A. finlandiensis</i> (n = 6) <i>A. jandaei</i> (n = 4) <i>A. tecta</i> (n = 2) <i>A. rivuli</i> (n = 1) <i>A. eucrenophila</i> (n = 1) Atypical RFLP pattern (n = 7)
Pet shop	149	117	116	116	80	60	<i>A. veronii</i> (n = 15) <i>A. dhakensis</i> (n = 6) <i>A. caviae</i> (n = 5) <i>A. finlandiensis</i> (n = 4) <i>A. hydrophila</i> (n = 4) <i>A. rivuli</i> (n = 2) <i>A. enteropelogens</i> (n = 2) <i>A. allosaccharophila</i> (n = 1) <i>A. media</i> (n = 1) <i>A. diversa</i> (n = 1) Atypical RFLP pattern (n = 19)
Total	298	258	256	255	154	114	Identified (n = 88) Unidentified (n = 26)

at 95 °C for 5 min. The suspension was immediately snap-incubated on ice for 10 min to precipitate the DNA. Following centrifugation, the supernatant containing the bacterial DNA was transferred into new labelled tubes. DNA concentration and purity were determined using a nanophotometer (Implen, Germany). Extracted genomic DNA was diluted to the final concentration of 50 and 100 ng/μL for subsequent analysis.

Genus identification

Isolates were subjected to genus confirmation using the *GCAT* gene as described by Chacón et al. (2002). The DNA amplification reaction was carried out in a PCR mixture that

contained 1U Dream Taq polymerase (Fermentas, Thermo Fisher), 0.2 μM forward primer, 0.2 μM reverse primer, 50 ng of DNA template and RNase-free water. The mixture was denatured at 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products were then electrophoresed in a 1.5% (w/v) agarose gel. *Aeromonas hydrophila* ATCC 7966 and *E. coli* ATCC 25922 were used as positive and negative controls, respectively.

ERIC-PCR analysis

GCAT-positive samples were subjected to further investigation using ERIC-PCR to exclude clonal types. ERIC-PCR

was carried out with the primer pairs described by Versalovic et al. (1991) using the method by Szczuka and Kaznowski (2004) as well as Yano et al. (2015) with some modifications. DNA amplification was carried out in a PCR mixture that contained 2.4 U of Taq DNA polymerase (Fermentas, Thermo Fisher), 100 ng of DNA template, 30 pmol of primers, 250 μ M dNTP mixture, 3.75 mM MgCl₂ and 1 $\times$ buffers with (NH₄)₂SO₄. The modified PCR conditions for optimal DNA amplification were initial denaturation at 95 °C for 7 min followed by 30 cycles at 90 °C for 30 s, 52 °C for 1 min and 65 °C for 8 min, and a final extension at 65 °C for 16 min. The amplified products were electrophoresed in 1.5% (w/v) agarose gels with 10 mg/mL ethidium bromide at 56 V for 4 h. ERIC fingerprints and banding patterns captured as pictures were input into GelJ software (Heras et al., 2015). Digitised profiles were labelled and analysed for construction of a dendrogram. The Dice coefficient was used to calculate the similarity between fingerprints, while cluster analysis was performed using the unweighted pair-group method with arithmetic mean (UPGMA) with a tolerance of 4.0% for matching. All isolates showing unique ERIC-PCR patterns were subjected to species identification.

Species identification

Aeromonas isolates were subjected to a lab-on-a-chip-based PCR-RFLP of the *rpoD* gene fragment for species identification (Puah et al., 2018). Briefly, partial *rpoD* amplicons were cut by the AluI restriction enzyme for separation on DNA 1000 chip using Agilent 2100 Bio-analyser. Digested patterns were compared with the *in silico* digestion patterns of 27 type strains for

identification. Differentiation between *A. jandaei* and *A. sobria* required further confirmation through RsaI restriction enzyme cut profile. The PCR-RFLP was carried out in duplicate.

RESULTS

Bacterial isolation

Water samples from ten ornamental fish tanks were successfully collected from home environments ($n = 5$, 3 koi ponds and 2 home aquaria) and commercial pet shops ($n = 5$) located in the Klang Valley (Malaysia). The processed water samples subsequently yielded 298 colonies on the selective agar. The number of selected colonies depended on the diversity of the colonies in each sample (Table 1).

Biochemical tests

Upon screening, 40 colonies were excluded due to their inability to oxidise the test reagent to indophenols. Two other isolates were neglected on the basis of the results of the 6.5% salt tolerance test. The remaining 256 colonies were further characterised based on their ability to ferment lactose. A total of 255 isolates presented typical aeromonad characteristics with colourless, non-lactose fermenting colonies and they were selected for molecular analysis (Tables 2 and 3).

Genus identification and ERIC-PCR analysis

One hundred and fifty-four out of the 255 presumptive *Aeromonas* isolates produced a distinct amplified 247-bp

Table 3. The distribution of *Aeromonas* species (in percentage) in tank water inhabited by different fish species. Source ID: W: home aquarium, P: pet shop (for more details see Table 1)

<i>Aeromonas</i> species	Strains, n (%)					
	Fish tank with koi fish $n = 45$	Fish tank with goldfish $n = 16$	Fish tank with arowana $n = 16$	Fish tank with pictus catfish $n = 19$	Fish tank with both Silver shark and dollar fish $n = 4$	Fish tank with both koi and catfish $n = 10$
Source ID	(W1, W4, P2, P4)	(W2, P1)	(W3)	(P3)	(P5)	(W5)
<i>A. veronii</i>	16 (36)	7 (35)	0	0	3 (75)	5 (50)
<i>A. dhakensis</i>	2 (4)	1 (5)	11 (69)	5 (26)	0	0
<i>A. caviae</i>	4 (9)	1 (5)	0	3 (16)	1 (25)	0
<i>A. finlandiensis</i>	4 (9)	0	1 (6)	0	0	5 (50)
<i>A. hydrophila</i>	0	0	0	4 (21)	0	0
<i>A. jandaei</i>	0	0	4 (25)	0	0	0
<i>A. tecta</i>	2 (4)	0	0	0	0	0
<i>A. rivuli</i>	3 (7)	0	0	0	0	0
<i>A. enteropelogens</i>	0	2 (10)	0	0	0	0
<i>A. allosaccharophila</i>	0	1 (5)	0	0	0	0
<i>A. eucrenophila</i>	1 (2)	0	0	0	0	0
<i>A. media</i>	0	0	0	0	0	0
<i>A. diversa</i>	0	0	0	1 (5)	0	0
Unclassifiable	12 (27)	8 (40)	0	6 (32)	0	0

product of the *GCAT* gene, and thus these isolates were confirmed to belong to the genus *Aeromonas*. These isolates were then subjected to ERIC-PCR (Fig. 1a and b). Using ImageJ software, ERIC-fingerprinting revealed 114 clusters of which 20 clusters (rectangle box) contained more than one isolate (Fig. 1a and 1b). The members of the clusters (60 isolates in total) were regarded as clones of each cluster. Thus one representative of each cluster (114 isolates in total) were subjected to species identification.

Species identification

All 114 isolates produced the 770–782-bp-sized target amplicon from the *rpoD* gene with the *AluI* restriction enzyme site. The PCR-RFLP products were resolved on a microfluidic-based lab-on-a-chip, and 13 *Aeromonas* species were identified (Fig. 2a and b) according to the expected fragment pattern described by Puah et al. (2018). Overall, the most prevalent species was *A. veronii* (27%, 31/114), followed by *A. dhakensis* (17%, 19/114), *A. finlandiensis* (9%, 10/114), *A. caviae* (8%, 9/114), *A. hydrophila* (4%, 4/114), *A. jandaei* (4%, 4/114), *A. rivuli* (3%, 3/114), *A. enteropelogens* (2%, 2/114), *A. tecta* (2%, 2/114), *A. allosaccharophila* (1%, 1/114), *A. eucrenophila* (1%, 1/114), *A. media* (1%, 1/114) and *A. diversa* (1%, 1/114) (Table 3, Fig. 3). The RFLP fragment patterns of *A. jandaei* and *A. sobria* were very similar, hence an additional restriction enzyme, *RsaI*, was used to validate the identity of *A. jandaei* (Fig. 2b). However, 26 different isolates, clustering into 6 types of RFLP patterns, were not identifiable with any of the 27 known species.

DISCUSSION

A total of 298 presumptive aeromonad strains were isolated from ornamental water samples. Using three biochemical assays – oxidase activity, lactose fermentation and salt tolerance tests – 43 presumptive strains were excluded (255/298). A low efficiency (60%) was noted for the biochemical identification of *Aeromonas* in comparison to the molecular method targeting the *GCAT* gene (154/255). The lack of accuracy in the biochemical tests is probably due to the limited number of phenotypic tests employed in this study. Carriero et al. (2016) previously described that the use of a wide variety of phenotypic tests can be useful for the identification of aeromonads. During the isolation of *Aeromonas* spp. from environmental and clinical habitats, biochemical tests are often employed for initial screening prior to taxonomic confirmation by molecular assays. Khor et al. (2015) applied the API 20E system (bioMérieux, France) for the characterisation of environmental aeromonads, while Soltan Dallal et al. (2016) used various biochemical tests including oxidase test, production of acetylmethylcarbinol, fermentation of glucose and lactose, gas production from glucose, acid production from arabinose and API 20E on clinical

isolates of aeromonads from stool specimens of children with diarrhoea. Thus, biochemical tests are still applicable for the pre-screening of *Aeromonas* spp. isolated from water sources of ornamental fishes.

In our study, ERIC fingerprints indicated that the *Aeromonas* spp. were highly diverse across different water samples. These results supported the observations of Jagoda et al. (2014) who recognised increased intraspecific diversity of *A. veronii* by this method. The ability of ERIC-PCR to reveal the clonality of isolates was reported by Khor et al. (2015), where 30 isolates out of the 122 aeromonads isolated from Malaysian lakes successfully formed 10 clusters. Furthermore, ERIC-PCR was performed to identify new species of *Aeromonas* isolated from water sources in Finland (Beaz-Hidalgo et al., 2015b). Some isolates showed remarkably different ERIC fingerprint patterns to each other and to all previously unidentified species. They were subsequently identified as new species, called *A. aquatica*, *A. finlandiensis* and *A. lacus* (Beaz-Hidalgo et al., 2015b). In the present study, three isolates of unknown species produced an absence of bands in ERIC-PCR despite multiple repetitions of amplification. This observation has not been reported by previous studies and thus requires further investigation. The lack of amplification may be due to the atypical ERIC sequences, and thus to the lack of binding sites of the currently used primers.

The number of species identified in this study was higher than that detected in previous published investigations (Table 3). Yano et al. (2015) reported five *Aeromonas* species isolated from shrimps from 16 inland ponds in Thailand, while Khor et al. (2015) reported the recovery of nine species of *Aeromonas* isolated from five freshwater lakes in Malaysia. In the study conducted by Dias et al. (2012), only 7 *Aeromonas* species (*A. aquariorum*, *A. caviae*, *A. culicicola*, *A. hydrophila*, *A. jandaei*, *A. media* and *A. veronii*) were identified from the skin of 30 ornamental fish and 14 water samples from pet shops in Portugal. Of these 7 species, all species except *A. culicicola* were detected in our study. The diversity of *Aeromonas* species isolated in our study may be related to the fish species composition of the water tanks, fish ponds and home aquaria. The most abundant species, *A. veronii* was detected in water samples associated with either one fish species (koi or goldfish) or with the parallel presence of two fish species (silver shark and dollar fish; koi and catfish). *Aeromonas veronii* is a known fish pathogen (Sreedharan et al., 2013; Walczak et al., 2017), thus its presence in the home aquaria is a potential health risk to ornamental fish species. Our results showed that *A. dhakensis* was the most common species in aquaria with silver arowana, one of the world's most expensive ornamental fishes. Jagoda et al. (2014) isolated *A. dhakensis* from koi fish and giant gourami showing signs of septicaemia, which indicated its ability to infect ornamental fish. Furthermore, *A. sobria* was detected from moribund silver arowana in Korea (Han et al., 2012), although the first identification of

Fig. 1. Dendrogram showing ERIC-PCR profiles of the *Aeromonas* isolates using UPGMA cluster method based on Dice coefficient with a tolerance of 4.0%. Red rectangles indicate similar ERIC-PCR patterns, white and black boxes denote unidentified and identified species, respectively, using *rpoD*-RFLP analysis. (a) ERIC-PCR dendrogram part 1/2 with 71 samples; (b) ERIC-PCR dendrogram part 2/2 with 83 samples

Fig. 3. Distribution of *Aeromonas* spp. in the tank water of ornamental fishes in home environment and commercial pet shops. Thirteen species of *Aeromonas* were successfully identified among the 114 isolates and are presented on the diagram in the order of their prevalence, while 26 isolates were not identifiable at species level

the species might have been incorrect (Beaz-Hidalgo et al., 2015a).

In the present study, the most commonly isolated species were *A. veronii*, *A. dhakensis*, *A. caviae* and *A. finlandiensis*. Of these 4 species, *A. veronii* was the most prevalent species identified in the water samples (27%, 31/114). Similar findings were reported in Portugal (42%, 94/225) with aeromonads isolated from ornamental fishes (Dias et al., 2012) and in Korea (17%, 13/70) from diseased fish at eel farms (Yi et al., 2013). Besides environmental samples, *A. veronii* was reported to be highly prevalent also in clinical specimens, especially in Taiwan, where this species constituted 44% of bacteraemia cases in the Chi Mei Medical Centre (Tang et al., 2014). *Aeromonas dhakensis* was the second most prevalent species isolated in this study (17%, 19/114). This species was first isolated from the tank water and skin of ornamental fishes (Martinez-Murcia et al., 2008). *Aeromonas dhakensis* was mostly identified from home aquaria in SS20 Petaling Jaya city with only arowana fish (10%, 11/114). Its prevalence in environmental samples has also been reported by Dias et al. (2012) from pet shops in Portugal (19%, 43/225), and from tank water and fishes in Australia (50%, 26/52) (Aravena-Román et al., 2011). *Aeromonas dhakensis* has been recognised as an emerging pathogen for both humans and fishes (Esteve et al., 2012; Chen et al., 2014). *Aeromonas finlandiensis* (9%, 10/114) and *A. caviae* (8%, 9/114) were isolated from tank waters of ornamental fishes in the present study. While *A. caviae* has been observed in water samples, *A. finlandiensis* has not been reported in ill fishes (Sreedharan et al., 2012; Jagoda et al., 2014). *A. finlandiensis* is a new member of the *Aeromonas* family. It was first isolated from Lake Pyhälampi in Finland where waters in the lake showed

an elevated nitrogen content (Beaz-Hidalgo et al., 2015b). In that study, this species was isolated from a koi pond from the Second Residential College in University of Malaya (50%, 5/10). To date, *A. finlandiensis* has not been reported to cause any human clinical infections.

The species identification of 26 isolates possessing yet unknown *rpoD*-RFLP patterns was unsuccessful. The inability to classify these isolates could be due to the lack of a complete *rpoD*-RFLP database for all 36 established *Aeromonas* species. As yet, no *rpoD* sequence information is available for *A. aquatilis*, *A. cavernicola*, *A. enterica*, *A. crassostreae*, *A. intestinalis* and *A. lusitana* in the public databases. Thus, the 26 isolates could potentially belong to these species or even to new species that have not been described so far. For the identification of these isolates at species level, other platforms like multilocus sequence typing or whole genome sequencing should be performed.

In summary, the results of the present study highlight the potential risks of the detected *Aeromonas* species to the health of ornamental fishes. In freshwater tropical ornamental fishes, haemorrhagic septicaemia, red pest and red sore have been reported in association with motile *Aeromonas* species such as *A. veronii*, *A. caviae*, *A. hydrophila* and *A. jandaei* (John and Hatha, 2013; Dumen et al., 2018). Considering the fast-growing ornamental fish industry, the elaboration of fish health strategies is needed for the effective control of these microorganisms.

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