Short Notes

Identification error of *Aeromonas aquariorum*: a causative agent of septicemia

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Running title: Clinical misidentification of *Aeromonas aquariorum*

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24 **ABSTRACT**

25 A genetic re-identification of seven *Aeromonas* blood isolates revealed that phenotype-based
26 identification systems misidentified 5 (71.4%) isolates. In *Aeromonas* strains, *A. aquariorum*
27 was the most common misidentified-organism and showed the most potent cytotoxic
28 activities against human blood cell lines, suggesting that the correct identification of *A.*
29 *aquariorum* is important.

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The genus *Aeromonas* is comprised of oxidase-positive, glucose-fermenting, gram-negative rods that are widely distributed in freshwater, estuarine and marine environments and causes a variety of human illnesses such as enterocolitis and septicemia (Figueras, 2005; Janda & Abbott, 2010; Morinaga, et al., 2011). We previously reported that *Aeromonas* septicemia occurred frequently in hospitalized patients with underlying diseases including malignancy in Japan (Morinaga, et al., 2011). Phenotypical differentiation of the genus *Aeromonas* or its species has been recognized to be difficult because of the non-uniform biochemical reactions, and can lead to misidentification (Abbott, Cheung, & Janda, 2003; Aravena-Roman, Harnett, Riley, Inglis, & Chang, 2011; Figueras, 2005; Figueras, Beaz-Hidalgo, Senderovich, Laviad, & Halpern, 2011). The acid production from arabinose and the production of urocanic acid and sodium dodecyl sulfate have been proposed for discriminating *A. aquariorum* from other species (Esteve, Alcaide, & Blasco, 2012), but the molecular identification has been emphasized as an alternative method for the correct identification (Figueras, Beaz-Hidalgo, Collado, & Martínez-Murcia, 2011; Puthucheary, Puah, & Chua, 2012). To examine the clinical importance of the correct identification, we molecularly re-identified the isolates included in our previous report and evaluated the virulence genes and cytotoxicity on human cell lines.

Seven *Aeromonas* strains recovered from 7 cases with septicemia (one strain did not regrowth) (Morinaga, et al., 2011) were re-identified by sequencing the *rpoD* gene using

primers and conditions previously described (Soler, et al., 2004). The sequences obtained were independently aligned using Mega5 program with the sequences of the type and reference strains of all members of the genus *Aeromonas* taken from our in-house data base (A. J. Martinez-Murcia, et al., 2011). Percentage similarity was calculated using the Ez Taxon pairwise alignment tool (Chun, et al., 2007). Genetic distances and clustering were determined using Kimura's two parameter model (Kimura, 1980) and the evolutionary tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the Mega5 program (Tamura, et al., 2011). Stability of the relationship was assessed by the bootstrap method (1000 replications).

Sequence-based analysis identified 4 of the 7 isolates as *A. aquariorum* and the rest as *A. hydrophila*, *A. caviae* and *A. veronii* (Fig. 1 and Table 1). Only two of seven strains (28.6%) were correctly identified by phenotypic identifications (Vitek2 (bioMerieux, Hazelwood, MO, USA) and Phoenix100 systems (BD, Franklin Lakes, NJ, USA)). The species, *A. aquariorum* was phenotypically misidentified as *A. hydrophila* in agreement with a previous study (Aravena-Roman, et al., 2011), but also as *A. veronii* bv *sobria*, despite misidentification with *A. caviae* (Figueras, et al., 2009).

A. aquariorum is a novel species of the genus *Aeromonas* (Esteve, et al., 2012; A. Martinez-Murcia, Monera, Alperi, Figueras, & Saavedra, 2009; A. J. Martinez-Murcia, et al., 2008) and has been indicated as a pathogenic bacterium in humans (Aravena-Roman, et al.,

2011; Figueras, et al., 2009). While *A. hydrophila*, *A. caviae* and *A. veronii* bv. *sobria* have been recognized as the most common *Aeromonas* species associated with septicemia (Janda, Guthertz, Kokka, & Shimada, 1994), we identified *A. aquariorum* strains with a higher frequency (57.1% including a case with two strains) compared to previous reports in Australia (30.4%) and Malaysia (50.0%) (Aravena-Roman, et al., 2011; Puthucheary, et al., 2012). These indicate that *A. aquariorum* should be noticed globally as a causative agent of septicemia. *A. aquariorum* can lead to invasive infectious diseases (Figueras, Beaz-Hidalgo, Senderovich, et al., 2011), however, there are still no report about the toxicity against human cells despite the virulence genes has been explored.

For the cytotoxicity experiments, bacteria were cultured in 3 mL of Luria-Bertani broth (37°C, at 250 rpm for 10 h) and each bacterial culture broth was filtered through 0.22 µm to remove bacteria. The human monocytic cell line THP-1 and the human neutrophilic cell line HL-60 were plated into 96-well plates at a density of 5×10^4 cells/well and stimulated by filtered bacterial culture broth ($\leq 1\%$ v/v) or 1% Triton X-100 as a control for 100% cell death. After stimulation for 12 h, the cell viability was measured using an XTT assay kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. *A. hydrophila* ATCC 7966^T, *A. caviae* ATCC 15468^T and *Pseudomonas aeruginosa* PAO-1 and NUS10 strains were used as the reference strains.

The cytotoxicity was expressed as the median lethal dose (LD₅₀) of triplicate experiments (Table 1). Whereas *A. hydrophila* ATCC 7966^T, *A. caviae* ATCC 15468^T and *P. aeruginosa* strains was not cytotoxic (LD₅₀ > 1%, data not shown), *A. aquariorum* strains (AEB03, AEB04, AEB06, AEB06R) showed strong cytotoxicity (LD₅₀ in THP-1, 0.04 ± 0.01 (means ± SEM, n = 3) - 0.25 ± 0.01 %; in HL-60, 0.11 ± 0.02 - 0.58 ± 0.06 %). *A. hydrophila* (AEB01) showed mild cytotoxicity (0.30 ± 0.07 % in THP-1, 0.53 ± 0.19 % in HL-60) compared to *A. aquariorum*. In contrast, LD₅₀ values of *A. veronii* (AEB02) and *A. caviae* (AEB05) were over 1 %. The potent cytotoxicity of *A. aquariorum* can justify the importance of differentiation of *A. aquariorum* from other prevalent *Aeromonas* clinical species (Figueras, 2005).

Furthermore, the pore-forming toxin gene *aerolysin/hemolysin*, and the cytotoxic enterotoxins genes, *ast*, *alt* and *act* were evaluated (Table 1) by PCR (Figueras, et al., 2009; Nawaz, et al., 2010). The *alt* gene was present in all of the cytotoxic strains (AEB01, AEB03, AEB04, AEB06, AEB06R). The *aerolysin/hemolysin* and *act* genes were observed in the cytotoxic strains except AEB03. The *ast* gene was detected only in the *A. hydrophila* AEB01 and no genes were detected in the *A. caviae* AEB05. The presence of *alt*, *aerolysin/hemolysin* or *act* genes seemed parallel to the cytotoxicity although we cannot exclude the involvement of other virulence genes not investigated (Chopra, Graf, Horneman, & Johnson, 2009). Prevalence of *alt*, *act*, *aerolysin/haemolysin* and *ast* in *A. aquariorum* strains in this study

was 100, 75, 75 and 50 %, respectively, while that in other study of 25 clinical strains was 28, 44, 100 and 84 %, respectively (Figueras, et al., 2009).

The severity of the septicemia in the studied patients was evaluated using SAPS II (fifteen scores for the type of admission, chronic diseases, consciousness, age, blood pressure, heart rate, temperature, oxygenation, urine output, serum urea, white blood cells, potassium, sodium, bicarbonate and bilirubin) and SOFA (consist of six scores for the respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems) scoring systems (Le Gall, Lemeshow, & Saulnier, 1993; Vincent, et al., 1996) (Table 1). The high scores were observed in the two dead patients (AEB02, AEB06 and AEB06R were isolated from these patients) but the scores varied to the patients. We believe that the differences are partly because of patients' various medical conditions as previously reported (Morinaga, et al., 2011). To clarify the clinical characteristics of *A. aquariorum* septicemia, further studies with a large number of cases will be required.

This study provides the first evidence of severe cytotoxicity of *A. aquariorum* strains to human cell lines and corroborates the high prevalence of virulence genes. Because *A. aquariorum* is uncovered by clinical automated identification systems and has a potential pathogenicity, *A. aquariorum* should be differentiated from other *Aeromonas* species.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Table 1. The phenotypic and molecular identification and the pattern of virulence genes of the clinical isolates of *Aeromonas* strains recovered from septicemia cases [1] in Japan.

Strains	Identification		PCR detection of virulence genes				LD ₅₀ (%) after stimulation with bacterial culture media ^a		Severity scoring	
	Phenotypic	<i>rpoD</i> gene	<i>aerolysin</i>	<i>act</i>	<i>alt</i>	<i>ast</i>	THP-1 cells	HL-60 cells	SOFA	SAPS II
			<i>/hemolysin</i>							
AEB01	<i>A. hydrophila</i>	<i>A. hydrophila</i>	+	+	+	+	0.30 ± 0.07	0.53 ± 0.19	1	18
AEB02	<i>A. hydrophila</i>	<i>A. veronii</i>	+	+	-	-	>1.0	>1.0	12	62
AEB03	<i>A. hydrophila</i>	<i>A. aquariorum</i>	-	-	+	-	0.25 ± 0.01	0.58 ± 0.06	8	51
AEB04	<i>A. hydrophila</i>	<i>A. aquariorum</i>	+	+	+	-	0.09 ± 0.01	0.25 ± 0.02	3	35
AEB05	<i>A. caviae</i>	<i>A. caviae</i>	-	-	-	-	>1.0	>1.0	10	58
AEB06 ^b	<i>A. veronii</i> bv. <i>sobria</i>	<i>A. aquariorum</i>	+	+	+	+	0.08 ± 0.01	0.21 ± 0.04	13 ^c	73 ^c
AEB06R ^b	<i>A. veronii</i> bv. <i>sobria</i>	<i>A. aquariorum</i>	+	+	+	+	0.04 ± 0.00	0.11 ± 0.02		

^a Values were expressed as mean ± SEM (n = 3). ^b Strains AEB06 and AEB06R were isolated from the same patient on different days. ^c Scores at the first onset of the patient.

FIGURE LEGENDS

Figure 1. Unrooted neighbor-joining phylogenetic tree based on the combined stretch of *rpoD* (790 bp) gene sequences showing the relationship within the genus *Aeromonas* of 7 clinical strains. Type strains are in bold font. Numbers at nodes indicate bootstrap values (1,000 replicates). The scale bar represents 1 nucleotide substitution per 50 nucleotides.

Figure 1