Original Article

Detection of putative virulence genes in *Aeromonas* isolates from humans and animals

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Abstract

Introduction: Aeromonas are food- and water-borne bacteria that are considered to be zoonotic human pathogens. This study aimed to investigate the presence of genes associated with virulence in human and animal *Aeromonas* isolates and the potential role of animal isolates with regards to human *Aeromonas* infections.

Methodology: The presence of *aer*A, *hly*A, *alt, ast, laf, asc*F-G, *stx*1 and *stx*2 putative virulence genes in 40 human and animal *Aeromonas* isolates (16 human and 24 animal isolates) were examined by polymerase chain reaction (PCR). DNA fragments of expected sizes were purified and sequenced. BLAST in the NCBI was used to verify any amplified products.

Results: PCR screening showed that *hly*A, *alt,* and *laf* genes were determined at ratios of 6.25%, 50%, and 6.25%, respectively, in human isolates. The ratios of *hly*A, *alt, asc*F-G, *laf, stx*2, and *stx*1 genes in animal isolates were 58.3%, 20.83%, 33.3%, 20.83%, 8.33%, and 4.17%, respectively. Neither *aer*A nor *ast* genes were detected in any isolates. Any one of eight putative virulence genes was not detected in seven human and eight animal isolates in the study.

Conclusions: The current study is the first to investigate the presence of the virulence gene in gull *Aeromonas* isolates. The manifestation of the presence of the virulence gene and gene combinations was considerable, especially in fish and gull isolates when compared with clinical human isolates. The current study demonstrates the potential importance of fish and gulls in terms of human *Aeromonas* infections.

Key words: *Aeromonas* spp.; human; animal; putative virulence genes; PCR.

J Infect Dev Ctries 2014; 8(11):1398-1406. doi:10.3855/jidc.4879

(Received 19 February 2014– Accepted 31 August 2014)

Introduction

Aeromonads are food- and water-borne bacteria. However, these bacteria are considered to be zoonotic human pathogens that can cause severe diarrhea, dysentery, and bacteremia. In human and veterinary medicine, *Aeromonas* strains are isolated mainly from fecal, wound, and abort samples [1]. Detection of aeromonads in animals constitutes a potential risk [2,3]. Cumulative data strongly allege that animals are subject to an ever-present reservoir [3]. Most of the studies on putative virulence genes of animal *Aeromonas* isolates are related to fish isolates [4-10]. Gulls are widespread coastal bird species that may contaminate coastal and lake water by their feces, which is a major source of contamination [11]. In our previous studies, we determined a clonal relationship by pulsed-field gel electrophoresis between a cattle *A. caviae* isolate and a human *A. caviae* isolate [12]. The determination of such a relationship reveals a possibility that animals other than fish have potential roles in human *Aeromonas* infections. The pathogenicity of *Aeromonas* spp. is multifactorial and complex and may include products of a number of different genes acting individually or collectively.The presence of *aer*A, *hly*A, *alt*, and *ast* genes could contribute to diarrhea-related virulence [13]. Shiga toxins (STX1 and STX2) coded by *stx*1 and *stx*2 genes are important virulence factors in the pathogenesis of gastroenteritis, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) [14], and the *asc*F gene-coded putative type III secretion system also plays important role in pathogenicity [15]. The *Aeromonas* species that possess lateral flagella are generally related to persistent or dysenteric infections [16]. It has been reported that the presence of aeromonads that carry the virulence gene in animals is a risk factor for

public health [5,17]. In the literature review, no study was found that investigated the presence of these genes in gull *Aeromonas* strains. Most studies focused on *Aeromonas* strains isolated from fish, but there are studies that investigated the presence of some of these genes in *Aeromonas* strains isolated from other animal species [4-10,17,18]; however, among the studies in which human and animal *Aeromonas* isolates were examined, no study was found that aimed to determine the presence of *aer*A, *hly*A, *alt*, *ast, asc*F-G, laf, *stx*1 and *stx*2 genes that are targeted in the current study. The current study aimed to investigate the presence of the eight virulence genes in human and animal *Aeromonas* isolates and their potential importance in terms of human *Aeromonas* infections.

Methodology

Aeromonas isolates

In this study, 40 *Aeromonas* strains isolated previously from humans (14 clinical *A. caviae* and 2 non-clinical *A. sobria* isolates) and animals (15 *A. caviae*, 5 *A. sobria*, 3 *A. veronii* and 1 *A. media* isolates) were used. Human clinical strains were isolated from diarrheal stool specimens. Non-clinical strains were isolated from healthy human stool specimens. Nine clinical human strains, cattle and sheep strains were isolated in 2002; other human strains (2 non-clinical, 5 clinical strains), chicken strain, gull and fish strains were isolated at 2005. Strains were isolated as previously described [12].

Fecal samples obtained from humans (diarrheic and non-diarrheic), chickens, and gulls using sterile swabs, and from the intestinal contents of fish (*Chalcalburnus tarichi* PALLAS 1811) were inoculated into alkaline peptone water (APW, pH 8.4). After incubation of samples overnight at 28°C within APW, 0.1 mL was inoculated into 7% defibrine sheep blood agar (Blood Agar Base No. 2, Oxoid, Basingstoke, England, CM0271) containing 10 µg/mL ampicillin. An oxidase test was performed for suspicious colonies growing in the medium. Tests identified the bacteria as *Aeromonas* spp. when the bacteria were oxidase positive, Gram-negative, motile, fermented glucose (O/F:+/+), showed no growth in broth including 6% NaCl, grew in saltless broth, did not compose acid from inositol, and were resistant to vibriostatic agent O/129 (150 µg, Oxoid DD0015). Test bacteria were identified using BD Phoenix (Becton, Dickinson and Company, Sparks, Maryland, USA) panels at species level. Stock cultures were maintained frozen at -80°C in peptone water (1% peptone, 0.5% NaCl [pH 7]) with 30% (vol/vol) glycerol.

Detection of putative virulence genes by PCR

Using a GF-1 Bacterial DNA Extraction Kit (Vivantis Sdn Bhd, Selangor Darul Ehsan, Malaysia), bacterial genomic DNA isolation was conducted from colonies of *Aeromonas* isolates grown upon incubation on tryptic soy agar for 24 hours at 37°C and reproduced in a thermal cycler device.

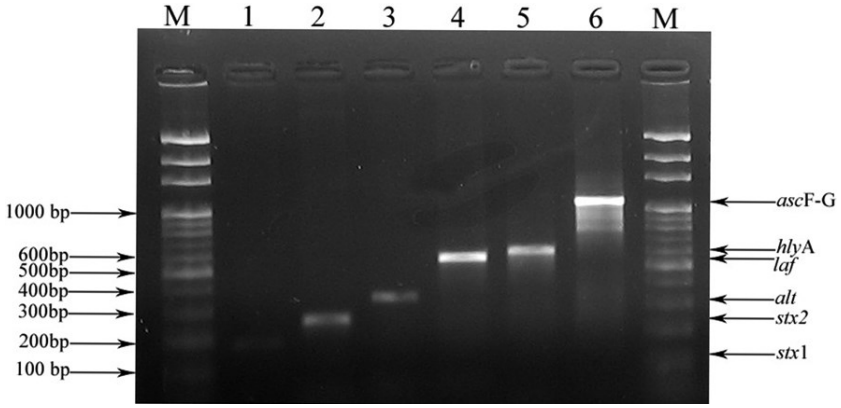
Table 1. Polymerase chain reaction primers used to detect the targeted genes, and applied annealing temperatures and PCR products anticipated for putative virulence genes

Target genes	Primer sequences (5'→3')	Annealing temperature (°C)	Amplicon length (bp)	References
*aer*A-F	GC(A/T)GA(A/G)CCC(A/G)TCTATCC(A/T)G	55	252	Santos *et al*. [22]
*aer*A-R	TTTCTCCGGTAACAGGATTG			
*hly*A-F	GGCCGGTGGCCCGAAGATACGGG	62	597	Wong *et al*. [34]
*hly*A-R	GGCGGCGCCGGACGAGACGGG			
alt-F	CCA TCC CCA GCC TTT ACG CCA T	63	338	Martínez *et al*. [35]
alt-R	TTT CAC CGA GGT GAC GCC GT			
ast-F	ATG CAC GCA CGT ACC GCC AT	66	260	Martínez *et al*. [35]
ast-R	ATC CGG TCG TCG CTC TTG GT			
laf-F	GGT CTG CGC ATC CAA CTC	60	550	Gavín *et al*. [4]
laf-R	GCT CCA GAC GGT TGA TG			
*asc*F-G-F	ATG AGG TCA TCT GCT CGC GC	55	900	Chacón *et al*. [15]
*asc*F-G-R	GGA GCA CAA CCA TGG CTG AT			
*stx*1-F	ATA AAT TGC CAT TCG TTG ACT AC	61	180	Paton and Paton [36]
*stx*1-R	AGA ACG CCC ACT GAG ATC ATC			
*stx*2-F	GGC ACT GTC TGA AAC TGC TCC	61	255	Paton and Paton [36]
*stx*2-R	TCG CCA GTT ATC TGA CAT TCT G			

Table 2. Distribution of eight putative genes by species and isolation sources in *Aeromonas* strains

Isolation source	*Aeromonas* species	Number of isolates	Putative virulence genes (%)							
			alt	***ast***	***aer*A**	***hly*A**	***Stx*1**	***Stx*2**	***laf***	***asc*F-G**
Human stool with diarrhea	*A. caviae*	7	+	-	-	-	-	-	-	-
		1	+	-	-	-	-	-	+	-
		6	-	-	-	-	-	-	-	-
Subtotal (%)		14	8 (57.14)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (7.14)	0 (0)
Healthy human stool	*A. sobria*	1	-	-	-	+	-	-	-	-
		1	-	-	-	-	-	-	-	-
Subtotal (%)		2	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)
Sheep	*A. caviae*	2	-	-	-	-	-	-	-	-
	A. sobria	1	-	-	-	-	-	-	-	-
Subtotal (%)		3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cattle	*A. caviae*	1	-	-	-	-	-	-	-	-
	A. sobria	1	-	-	-	-	-	-	-	-
Subtotal (%)		2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fish	*A. caviae*	1	+	-	-	-	-	-	-	+
	A. media	1	-	-	-	+	-	-	-	+
	A. sobria	1	+	-	-	+	-	-	+	-
		1	+	-	-	-	-	-	-	+
		1	-	-	-	+	-	-	+	+
	A. veronii	1	-	-	-	+	-	-	-	-
		1	+	-	-	+	-	-	-	+
Subtotal (%)		7	4 (57.14)	0 (0)	0 (0)	5 (71.43)	0 (0)	0 (0)	2 (28.57)	5 (71.43)
Gull	*A. caviae*	3	-	-	-	-	-	-	-	-
		1	+	-	-	+	-	-	-	-
		3	-	-	-	+	-	-	-	-
		1	-	-	-	+	-	+	-	+
		2	-	-	-	+	-	-	+	+
		1	-	-	-	+	+	+	+	-
Subtotal (%)		11	1 (9.09)	0 (0)	0 (0)	8 (72.73)	1 (9.09)	2 (18.18)	3 (27.27)	3 (27.27)
Chicken	*A. sobria*	1	-	-	-	+	-	-	-	-
Subtotal (%)		1	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Total		**40**	**13 (32.5)**	**0**	**0**	**15 (37.5)**	**1 (2.5)**	**2 (5)**	**6 (15)**	**8 (20)**

Figure 1. Detection of 180, 255, 338, 550, 597, and 900 bp fragments in selected *Aeromonas* isolates using PCR. Lane 1 contains *stx*1 gene produced by *Aeromonas* isolate. Lanes 2-6 contain *stx*2, *alt*, *laf*, *hly*A, and *asc*F-G genes produced by representative *Aeromonas* isolates, respectively. M molecular weight standard (VC 100 bp Plus DNA Ladder, Vivantis, Malaysia).



The following PCR protocol was followed: 5 µL bacterial DNA, 200 µM deoxy-nucleotide triphosphate (dNTP), 1.25 mM magnesium chloride ($MgCl_2$), 1.5 µM primer (forward-reverse for each primer), 5 µL 10X PCR buffer solution (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0) and 1.25 unit of Taq DNA Polymerase (New England BioLabs, Ipswich, USA,) were added into 0.5mL PCR tubes autoclaved previously and were completed up to 50 µL with a final volume of distilled water. Samples were subject to the following standard cycles to detect the target virulence genes (*aer*A, *hly*A, *alt, ast, laf, asc*F-G, *stx*1, and *stx*2). Different annealing temperatures of each primer are presented in Table 1. First, thermal cycles were applied for 3 minutes at 94°C, and then 30 cycles were applied individually as follows: 30 seconds at 92°C (denaturation), 30 seconds at an annealing temperature as presented in Table 1for each primer, 1 minute at 72°C (extension), and finally for 2 minutes at 72°C. PCR products to be used were stored at +4°C. Each product was loaded into a gel well for purposes of determining the presence of any amplified gene zone and verifying the presence or absence of a target gene in each PCR product. In TBE buffer, electrophoresis (80V, 70 minutes) was implemented in 1% agarose gel. After the gel was painted by ethidium bromide, it was monitored and analyzed under ultraviolet light (Figure 1). For this purpose, gel images obtained in this study were compared to lengths of base pairs given in Table 1by means of a marker. Representative PCR products selected arbitrarily from PCR products belonging to each gene were sequenced after PCR amplification. PCR products were purified for sequencing using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany), according to the protocol of the manufacturer. PCR products were sequenced on both strands using the virulence gene primers (2 pmol/µL) in an ABI 3130*xl* genetic analyzer (Applied Biosystems, Foster City, USA). BLAST in the NCBI was used for purpose of verifying the amplified products.

Statistical analysis

The statistical significance of the data was detected by a Chi-square test (χ^2) and probability value (p) < 0.05 was considered to be statistically significant.

Results

Of 40 isolates analyzed, 25 (62.5%) were positive for at least one of the putative virulence genes, 9/16 (56.25%) and 16/24 (66.66%) from human and animal samples, respectively. *aer*A and *ast* genes were not detected in any of isolates. When the sequence data were compared with the existing sequences present in GenBank (http://www.ncbi.nlm.nih.gov/) using the BLAST program, the sequence identity for *hly*A, *alt*, *asc*F-G, *laf*, *stx*2, and *stx*1 genes were 90%, 88%, 90%, 94%, 97%, and 97%, respectively.In all isolates, *hly*A, *alt, asc*F-G, *laf, stx*2 and *stx*1 genes were detected at ratios of 37.5% (15/40), 32.5% (13/40), 20% (8/40), 15% (6/40), 5% (2/40), and 2.5% (1/40), respectively. The *stx*2 gene was detected in only two gull isolates. Namely, both *stx*1 and *stx*2 genes ($stx1^+/stx2^+$) were detected in one gull isolate, and the *stx*1 gene was detected in the other gull isolate. In other animal and human isolates, no *stx*1 and *stx*2 genes were detected. The *alt* gene was detectedin five animal isolates (5/24; 20.83%) and eight clinical isolates (8/14; 57.14%). The *laf* gene was detected in five (5/24; 20.83%) animal isolates and one (1/14; 7.14%) clinical isolate. The *hly*A gene was detected in

Table 3. Distribution of putative virulence gene combinations (genotypes) by isolation sources

Number	Genotype	Number of isolates (%)	Isolation source (n)
1	alt^+ (*aer*A$^-$/*hly*A$^-$/*ast*$^-$/*laf*$^-$/*asc*F-G$^-$/*stx*1$^-$/*stx*2$^-$)	7/40 (17.5%)	Hc
2	*hly*A$^+$ (*aer*A$^-$/*alt*$^-$/*ast*$^-$/*laf*$^-$/*asc*F-G$^-$/*stx*1$^-$/*stx*2$^-$)	6/40 (15%)	Hnc (1), G (3), F (1), Ch (1)
3	alt^+/laf^+(*aer*A$^-$/*hly*A$^-$/*ast*$^-$/*asc*F-G$^-$/*stx*1$^-$/*stx*2$^-$)	1/40 (2.5%)	Hc
4	*asc*F-G$^+$/*hly*A$^+$ (*aer*A$^-$/*alt*$^-$/*ast*$^-$/*laf*$^-$/*stx*1$^-$/*stx*2$^-$)	1/40 (2.5%)	F
5	alt^+/*asc*F-G$^+$ (*aer*A$^-$/*hly*A$^-$/*ast*$^-$/*laf*$^-$/*stx*1$^-$/*stx*2$^-$)	2/40 (5%)	F
6	alt^+/*hly*A$^+$ (*aer*A$^-$/*ast*$^-$/*laf*$^-$/*asc*F-G$^-$/*stx*1$^-$/*stx*2$^-$)	1/40 (2.5%)	G
7	alt^+/*hly*A$^+$/laf^+ (*aer*A$^-$/*ast*$^-$/*asc*F-G$^-$/*stx*1$^-$/*stx*2$^-$)	1/40 (2.5%)	F
8	alt^+/*asc*F-G$^+$/*hly*A$^+$ (*aer*A$^-$/*ast*$^-$/*laf*$^-$/*stx*1$^-$/*stx*2$^-$)	1/40 (2.5%)	F
9	*asc*F-G$^+$/*hly*A$^+$/laf^+ (*aer*A$^-$/*alt*$^-$/*ast*$^-$/*stx*1$^-$/*stx*2$^-$)	3/40 (7.5%)	G (2), F (1)
10	*asc*F-G$^+$/*hly*A$^+$/*stx*2$^+$ (*aer*A$^-$/*alt*$^-$/*ast*$^-$/*laf*$^-$/*stx*1$^-$)	1/40 (2.5%)	G
11	*hly*A$^+$/laf^+/*stx*1$^+$/*stx*2$^+$ (*aer*A$^-$/*alt*$^-$/*ast*$^-$/*asc*F-G$^-$)	1/40 (2.5%)	G
12	*aer*A$^-$, *hly*A$^-$, *alt*$^-$, *ast*$^-$, *laf*$^-$, *asc*F-G$^-$, *stx*1$^-$, *stx*2$^-$	8/40 (20%) 7/40 (17.5%)	S (3), Ca (2), G (3) Hnc (1), Hc (6)

Hc: human clinical; Hnc: human non-clinical; G: gull; F: fish; S: sheep; Ca: cattle; Ch: chicken; n: number of isolates

one of two non-clinical human isolates, while it was not detected in clinical isolates. The same gene was detected in 14 animal isolates (14/24; 58.33%). The *asc*F-G gene was not detected in human isolates, but it was detected in eight (8/24; 33.33%) animal isolates. Any one of eight putative virulence genes investigated in the study was not detected in fifteen isolates (15/40; 37.5%), of which seven were human (one non-clinical, six clinical) and eight were animal isolates. When animal species were considered, none of the eight genes were detected in cattle and sheep isolates. In the chicken isolate, only the *hly*A gene was detected. The distribution of *hly*A, *alt, asc*F-G, and *laf* genes in fish isolates was determined at ratios of 71.43% (5/7), 57.14% (4/7), 71.43% (5/7), and 28.57% (2/7), respectively. The same genes were determined at ratios of 72.73% (8/11), 9.09% (1/11), 27.27% (3/11), and 27.27% (3/11) in gull isolates, respectively (Table 2). The presence of *hly*A and *asc*F-G genes in human isolates was found significantly less frequently than it was in animal isolates ($p < 0.05$). No statistically significant difference was determined for the other detected genes. Comparing the detection rates of genes in fish and gull isolates to the rates detected in human isolates, *hly*A and *asc*F-G genes were detected considerably more frequently ($p < 0.05$) in fish isolates than in human ones. While the *hly*A gene was detected considerably more frequently in gull isolates than in human isolates, the *alt* gene in gull isolates was detected considerably less frequently than in human isolates. No statistically significant difference was found between bacterial species and the presence of the virulence gene. Finally, when any findings were analyzed by considering combinations of putative virulence genes, isolates were determined to be divided into 12 genotypes (Table 3). Among significant genotypes in human and animal isolates, alt^+/ast^+ and $aerA^+/hlyA^+$ genotypes were not detected. Genotypes 1 and 12 were dominant in human isolates, and genotype 12 was dominant in animal isolates. While genotype 2 and 12 were detected in both human and animal isolates, genotype 1 and genotype 3 were found in only human isolates and genotypes 4 through 11 were found in only animal isolates.

Discussion

In our study, 62.5% of all isolates and 57.14% (8/14) of clinical isolates harbored at least one putative virulence gene, while 42.86% (6/14) of clinical isolates did not harbor any of these genes. Ottaviani *et al*. [19] found that 60.6% of the isolates from food, clinical and environmental isolates harbored at least one putative virulence gene in 142 *Aeromonas*isolates, and this percentage was 56.2% in clinical isolates in the studies to determine *act*, *ast*, *alt* and *aer*A genes. The same researchers determined that 14 (43.75%) of 32 clinical samples were negative in terms of all virulence aspects. The percentages obtained in our study matched the percentages specified in the study of Ottaviani *et al*. [19].

The presence of *aer*A, *hly*A, *alt*, and *ast* genes encoding hemolytic, cytotoxic, cytotonic, and enterotoxic activities may contribute to diarrheal-related virulence [13]. Cytotoxins (aerolysin, hemolysin and multifunctional repeat-in-toxin) produced by *A. hydrophila*, a node-like receptor family, trigger caspase-1 activation by means of pyrin domain containing 3 ($NLRP_3$) inflammasomes in macrophages, and this triggers pyroptosis as a form of proinflammatory necrosis in macrophages [20]. Gray *et al*. [21] found cytotoxins in 10 of 16 cattle isolates. In our study, no virulence genes were detected in two cattle isolates.

Aerolysin is the best-studied hemolysin, but its *Aeromonas* isolates may produce more hemolytic toxins having any virulence properties [22]. HlyA is a hemolysin like β-hemolytic *Vibrio cholera* [13]. In a previous study, Ottaviani *et al*. [19] found the detection rate of the *aer*A gene to be 92.7% and 88% in food and clinical *Aeromonas* isolates, respectively. Baloda *et al*. [8] reported that they detected an aerolysin-specific PCR product in 8 (66.66%) of 12 fish isolates. Nawaz *et al*. [10] noted that the detection rate of *aer*A gene as 96% in fish isolates. Wu *et al*. [23] detected *aer*A and *hly*A genes, respectively, in 31% and 40% of 116 consecutive clinical *Aeromonas* isolates. Pablos *et al*. [13] reported that they detected the *aer*A and *hly*A genes in 25.0% and 28.1% clinical isolates, respectively. Abdullah *et al*. [18] detected the *hly*A gene in 7 of 8 (87.5%) isolates from children with diarrhea and in 21 of 32 (65.63%) chicken carcass isolates. Khajanchi *et al*. [24] detected the *hly*A gene in 15.09% of 53 stool clinical *Aeromonas* isolates obtained from different regions of the world and from different geographical locations of the United States. Contrary to the findings of previous studies, in our study, we did not detect the *aer*A gene in human isolates and animal isolates, and we did not detect the *hly*A gene in clinical strains. However, we did detect the *hly*A gene in a chicken isolate.

*Aeromonasstx*1 and *stx*2 gene series are quite similar to most virulent *stx* gene variants of Shiga toxin-producing *Escherichia coli*. It has been suggested that a horizontal transfer can be realized

among such microorganisms having these genes. Shiga toxins (STX1 and STX2) include significant virulence factors in pathogenesis of gastroenteritis, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) [14]. In previous studies conducted to detect the *stx*1 and *stx*2 genes, Pablos *et al*. [13] did not detect the *stx*1 and *stx*2 genes in clinical isolates. Alperi and Figueras [14] reported that they detected the *stx*1 gene in 19 (23.8%) of 80 human isolates, and that 1 (1.3%) of these was positive for both *stx*1 and *stx*2 genes ($stx1^+/stx2^+$). Contrary to any findings obtained in Alperi and Figueras's studies [14], in our study, we did not detect *stx*1 and *stx*2 genes in human isolates. However, the relevant findings obtained in our study were found to be compatible with the findings obtained in the studies of Pablos *et al*. [13].

The *asc*F-G gene is one of the genes that encode the components of the putative type III secretion system (TTSS). Since TTSS facilitates delivery of toxins directly into host cells, it plays a principal role in pathogenicity [15]. In our study, no *asc*F-G gene was found in clinical isolates. This was compatible with the findings from the study of Pablos *et al*. [13]. Contrary to the findings obtained in our study, Chacón *et al*. [15] detected the *asc*F-G genein 50%of 84 clinical isolates. Senderovich *et al*. [25] detected the same gene in 12% of 17 clinical isolates. Silver and Graf [26] detected the same gene in all 20 environmental and human isolates they studied. Wu *et al*. [23] detected the same gene in 18% of the isolates they studied.

Gavín *et al*. [4] noted that the presence of lateral flagella and swarmer motility was a pathogenic factor in mesophilic *Aeromonas* isolates. The same researchers reported that they detected the *laf* gene in 62% of clinical *Aeromonas* isolates and in 70% of fish isolates. Kirov *et al*. [16] detected the *laf* gene in approximately 50% of clinical, environmental, and reference strains. Aguilera-Arreola *et al*. [6] detected the same gene in 77.27% (17/22) of clinical isolates; they detected the *laf*A gene in two fish isolates as well. Pablos *et al*. [13], Senderovich *et al*. [25], and Aguilera-Arreola *et al*. [27] detected this gene in 9.4%, 41%, and 36.7% isolates, respectively. Contrary to previous studies, in our study, the *laf* gene was not detected in 14 clinical isolates. However, the same gene was detected in 28.57% (2/7) of fish isolates. In our study, the percentage of the same gene detected in fish isolates was found to be lower than that found by Gavín *et al*. [4] and Aguilera-Arreola *et al*. [6].

Aeromonads may produce any heat-labile cytotonic enterotoxin (Alt) and heat-stable cytotonic enterotoxins (Ast). These toxins cause extension of Chinese hamster ovary (CHO) cells, a liquid secretion in rat ligated ileal loop, and an increase in prostaglandins and cyclic adenosine monophosphate (AMP) levels of the intestinal mucosa [28]. Abdullah *et al*. [18] detected the *alt* gene at ratios of 87.5% and 62.5% in isolates of children with diarrhea and chicken carcasses, respectively. Aguilera-Arreola *et al*. [6] detected the *ast* and *alt* genes at ratios of 95.45% (21/22) and 81.82% (18/22) in clinical isolates, respectively; furthermore, they detected the *ast* gene in two fish isolates, but did not detect the *alt* gene. Wu *et al*. [23] detected the presence of the *ast* and *alt* genes at ratios of 13% and 44%; Pablos *et al*. [13] detected them at ratios of 18.8% and 71.9%; Khajanchi *et al*. [24] detected them at ratios of 13.21% and 90.57%; Yi *et al*. [5] detected them at ratios of 27.1% and 55.7%; Senderovich *et al*. [25] detected them at ratios of 6% and 18%; and Aguilera-Arreola *et al*. [27] detected them at ratios of 28.4% and 34.9%, respectively. Morinaga *et al*. [29] detected the *alt* and *ast* genes in five and three of seven clinical *Aeromonas* isolates, respectively, from septicemia cases. Ottaviani *et al*. [19] reported that they detected the *alt* gene in 76.4% and 55.5% of food and clinical isolates, respectively, and the *ast* gene in 16.4% and 61.1% of food and clinical isolates, respectively. Albert *et al*. [30] detected the *alt* and *ast* genes at ratios of 16.5% and 15.7%, respectively, in isolates of children with diarrhea. Also, they detected the same two genes at ratios of 33.3% and 25.9%, respectively, in a control group of children. Nawaz *et al*. [10] did not detect the *alt* and *ast* genes. In our study, the *alt* gene was detected at a ratio of 57.14% in clinical isolates. While this percentage is compatible with the findings obtained in the studies of Yi *et al*. [5] and Ottaviani *et al*. [19], it was higher than the findings obtained in the studies of Senderovich *et al*. [25], Albert *et al*. [30], Aguilera-Arreola *et al*. [27] and Wu *et al*. [23], and lower than the findings obtained in the studies of Abdullah *et al*. [18], Pablos *et al*. [13], Khajanchi *et al*. [24] and Morinaga *et al*. [29]. Contrary to any findings obtained by Nawaz *et al*. [10] and Aguilera-Arreola *et al*. [6], in our study, the *alt* gene was detected at a ratio of 57.14% (4/7) in fish isolates. Contrary to the findings of Aguilera-Arreola *et al*. [6], in our study, we did not detect the *ast* gene in fish isolates, in agreement with the findings of Nawaz *et al*. [10]. However, we did not detect the *ast* gene in any human or animal isolates.

Putative virulence genes may occur in different combinations in *Aeromonas*. Ottaviani *et al*. [19]

found that 33.7% of all isolates had three toxins, and when sources of the isolates were considered, the ratio was 38.8% in clinical isolates and 34.5% in food isolates. In these isolates, they found percentages of isolates with two toxins to be 22.2% and 41.8% in clinical isolates and food isolates, respectively. Contrary to the studies of Ottaviani *et al*. [19], in our study, the *act* gene was not examined, and the *ast* and *aer*A genes were not detected; the gene combinations detected by the other researchers were therefore not detected in our study. Senderovich *et al*. [25] reported that, as virulence genotypes, three isolates (18%) had five different genes; four isolates (24%) had three or four different genes; and two isolates (12%) had two different genes. Contrary to the findings of Senderovich *et al*. [25], in our study, any isolate having five different genes was not detected. While three or four different genes were detected in seven isolates (17.5%; 7/40), two different genes were detected in five isolates (12.5%; 5/40). In our study, the 12.5% isolates obtained from strains having two different genes was found to be compatible with the findings of Senderovich *et al*. [25]; however, the percentage obtained by them for the isolates having three or four genes was higher than the percentage obtained in the current study. Hu *et al*. [9] noted that all fish and water isolates had three or more genes in different combinations. Contrary to Hu *et al*. [9], in our study, these gene combinations were detected only in seven isolates. Aguilera-Arreola *et al*. [27] reported that they detected seven different gene combinations – *alt* alone, *ast* alone, *aer*/*hem* alone, *alt*+*aer*/*hem*, *ast*+*aer*/*hem*, *alt*/*ast*, *alt*+*ast*+*aer*/*hem* – at ratios of 0.9%, 0%, 61.5%, 6.4%, 0.9%, 7.3%, and 20.2%, respectively, by PCR and dot blot. Yi *et al*. [5] noted that the isolates investigated in their study were divided into seven genotypes based on the enterotoxin genes (*act*/*alt*/*ast*). In our study, 12 different gene combinations were detected. It has been reported that the *aer*A^+/*hly*A^+ genotype provided the best estimation of virulence for *A. hydrophila* in an animal model [31]. However, a study found that, when the distribution of hemolytic genes was compared to extracellular products, three genotypes (*aer*A^+/*hly*A^+, *aer*A^+/*hly*A^-, *aer*A^-/*hly*A^-) were enterotoxic in the suckling-mouse test and had a capacity to express other virulence properties at different temperatures [32]. Pablos *et al*. [13] stated that they detected the *aer*A^+/*hly*A^+ gene combination in two clinical isolates (6.25%; 2/32). Albert *et al*. [30] suggested that the enterotoxigenic aeromonads having the *alt*$^+$/*ast*$^+$ genotype could be true diarrheal pathogens. Pablos *et al*. [13] detected the *alt*$^+$/*ast*$^+$ genecombination in four clinical isolates (12.5%; 4/32). Albert *et al*. [30] reported that they detected the *alt*$^+$/*ast*$^+$ genotype at ratios of 55.7% and 22.2% in isolates of children with diarrhea and control children, respectively. Aguilera-Arreola *et al*. [6] found the *alt*$^+$/*ast*$^+$ genotype in 4.55% (1/22) of isolates. Contrary to previous studies, in our study, no gene combinations were detected in *aer*A^+/*hly*$A^+$and *alt*$^+$/*ast*$^+$genotypes. Abdullah *et al*. [18] stated they detected the *alt*$^+$/*hly*A^+genotype in 75% and 37.5% of isolates from children with diarrhea and from chicken carcasses, respectively. In our study, the *alt*$^+$/*hly*A^+ genotype was detected in only one gull isolate.

In the current study, the virulence gene was not detected in 43.75% of human isolates and in 33.3% of animal isolates (in all cattle and sheep isolates). The multifactorial and complex pathogenicity of *Aeromonas* or its involvement in many different gene products, which work alone or in cooperation [13], necessitates detailed studies on this subject. In the present study, the *alt* gene was found as the main gene at a rate of 57.14% in human diarrhea-originated strains. This finding was consistent with the study of Aguilera-Arreola *et al*. [6]. In the present study, at least one virulence gene was detected in all fish isolates. Nawaz *et al*. [10] reported that consumption of improperly cooked catfish contaminated with *A. veronii*, which carries the virulence gene, produces a potential health risk. Thus, the presence of virulence genes in fish is important for public health [5-7,10,30,32]. Albert *et al*. [30] reported that the prevalence of the *alt* gene is similar among environmental isolates and isolates of children with diarrhea; however, the prevalence of the *ast* gene is significantly higher in environmental isolates when compared with the isolates from children with diarrhea. The present study revealed that the eight genes are not equally distributed in human and animal isolates and that the prevalence of the *alt* gene is higher in human isolates, whereas the prevalence of *hly*A, *stx*1, s*tx*2, *laf*, and *asc*F-G genes is higher in animal isolates. Lévesque *et al*. [33] reported that the detection of bacteria, including *Aeromonas* in gulls, would contribute to the microbiological contamination of recreational water and that there is a need for additional studies investigating the potential roles of the birds in the contamination of humans. In the literature review, no study was found related to the detection of virulence genes in gull *Aeromonas* strains. In the current study, for the first time, *alt*, *hly*A, *stx*1, *stx*2, *laf*, and *asc*F-G virulence genes were detected at

various ratios (9.09%, 72.73%, 9.09%, 18.18%, 27.27%, and 27.27%, respectively) in gull *Aeromonas* isolates. Furthermore, again in gull isolates, *hly*A$^+$/*laf*$^+$/*stx*1$^+$/*stx*2$^+$, *asc*F-G$^+$/*hly*A$^+$/*stx*2$^+$, *asc*F-G$^+$/*hly*A$^+$/*laf*$^+$, and *alt*$^+$/*hly*A$^+$ virulence gene combinations were found at rates of 9.09%, 9.09%, 18.18%, and 9.09%, respectively.

Conclusions

The current study investigated for the first time the presence of the virulence gene in gull *Aeromonas* isolates. The virulence gene and gene combinations were found in considerable numbers, especially in fish and gull isolates when compared with clinical human isolates. Fish and gulls are potentially important in human *Aeromonas* infections.

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Conflict of interests: No conflict of interests is declared.