

Prevalence, virulence and antimicrobial resistance patterns of *Aeromonas* spp. isolated from children with diarrhea

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Abstract

Background *Aeromonas* spp. cause various intestinal and extraintestinal diseases. These bacteria are usually isolated from fecal samples, especially in children under five years old. The aim of this study was to assess the prevalence of *Aeromonas* spp. and their antimicrobial resistance profile in children with diarrhea referred to the Children Medical Center in Tehran, between 2013 and 2014.

Methods A total number of 391 stool samples were collected from children with ages between 1 day and 14 years old, with diarrhea (acute or chronic), referred to the Children Hospital, Tehran, Iran, between 2013 and 2014. Samples were enriched in alkaline peptone water broth for 24 hours at 37 °C and then cultured. Suspicious colonies were analyzed through biochemical tests. Furthermore, antimicrobial susceptibility tests were carried out for the isolates. Isolates were further studied for *act*, *ast*, *alt*, *aerA* and *hlyA* virulence genes using polymerase chain reaction.

Results In total, 12 isolates (3.1%) were identified as *Aeromonas* spp.; all were confirmed using the API-20E test. Of these isolates, five *A. caviae* (42%), four *A. veronii* (33%) and three *A. hydrophila* (25%) were identified in cases with gastroenteritis. Second to ampicillin (which was included in the growth medium used), the highest rate of antimicrobial resistance was seen against nalidixic acid and trimethoprim-sulfamethoxazole (5 isolates each, 41.6%) and the lowest rate of antimicrobial resistance was seen against gentamicin, amikacin and cefepime (none of the isolates). Results included 76.4% *act*, 64.7% *ast*, 71.5% *alt*, 83.3% *aerA* and 11.7% *hlyA* genes.

Conclusion *Aeromonas* spp. are important due to their role in diarrhea in children; therefore, isolation and identification of these fecal pathogens should seriously be considered in medical laboratories. Since virulence genes play a significant role in gastroenteritis symptoms caused by these bacteria, *Aeromonas* species that include virulence genes are potentially suspected to cause severe infections. Moreover, bacterial antimicrobial resistance is increasing, especially against trimethoprim-sulfamethoxazole and nalidixic acid.

Keywords *Aeromonas* spp., antimicrobial resistance, diarrhea, children, Iran

Background

Aeromonas species are aerobic and anaerobic Gram-negative bacilli, which grow under alkaline

conditions (optimum pH 5.5-9.0).^{1,2} Some clinically isolated *Aeromonas* spp. are pathogenic to humans. Gastroenteritis is the most common type of *Aeromonas* infection in humans. The clinical picture varies from simple diarrhea to

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massive invasive dysentery. Furthermore, these bacteria have been isolated from fecal samples of children aged under five years and from other organs such as meninges, peritoneum and endocardium in adults.³ *Aeromonas* spp. can cause short-term or long-term diarrhea in older or immunosuppressed children.⁴ However, evidence for the enteropathogenic role of *Aeromonas* spp. has been controversial.¹ The bacteria produce a wide range of exoenzymes (e.g., enterotoxins); however, some species do not express toxin genes. Two groups of enterotoxins have been described in *Aeromonas* spp., including cytotoxic enterotoxin and cytotoxic enterotoxin.⁵ Cytotoxic enterotoxin (encoded by *act*) can cause hemolysis and cytotoxicity as well as enterotoxicity.⁶ The toxin plays an important role in the pathogenesis of *A. hydrophila* and is associated with aerolysin;⁷ it creates pores in the cell membrane, resulting in the cell death. In contrast, *Ast* and *Alt* cytotoxic enterotoxins do not affect epithelial tissues. The action mechanism of these toxins is mostly similar to the cyclic adenosine monophosphate and prostaglandin-mediated mechanism of cholera toxin in intestinal epithelial cells.⁸ Two hemolytic toxins, including hemolysin *AHH*₁ (*hlyA*) and aerolysin (*aerA*), have been described in the bacteria. Hemolysin *AHH*₁ is similar to *hlyA* hemolysin in *Vibrio cholerae* and aerolysin to *aerA* in *V. cholerae*. The effects of these toxins include hemolysis, cytotoxicity and enhanced virulence.⁹ The aim of the current study was to assess the prevalence of *Aeromonas* spp. and their antimicrobial susceptibility pattern in children with diarrhea referred to the Children Medical Center in Tehran, between 2013 and 2014. Furthermore, *act*, *ast*, *alt*, *aerA* and *hlyA* virulence genes were characterized in the isolated *Aeromonas* spp.

Methods

A total number of 391 stool samples were collected from children with ages between 1 day and 14 years old, with diarrhea (acute or chronic), referred to the Children Medical Center, Tehran, Iran, from July 2013 to December 2014. Samples were enriched first in

alkaline peptone water broth and incubated at 37 °C for 18-24 hours. A loop of this mixture was subcultured in CIN (cefsulodin, irgasan, novobiocin) agar, MacConkey agar and blood agar containing 5% of sheep blood and 10 µg/mL of ampicillin. Colonies on ampicillin blood agar (ABA) were checked for hemolysis and results were recorded (except for *A. enteropelogenes*, which is not resistant to ampicillin). Oxidase test and Gram staining were carried out for the colonies. Negative lactose colonies grown on CIN agar and MacConkey agar were also subcultured on ABA. Tests for species identification included production of acetylmethylcarbinol (Voges-Proskauer), fermentation of glucose and lactose (Kligler), gas production from glucose, acid production from arabinose, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase and esculin hydrolase. Furthermore, 20E-API system biochemical tests (BioMérieux, Marcy-l'Étoile, France) were used to identify Enterobacteriaceae and Gram-negative bacilli. Results were analyzed in comparison with the 23S rRNA results. All culture media were purchased from Merck (Darmstadt, Germany).

Antimicrobial susceptibility testing

Bacterial susceptibility to antimicrobials was carried out using the Kirby-Bauer method according to CLSI guidelines. A colony of the bacteria was inoculated in a test tube containing broth media. Broth culture was incubated at 35 °C until it reached 0.5 McFarland turbidity. Broth was recultured on Mueller-Hilton agar plates (Merck). Antimicrobial disks were placed on the plates and incubated at 37 °C for 16-18 h before results were read. The antimicrobials were also selected according to CLSI guidelines.¹⁰ Antimicrobial disks (Mast, Bootle, UK) used for the susceptibility test included chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), nalidixic acid (30 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (TMP-SMX, 25 µg), streptomycin (10 µg), ampicillin (10 µg), ceftiofloxacin (30 µg), amikacin (30 µg), ceftiofloxacin (30 µg), cefotaxime (30 µg) and cefepime (30 µg).

DNA extraction and polymerase chain reaction

DNA was extracted from the bacterial colonies using boiling method and stored at -20 °C until use. A 23S rRNA gene was used to confirm the isolated *Aeromonas* spp., generating a 550 bp fragment.¹¹ Polymerase chain reaction (PCR) for the virulence genes was carried out based on an original protocol by Sreedharan et al. (2012).⁹ The primers used in this study are described in Table 1. PCR master mix (SinaClon BioScience Co, Tehran, Iran) was prepared in a final volume of 20 µL including 1X buffer, 1.5 mM of MgCl₂, 0.2 mM of dNTPs, 10pM of each primer, 0.5 IU/µL of Taq DNA polymerase, 1 µL of the template DNA and sufficient amount of sterile distilled water. PCR reaction was thermally cycled (Peqlab Primus 96, Peqlab Biotechnologie GmbH, Erlangen, Germany) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles, each cycle including denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec. Final extension included incubation at 72 °C for 5 min. Amplicons were electrophoresed on 1.5% agarose gels and visualized under UV. Thermal cycle conditions for other genes were similar to those of 23S rRNA gene except that the annealing temperature included 56 °C for *act* and *ast*, 58 °C for *alt* and *aerA* and 68 °C for *hlyA*.

Statistical analysis

Statistical analysis was carried out using Chi-Square (Exact method) analysis on SPSS Statistics for Windows, version 14 (SPSS Inc., Chicago, IL, USA). P-values ≤0.050 were considered as significant.

Results

Of 391 samples from children suffering from diarrhea (223 males, 57%; 168 females, 43%), 12 *Aeromonas* spp. were isolated including three (0.8%) *A. hydrophila*, five (1.3%) *A. caviae* and four (1%) *A. veronii*. Results showed no significant correlation between the pathogens and the participants' age ($p=0.730$, $\chi(3)=1.56$).

No significant correlation was seen between the pathogen and the participants' sex ($p=0.160$, $\chi(1)=2.23$). No significant correlation was observed between bacterial isolation and the duration of diarrhea in participants ($p=0.910$, $\chi(2)=0.801$), nor the feces pH ($p=0.600$, $\chi(3)=2.02$). Five patients (41.7%) showed watery diarrhea, six (50%) dysentery and ten (83.3%) mucoid diarrhea. No significant correlation was seen between bacterial isolation and the number of white or red blood cells in feces of the participants ($p=0.379$, $\chi(4)=3.354$ and $p=0.300$, $\chi(4)=4.344$, respectively). Furthermore, no significance was observed between bacterial isolation and the diarrheal form (Table 2).

Antimicrobial susceptibility testing

Second to ampicillin (which was included in the growth medium used), the highest rate of antimicrobial resistance was seen against nalidixic acid and trimethoprim-sulfamethoxazole (5 isolates each, 41.6%). The lowest rate of antimicrobial resistance was seen against gentamicin, amikacin and cefepime (none of the isolates) (Table 3). *A. veronii* showed antimicrobial resistance to TMP-SMX and streptomycin (50%) and susceptibility to cefoxitin (100%), compared to other species which showed no resistance to TMP-SMX and streptomycin and resistance to cefoxitin. *A. hydrophila* showed antimicrobial resistance to tetracycline (33%) and susceptibility to cefotaxime (100%), compared to other species which showed no resistance to tetracycline and resistance to cefotaxime. *A. caviae* demonstrated resistance to ceftazidime (20%) and susceptibility to nalidixic acid (100%), compared to other species which demonstrated no resistance to ceftazidime and resistance to nalidixic acid.

Polymerase chain reaction

PCR results for 23S rRNA genes confirmed the identity of all *Aeromonas* isolates. Molecular identification of the virulence genes showed that 76.4%, 64.7%, 71.5%, 83.3% and 11.7% of the *Aeromonas* isolates included *act*, *ast*, *alt*, *aerA* and *hlyA* genes, respectively (Table 4).

Table 1. Primers used in PCRs

Gene	Sequence	bp	Ref.
23s rRNA	F 5'-GGAACTTCTTGGCGAAAAC R 5'-GGTTCTTTTCGCCTTTCCCT	550	10
<i>hlyA</i>	F 5'-GGCCGGTGGCCCCGAAGATACGGG-3' R 5'-GGCGGCGCCGGACGAGACGGG-3'	597	9
<i>aerA</i>	F 5'-GAGCGAGAAGGTGACCACCACCAAGAAC-3' R 5'-TTCCAGTCCCACCACCTTCACTTCAC-3'	417	9
<i>act</i>	F 5'-AGAAGGTGACCACCAAGAACA-3' R 5'-AACTGACATCGGCCTTGAAGTC-3'	232	9
<i>alt</i>	F 5'-TGACCCAGTCCTGGCACGGC-3' R 5'-GGTGATCGATCACCACCAGC-3'	442	9
<i>ast</i>	F 5'-TCTCCATGCTTCCCTTCCACT-3' R 5'-GTGTAGGGATTGAAGAAGCCG-3'	331	9

Table 2. Association of diarrheal form and *Aeromonas* spp. isolated in the current study

Diarrheal form		<i>Aeromonas</i> positive n (%)	<i>Aeromonas</i> negative n (%)	Total n (%)	P-value
Watery diarrhea	Yes	5 (41.7)	202 (53.4)	207 (53.1)	0.056
	No	7 (58.3)	176 (46.6)	183 (46.9)	
Dysentery	Yes	6 (50)	125 (33.1)	131 (33.6)	0.220
	No	6 (50)	253 (66.9)	259 (66.4)	
Mucoid diarrhea	Yes	10 (83.3)	289 (76.3)	299 (75.5)	0.740
	No	2 (16.7)	90 (23.7)	92 (23.5)	

Table 3. Results of antibacterial susceptibility tests

Antibiotic	S (%)	I (%)	R (%)*
Ampicillin**	0 (0)	0 (0)	12 (100)
Gentamicin	12 (100)	0 (0)	0 (0)
Tetracycline	8 (66)	1 (8.3)	3 (25)
Cefoxitin	6 (50)	3 (25)	3 (25)
Nalidixic acid	7 (58.3)	0 (0)	5 (41.6)
Amikacin	12 (100)	0 (0)	0 (0)
Ceftazidime	7 (58.3)	4 (33.3)	1 (8.3)
Chloramphenicol	11 (91.6)	0 (0)	1 (8.3)
TMP-SMX	5 (41.6)	2 (16.6)	5 (41.6)
Cefotaxime	8 (66.6)	2 (16.6)	2 (16.6)
Cefepime	12 (100)	0 (0)	0 (0)
Streptomycin	7 (58.3)	3 (25)	2 (16.6)
Ciprofloxacin	9 (75)	3 (25)	0 (0)

I - intermediate; R - resistant; S - sensitive; TMP-SMX - trimethoprim-sulfamethoxazole.

*The total sum percentages do not produce 100 in some cases due to decimal rounding.

**Ampicillin was included in the growth medium used for culturing the isolates.

Table 4. Prevalence of virulence genes in bacterial species

Gene	<i>A. veronii</i> n (%)	<i>A. hydrophila</i> n (%)	<i>A. caviae</i> n (%)	Total (%)
<i>alt</i>	5 (71.4)	4 (100)	3 (50)	71.5
<i>ast</i>	3 (42.8)	4 (100)	4 (66.6)	64.7
<i>act</i>	5 (71.4)	4 (100)	4 (66.6)	76.4
<i>aerA</i>	7 (100)	4 (100)	3 (50)	82.3
<i>hlyA</i>	0 (0)	2 (50)	0 (0)	11.7

Discussion

Gastroenteritis is the most common type of infection caused by *Aeromonas* spp. in humans. Clinical symptoms vary from chronic watery diarrhea to massive dysentery.¹² Clinical symptoms include fever, stomach ache, nausea and vomiting. In the current study, patients had at least two clinical symptoms and 50% of them had numerous red blood cells in their feces. These results were similar to the results of another study by Soltan Dallal et al. (2004) in which 14 (5.4%) *Aeromonas* spp. including eight *A. veronii*, five *A. caviae* and one *A. hydrophila* were isolated from a total sum of 310 bacterial samples.¹³ Several virulence genes can influence the pathogenicity of *Aeromonas* spp.¹⁴ The cytotoxic enterotoxin *act* is closely associated with *aerA*. Products of both genes include hemolytic and cytotoxic effects and are lethal to mice.¹⁵ In 1997, Albert et al. reported differences between control and environmental samples.¹⁶ In samples isolated from children with diarrhea, *ast*, *alt* and both genes were found in 15.7%, 16.5% and 55.7% of the isolates, respectively. Albert et al. also identified *ast* alone, *alt* alone and both genes in 25.9%, 33.3% and 22.2% of the control isolates, respectively. Furthermore, *ast* alone, *alt* alone and both *ast* and *alt* were found in 30%, 16.7% and 33.3% of the environmental isolates, respectively.¹⁵ Albert suggested that isolates which included *ast* and *alt* genes caused watery diarrhea and isolates which included *alt* gene caused loose stools. In the present study, *act*, *ast*, *alt*, *aerA* and *hlyA* genes were identified in 76.4%, 64.7%, 71.5%, 83.3% and 11.7% of the isolates, respectively. Of 191 *A. caviae* isolates studied by Kingombe et al. in 1999, *alt* was found in 164 (86%) and *act* in 68 (36%) isolates.¹⁷ Moreover, of 206 *A. hydrophila* isolates, *act* was detected in 171 (83%) and *alt* in 181 (88%) isolates, while *ast* was limited to the control strains.

Aeromonas spp. are not usually identified in clinical laboratories; also, prescription of ineffective antimicrobials has increased the antimicrobial resistance pattern of these bacteria. In the current study, antimicrobial resistance was reported in nearly 21.77% of the total isolates; most displayed multiple resistance.

Resistance to nalidixic acid and trimethoprim-sulfamethoxazole was detected in five isolates each (41.6%). Furthermore, three isolates (25%) were moderately resistant to ciprofloxacin. In a study by Soltan Dallal et al. (2004),¹³ resistance to nalidixic acid and trimethoprim-sulfamethoxazole was reported in 7.1% and 28.6% of the isolates respectively while no resistance to ciprofloxacin was reported. In another study by Sinha et al. during 2000-2001, resistance to nalidixic acid was detected in 62.8% and 54.4% and to ciprofloxacin in 22.4% and 12.3% of the isolates.¹⁸ In a 9-year study by Yamada et al. (1986-1995), resistance to nalidixic acid was reported in nearly 3% of the isolates.¹⁹ Therefore, these studies have shown that resistance to trimethoprim-sulfamethoxazole and nalidixic acid has increased over the past decades. In the present study, three isolates (25%) were resistant to tetracycline and cefoxitin each. Resistance to tetracycline has previously been identified in 25% of *A. veronii* isolates by Yamada et al.¹⁹ Vila et al.²⁰ (2003) detected tetracycline resistance in 55.6% and 71.4% of *A. veronii* and *A. caviae* isolates, respectively. Moreover, they found that no isolates were resistant to cefoxitin while all were resistant to ampicillin.

Conclusion

In summary, the results of the present study have demonstrated that clinically important *Aeromonas* spp. include multiple virulence genes. Furthermore, antimicrobial resistance to the selected antimicrobials has increased in recent years; similar to that in other countries. However 25% of the isolates were intermediately resistant to ciprofloxacin, and resistance to quinolones should carefully be considered due to the importance of these antimicrobials in the treatment of infections caused by *Aeromonas* spp.

Authors' contributions statement: MMSD designed and supervised the study, provided research laboratory and master edited the manuscript. RMNF scientifically advised the study, reviewed the literature and edited the final version of the manuscript. MKT prepared the proposal and practically carried out the study. LA preliminarily drafted the manuscript. ZS collected and statistically analyzed data. All

authors reviewed and approved the final version of the manuscript.

Conflicts of interest: All authors – none to declare.

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