Libyan Journal of Medicine

ISSN: 1993-2820 (Print) 1819-6357 (Online) Journal homepage: http://www.tandfonline.com/loi/zljm20

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To cite this article: Khalifa Sifaw Ghenghesh, Salwa F. Ahmed, Piero Cappuccinelli & John D. Klena (2014) Genospecies and virulence factors of *Aeromonas* species in different sources in a North African country, Libyan Journal of Medicine, 9:1, 25497, DOI: 10.3402/ljm.v9.25497

To link to this article: https://doi.org/10.3402/ljm.v9.25497

Published online: 09 Sep 2014.

ljm Libyan Journal of Medicine

COACTION PUBLISHING

ORIGINAL ARTICLE

Genospecies and virulence factors of *Aeromonas* species in different sources in a North African country

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Introduction: Aeromonads of medical importance have been reported from numerous clinical, food, and water sources, but identification of genospecies and virulence factors of *Aeromonas* species from countries in North Africa and the Middle East are few.

Methods: In total 99 *Aeromonas* species isolates from different sources (diarrheal children [$n = 23$], non-diarrheal children [$n = 16$], untreated drinking water from wells [$n = 32$], and chicken carcasses [$n = 28$]) in Tripoli, Libya, were included in the present investigation. Genus identification was confirmed by biochemical analysis, and genospecies were determined using a combination of 16S rDNA variable region and *gyrB* sequence analysis. Polymerase chain reaction (PCR) was used to detect genes encoding toxins from 52 of the isolates.

Results: We identified 44 isolates (44%) as *A. hydrophila* (3 [3.0%] subspecies *anaerogenes*, 23 [23%] subspecies *dhakensis*, and 18 [18%] subspecies *ranae*); 27 isolates (27%) as *A. veronii*; 23 isolates (23%) as *A. caviae*; and 5 isolates (5.0%) as other genospecies. The genes encoding aerolysin (*aer*), cytolytic enterotoxin (*act*), and *A. hydrophila* isolate SSU enterotoxin (*ast*) were detected in 45 (87%), 4 (7.7%), and 9 (17%) of the 52 isolates tested, respectively. The gene encoding an extracellular lipase (*alt*) was not detected.

Conclusion: The majority of aeromonads from Libya fall within three genospecies (i.e. *A. hydrophila*, *A. veronii*, *and A. caviae*), and genes coding for toxin production are common among them.

Keywords: Aeromonas; *genospecies*; *virulence factors*; *Libya*

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Received: 18 July 2014; Revised: 17 August 2014; Accepted: 17 August 2014; Published: 9 September 2014

Aeromonas species are Gram-negative, oxidase-positive, facultative anaerobic, rod-shaped bacteria of the family *Aeromonadaceae*. Almost all strains grow on MacConkey agar and some are lactose-positive. Natural waters are the main habitat of these organisms. Aeromonads have been associated with skin and soft-tissue infections, particularly in individuals exposed to untreated environmental water sources such as during flooding events (1). The role of different *Aeromonas* species in gastroenteritis is controversial (2), but epidemiological evidence suggests infection with some types can cause diarrhea. Recently, Kotloff et al. (3) carried out a 3-year prospective matched case-control study of moderate-to-severe diarrhea in children less than 5 years of age living in seven sites in sub-Saharan Africa and Asia. They reported that *Aeromonas* was a leading pathogen among children 2 to <5 years only in Pakistan and Bangladesh in Asia. In addition, these organisms have been recognized as a cause of foodborne and waterborne outbreaks of disease (4).

Although the genus *Aeromonas* taxonomy is continuously changing, 17 hybridization groups or genospecies and 14 phenospecies have been described (5). However, only *A. hydrophila*, *A. veronii* biovar sobria, and *A. caviae* are commonly isolated from clinical, food, and water sources worldwide (6, 7).

Several virulence factors have been associated with pathogenicity of aeromonads. These include production of toxins (enterotoxins, cytotoxins, and hemolysins); ability to adhere to and invade cells; and production of various enzymes that are regarded as mechanisms of pathogenicity. Chopra et al. identified distinct genes encoding enterotoxins from an *A. hydrophila* isolate associated with diarrhea (8–10). One gene encodes a cytotoxic enterotoxin (*Act*), and two genes encode cytotonic enterotoxins, one of which is heat labile at 56°C (*Alt*), and

Citation: Libyan J Med 2014, **9**: 25497 - http://dx.doi.org/10.3402/ljm.v9.25497

the other of which is heat stable at this temperature (*Ast*). *Alt* exhibits intriguing homology with lipases and phospholipase C.

Reports characterizing *Aeromonas* species from countries in North Africa and the Middle East are few. The aim of the present study was to determine the genospecies and virulence genes of *Aeromonas* isolated from diarrheal and non-diarrheal children, chicken carcasses, and untreated well water used for drinking.

Methods

Strains

In total 99 isolates identified biochemically as members of the genus *Aeromonas* randomly selected from a large collection of nearly 400 aeromonads isolated from different sources during the past two decades in Tripoli, Libya, were included in the present investigation. The strains were obtained from diarrheal children ($n=23$), non-diarrheal children ($n=16$), untreated drinking water from wells ($n=32$), and chicken carcasses ($n=28$). The organisms were isolated from stool, chicken, and water samples using standard bacteriological procedures as reported previously (11). All organisms were identified by biochemical tests and API 20E (bioMerieux, Marcy l'Etoile, France). Strains were then inoculated into semi-solid nutrient agar in duplicates and maintained at ambient temperature until further characterized.

Determination of Aeromonas genospecies by DNA sequence analysis

Genospecies was determined using a combination of 16S rDNA (12), and *gyrB* (13) sequencing analysis described previously (14).

Whole cell lysate preparation

A loopful of a fresh overnight growth from each *Aeromonas* isolate cultured on MacConkey-lactose agar (Oxoid, Hampshire, United Kingdom) was suspended in 400 µl sterile deionized water, boiled for 10 min and transferred to ice for 5 min. Cell debris was pelleted by centrifugation at $12{,}000 \times g$ for 3 min (15), the supernatant was transferred to a new tube and refrigerated until use.

DNA analysis

PCR amplicons were purified using the PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. Nucleotide sequence was determined using dye terminator chemistry and cycle sequencing products were purified prior to loading on an ABI Prism 3,100 genetic analyzer (Applied Biosystems, Foster City, CA) using a DyeEx purification kit (Qiagen). Sequence files were assembled using BioEdit version 7.0.1 (16) and aligned with CLUSTAL X (17). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 4.0 (18). Phylogenetic trees were constructed using the neighbor-joining method with genetic distance calculated using the Kimura two-step algorithm. Bootstrap analysis (19) was performed with 2,000 samplings and values below 70% were excluded as non-significant.

Determination of virulence factors

In total 52 aeromonads (12 from diarrheal children, 12 from non-diarrheal children, 17 from chicken carcasses, and 11 from untreated drinking water from wells) were examined for the genes *aer*, *act*, *ast*, and *alt* using PCR techniques and sequencing as reported previously (8, 20–22). In addition, isolates were tested for their cytotoxic activity in Vero cell tissue culture using a previously described procedure (23).

Results

Of the 99 isolates, we identified 44 isolates (44%) as *A. hydrophila* (3 [3.0%] subspecies *anaerogenes*, 23 [23%] subspecies *dhakensis*, and 18 [18%] subspecies *ranae*); 27 isolates (27%) as *A. veronii*; 23 isolates (23%) as *A. caviae*; and 5 isolates (5.1%) as other genospecies (Table 1). *A. hydrophila* was common in water samples (84.4%) compared with diarrheal and non-diarrheal stool (33.3%) and chicken (14.3%) samples; *A. veronii* in chicken samples (60.7%) compared with diarrheal and non-diarrheal stool (23.1%) and water (3.1%) samples; and *A. caviae* in stool samples from diarrheal and non-diarrheal children (41.0%) compared with water (6.3%) and chicken (17.9%) samples. The genes *aer*, *act*, and *ast* were detected in 45 (87%), 4 (7.7%), and 9 (17%), respectively (Table 2). The *alt* gene was not detected. Cytotoxicity to Vero cells was observed in 7 of 12 (58%) aeromonads from diarrheal, 4 of 12 (33%) from non-diarrheal children, 8 of 11 (73%) from water, and 10 of 17 (59%) from chicken carcasses.

Discussion

Previous studies conducted in Libya found *Aeromonas* species in 4.2 to 14.6% of diarrheal children (24–26). In one of these studies (24) phenotypic speciation using Aerokey II (27) showed predominance of *A. caviae*, followed by *A. veronii*, and *A. hydrophila*. Abdullah et al. (28) genotyped eight aeromonads from diarrheal Libyan children by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of 16S rRNA genes: four (50%) were *A. caviae*, three (37.5%) were *A. veronii*, and one (12.5%) was *A. hydrophila*.

In the present investigation, the genospecies *A. caviae* predominated, followed by *A. hydrophila* (mainly subspecies *dhakensis* and subspecies *ranae*), and *A. veronii*, among aeromonads from diarrheal and non-diarrheal children. Predominance of these three genospecies was also observed among aeromonads from chicken and water samples, accounting for 95% (94/99) of total isolates tested. These genospecies account for the majority of

Citation: Libyan J Med 2014, **9**: 25497 - http://dx.doi.org/10.3402/ljm.v9.25497

Table 1. Genospecies of aeromonads isolated from different sources in Tripoli, Libya

	No. (%) positive				
Aeromonas genospecies	Diarrheic children ($n=23$)	Non-diarrheic children ($n=16$)	Water ($n=32$)	Chicken carcasses ($n=28$)	Total ($n=99$)
Hydrophila	8 (34.8)	5 (31.3)	27 (84.4)[a]	4 (14.3)	44 (44.4)
Subspecies *anaerogenes*	1 (4.3)	0 (0.0)	1 (3.1)	1 (3.6)	3 (3)
Subspecies *dhakensis*	2 (8.7)	3 (18.8)	15 (46.9)	3 (10.7)	23 (23.2)
Subspecies *ranae*	5 (21.7)	2 (12.5)	11 (34.4)	0 (0.0)	18 (18.2)
Veronii	7 (30.4)[b]	2 (12.5)	1 (3.1)	17 (60.7)[c]	27 (27.3)
Culicicola	0 (0.0)	1 (6.3)	0 (0.0)	1 (3.6)	2 (2)
Caviae	8 (34.8)[d]	8 (50)[d,e]	2 (6.3)	5 (17.9)	23 (23.2)
Allosaccharophila	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.6)	1 (1)
Enteropelogenes CLX204	0 (0.0)	0 (0.0)	2 (6.3)	0 (0.0)	2 (2)

[a]Significantly higher than prevalence among diarrheal stool isolates, non-diarrheal stool isolates and chicken isolates ($P<0.0002$, OR = 10.13; $P<0.0003$, OR = 11.88 and $P<0.0000002$, OR = 32.40, respectively).
[b]Significantly higher than prevalence among water isolates ($P<0.005$, OR = 13.56).
[c]Significantly higher than prevalence among diarrheal children stool isolates and water isolates ($P<0.04$, OR = 3.53 and $P<000002$, OR = 47.91, respectively).
[d]Significantly higher among diarrheal stool isolates ($P<0.007$, OR = 8.00 and non-diarrheal stool isolates ($P<0.0005$, OR = 15.00) than among water isolates.
[e]Significantly higher than prevalence among chicken isolates ($P<0.03$, OR = 4.60).

aeromonads reported from different sources in developed and developing countries (6, 7). The remaining 5% (5/99) of aeromonads investigated belonged to genospecies *A. enteropelogenes* CLX204, *A. culicicola*, and *A. allosaccharophila*. These aeromonads are recognized *Aeromonas* species with uncertain taxonomic status, with the latter two not being isolated from clinical material (29), indicating they may have no role in human disease. In agreement with our findings, a previous study reported the predominance of *A. caviae* genospecies among aeromonads from diarrheal and non-diarrheal children attending Clinical Research and Service Centre of the International Centre for Diarrhoeal Disease Research in Dhaka, Bangladesh (ICDDR-B) (30).

Recently, Carvalho et al. (31) identified 80 distinct *Aeromonas* strains by *gyrB*-based phylogenetic analysis among a collection of 206 isolates from untreated waters used for human consumption in Portugal that were discriminated by Random Amplified Polymorphic DNA-PCR (RAPD-PCR). The most common genospecies detected was *A. hydrophila* (26%) followed by *A. media* (23%). In agreement with their finding,

Table 2. Virulence genes in *Aeromonas* from Libya

		No. (%) positive for[a]			
Aeromonas from	No. tested	*aer*	*act*	*ast*	*alt*
Children					
With diarrhea	12	10 (83.3)	0 (0.0)	0 (0.0)	0 (0.0)
Without diarrhea	12	10 (83.3)	0 (0.0)	0 (0.0)	0 (0.0)
Water	11	9 (81.8)	4 (36.4)[b]	6 (54.5)[c]	0 (0.0)
Chicken carcasses	17	16 (94.1)	0 (0.0)	3 (17.6)	0 (0.0)
Total	52	45 (86.5)	4 (7.7)	9 (17.3)	0 (0.0)

[a]*aer* = aerolysin, *act* = *Aeromonas hydrophila* cytolytic enterotoxin, *ast* = *Aeromonas hydrophila* isolate SSU enterotoxin, *alt* = *Aeromonas* extracellular lipase. Two *Aeromonas* strains from water were positive for *act* but negative for *aer*. Both strains caused complete destruction of Vero cells.
[b]Significantly higher than in diarrheal children, non-diarrheal children and chicken samples ($P<0.03$, odds ratio [OR] = unidentified; $P<0.03$, OR = unidentified and $P<0.08$, OR-undefined, respectively).
[c]Significantly higher than diarrheal children, non-diarrheal children and chicken samples ($P<0.003$, OR = unidentified; $P<0.003$, OR = unidentified and $P<0.05$, OR = 5.60, respectively).

we identified the majority of aeromonads in untreated well water as *A. hydrophila*, but at a much higher rate (84%).

Chicken carcasses may also become contaminated with aeromonads during the washing and cleaning of such carcasses with *Aeromonas*-contaminated water. Elkot et al. (32) found *Aeromonas* species in more than 75% (218/290) of frozen chicken carcasses sold at retail outlets in Tripoli, Libya. There is lack of data on genospeciation of aeromonads from chicken by *gyrB*-based phylogenetic analysis. However, Abdullah et al. (28) using PCR–RFLP analysis of 16S rRNA genes, identified 30 of 32 (94%) isolates from chicken carcasses in Libya as *A. veronii*. In the present study, more than 60% of isolates from chicken samples were *A. veronii*.

Aeromonas species are commonly isolated from non-diarrheal children in developing countries. Therefore, it is important to detect virulence factors in aeromonads isolated from children in a matched case-control study of diarrhea in such countries. Hemagglutination (HA) of erythrocytes is associated with the ability of enteric bacteria to adhere to human epithelial cells. Burke et al. (33) reported that enterotoxigenic *A. hydrophila* isolates showed HA resistant to mannose and to fucose, whereas non-enterotoxigenic *A. caviae* commonly isolated from the environment or non-diarrheal individuals showed mannose-sensitive HA.

Examination of *Aeromonas* isolated in Libya for genes coding for virulence factors indicated that the *aer* gene was commonly present across all sample sources (86%). However, we did not find statistical significance between the presence of *aer* and sample source or genospecies (data not shown). Aerolysin is a pore forming toxin and is regarded as the most important virulence factor in *Aeromonas* food poisoning and one of the major virulence factors in gastroenteritis (8, 34, 35).

A previous study from Libya (28) reported aerolysin-like hemolysin gene sequences in 100% of 52 *Aeromonas* isolates from children with diarrhea, chicken carcasses, and a hospital environment. Ottaviani et al. (36) observed *aer*, *act*, *alt*, and *ast* in 50, 31, 31, and 34%, respectively, in 32 aeromonads from diarrheal patients. Among the *act*, *alt*, *ast*, and *aer* genes, only *aer* (83%) was detected in stools of diarrheal and non-diarrheal children in the present study.

Albert et al. (30) examined *alt*, *ast*, and *act* genes in *Aeromonas* isolates from children with diarrhea, children without diarrhea, and environmental sources (including surface water) in Bangladesh. They found that aeromonads positive just for the *alt* gene had similar distributions in the three sources; aeromonads positive just for the *ast* gene were significantly more prevalent among environmental specimens than among diarrheal children specimens; and aeromonads positive just for the *act* gene were not found in any of the three sources. In agreement with their results we detected the *ast* gene significantly more frequently among aeromonads from water samples than among aeromonads from three other sources (i.e. diarrheal children, non-diarrheal children, and chicken). However, contrary to their findings we did not detect the *alt* gene in aeromonads from the four sources examined and detected the *act* gene in more than 36% of *Aeromonas* isolates from water. Differences in the reported rates of virulence genes among *Aeromonas* species from different regions may be related to differences in geographical location.

In conclusion, the majority of aeromonads from Libya fall within three genospecies (i.e. *A. hydrophila*, *A. veronii*, *and A. caviae*). Furthermore, genes coding for toxin production and cytotoxicity to Vero cells are common features among *Aeromonas* species isolated from food and water sources in Libya, which may pose a health risk to users of such sources, particularly to immunocompromised individuals.

Acknowledgements

Dr. Ghenghesh would like to acknowledge the assistance provided by the late Mr. Salah S. Abeid in the collection of some of the organisms used in the present work. The late Mr. Abeid was a research assistant in the Department of Microbiology and Immunology, Faculty of Medicine, Tripoli, Libya, who worked with Dr. Ghenghesh for nearly 20 years and sadly passed away in 2008 due to cancer.

Conflict of interest and funding

The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

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