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ISOLATION AND IDENTIFICATION OF *AEROMONAS* SPP. FROM DRINKING WATER IN BASRAH GOVERNORATE

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ABSTRACT

Two hundred and twenty two water samples were collected from August 2008 to April 2009, which including 49 samples from 13 water purification plants in Basrah governorate (13 samples prior entering to the plants, 13 from precipitation, 10 from filtration tanks and 13 samples from the water coming out of these plants). Furthermore 127 samples of tap water were collected from 18 districts around Basrah governorate. Also 46 samples were collected from tankers which supplying Reverse Osmosis (RO) in 19 different places and from five RO water supplying plants in the governorate. The concentration of residual chlorine was measured at the time of collection with a range of 0- 1.32 mg/L. The Membrane Filtration method was used to filtered 5ml of each sample of plants and tap water and 100 ml of water samples of RO plants and tankers. The filter papers were placed on both M-FC agar for fecal coli form and Ampicillin Dextrin Agar with Vancomycin (ADA-V) medium for *Aeromonas* and then incubated at different temperature according to the requirements of these bacteria. 208 isolates on ADA-V medium have been identified as *Aeromonas* spp. by morphological and biochemical characters. Six species of Aeromonas spp. were diagnosedas: *A. hydrophila* (64) isolates, *A. caviae* (53), *A. schubertii* (34), *A. eucrenophila* (25), *A. encheleia* (17) and *A. veronii* bv. *veronii* (15). Antibiotic susceptibility tests were done for the types of *Aeromonas* bacteria isolated from water samples. All Aeromonas spp displayed multiple drug resistance, particularly to Ampicillin, Vancomycin and Cefixime. The presence of such pathogens in drinking water is a strong indicator of unsuitability for drinking and requires appropriate solution.

INTRODUCTION

The final effects of water degradation are the limits as to the use of drinking water reservoirs. Frequently this state is coupled with microbiological contamination, resulting in the penetration of potentially pathogenic bacteria or microorganisms detrimental to waters (Gołaś *et al*., 2002). Some of the bacteria, such as *Aeromonas,* may be a threat to human health due to their ability to multiply in drinking waters (Ashbolt, 2004). *Aeromonas* spp. occur ubiquitously and autochthonously in aquatic environments (Rahman *et al*., 2001), and commonly isolated from a wide range of aquatic systems including drinking water at treatment plants and in water distribution systems, lakes and rivers (Joseph and Carnahan, 2000 and Alavandi *et al*., 2001) as well as a variety of foods (Chang *et al*., 2008) and have gained importance as human pathogens causing gastrointestinal and extra intestinal infections (Cabrera *et al*., 2007). The exact etiology of disease involving aero monads is complicated by the diverse genetic, biochemical, and antigenic heterogeneity that exist among members of this group (Canals *et al*., 2007). *Aeromonas* infection may occur by taking fecal contaminated water and food (Lindberg *et al*., 2008) and because of its potential to cause human disease, the Environmental Protection Agency listed *Aeromonas hydrophila* on the first and second Contaminant Candidate List (CCL 1 and CCL 2) of potential waterborne pathogens (Environmental Protection Agency, 1998 and USEPA, 2005) as well as The World Health Organization has proposed this organism as one of the contaminants of concern in waterborne diseases (WHO, 2004). The present study aimed at investigating the occurrence of *Aeromonas* in water purification stations in Basrah governorate, drinking water from different districts and Reverse Osmosis water in the governorate, as well as their susceptibility to several antibiotics.

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Table 1. Average of Logarithmic number of *Aeromonas* spp. and *Fecal* coli form isolated from sanitation stations in Basrah governorate

sanitation stations	Raw water		Precipitation tankers		filtration tankers		water coming out of station	
	Aeromonas	*Fecal*	*Aeromonas*	*Fecal*	*Aeromonas*	*Fecal*	*Aeromonas*	*Fecal*
Al-Baradhiya	4	4	3.67	4	2.64	3.54	1.47	3.67
Al-Jubaila I	4	4	4	4	4	2.46	2.32	2.42
Al-Jubaila II	4	4	4	3.68	4	4	4	3.68
Al-Rubat	2.55	3.18	1.30	2.82	1.02	2.34	0	1.73
Al-Fayhaa	4	2.93	3.69	2.91	ND	ND	1	2.47
Al-Labani	3.57	3.06	1.87	2.91	ND	ND	2.33	2.20
Al-Abbas	4	4	2	2.34	2.68	2.77	0	0
Al-Ma'aqal	3.07	3.49	4	3.40	2.48	3.02	0	0
Hamdan	0	2.74	1.38	1.30	1.30	1.60	0	1.53
Shatt Al-Arab	1.84	2.24	2.34	2.57	1.38	0	1	1.47
Uwasain	2.75	2.94	2.73	2.93	ND	ND	0	1.38
Muhaila	1.30	2.55	2.83	2.44	1.01	1.12	0	0
Muhajran	2.47	3.08	1.87	0	1.38	0	0	0
Average	2.89	3.24	2.74	2.71	2.19	2.08	0.93	1.58

ND: Not Done

Table 2. Average Logarithmic number of Aeromonas and Fecal coli form (CFU/100mL) isolated from tap water and average residual chlorine concentration

District	No. of Samples	Average of residual chlorine concentration Mg/l	*Fecal* Log number	*Aeromonas* spp. isolates No.	Average *Aeromonas* Log number
Old Basra	9	1.03	2.89	11	2.33
Al-Jame'eyat	3	0.05	3.03	5	2.05
Al-Ashar	17	0.04	3.44	20	2.24
Al-Ma'aqal	13	0.17	3.43	15	1.99
Al-Hakeemya	8	0.63	2.91	8	2.32
Shatt-Al-Arab	10	0.44	2.66	9	2.13
Al-Esmae'e	5	0	2.32	6	2.13
Al-Hussain	8	1.01	2.78	9	2.07
Al-Tuwaisa	6	0	3.18	9	2.64
Al-Hadi	4	0	3.60	6	2.54
Al-Abela	3	0	3.44	5	2.22
Al-Jubaila	5	1.4	4	4	2.10
Al-Jazae'er	3	0	2.87	4	1.66
Al-Jumhurya	3	0	2.33	3	2.19
Al-Junaina	5	0.02	3.72	5	1,55
Al-Mowafakai	5	0	2.66	5	2.13
Al-Fayhaa	9	0.31	2.32	7	1.72
Abu Al.Khaseeb	11	1.32	2.34	15	2.57

Table 3. Percentage of resistance of *Aeromonas* spp. for antibiotics tested.

antibiotics / *Aeromonas* spp.		Ampicillin 10mcg	Kanamycin 30mcg	Erythromycin 15mcg	Gentamicin 10mcg	Tetracycline 30mcg	Ceftriaxone 30mcg	Streptomycin10mcg	Chloramphenicol 30mcg	Vancomycin30mcg	Neomycin 30mcg	Ciprofloxacin 5mcg	Cefixime5mcg
A. hydrophila	S	0	0	0	33.3	33.3	0	16.6	16.6	0	50	100	0
	I	0	16.6	33.3	0	0	83.3	66.6	16.6	0	0	0	0
	R	100	83.3	66.6	66.6	66.6	16.6	16.6	66.6	100	50	0	100
A. caviae	S	0	100	0	33.3	66.6	0	33.3	33.3	0	0	100	0
	I	0	0	66.6	33.3	33.3	66.6	33.3	0	0	66.6	0	0
	R	100	0	33.3	33.3	0	33.3	33.3	66.6	100	33.3	0	100
A. eucrenophila	S	0	0	0	0	0	0	0	0	0	0	100	0
	I	0	0	100	0	50	50	0	0	0	50	0	0
	R	100	100	0	100	50	50	100	100	100	50	0	100
A. schubertii	S	0	33.3	0	66.6	66.6	33.3	33.3	66.6	0	33.3	100	0
	I	0	0	0	0	0	33.3	33.3	0	0	33.3	0	0
	R	100	66.6	100	33.3	33.3	33.3	33.3	33.3	100	33.3	0	100
A.veronii bv. *veronii*	S	0	0	0	0	0	0	0	0	0	0	100	0
	I	0	0	0	50	100	50	50	0	0	50	0	0
	R	100	100	100	50	0	50	50	100	100	50	0	100
A. encheleia	S	0	0	0	66.6	66.6	0	33.3	33.3	0	66.6	100	0
	I	0	0	33.3	33.3	33.3	66.6	33.3	66.6	0	33.3	0	0
	R	100	100	66.6	0	0	33.3	33.3	0	100	0	0	100

R: Resistant I: Intermediate S: Susceptible

MATERIALS AND METHODS

Water Samples

A total of 222 samples were collected from August 2008 to April 2009, including 49 samples from 13 water purification stations in Basrah governorate (13 samples prior entering to the stations, 13 from precipitation, 10 from filtration tanks and 13 samples from the water coming out of these stations). Furthermore, 127 samples of tap water were collected from 18 quarters in Basrah governorate. In addition, 46 samples collected from tankers which are supplying Reverse Osmosis (RO) in 19 different places and from five RO water supplying stations in the governorate. The samples were collected according to Standard Methods for Examination of Water and Wastewater (APHA, AWWA, WEF, 1995). The concentration of residual chlorine for each sample was measured using chlorine meter (Lovibond 2000, UK) at the time of collection.

Isolation

Duplicates of 5ml of each sample of stations and tap water and 100 ml of water samples of RO stations and tankers were filtered by membrane filtration technique using 47 mm cellulose acetate membrane filters with a nominal pore size of 0.45 µm (Sartorius, Germany). The membrane filter papers were placed on M-FC agar and incubated in water bath at 44.5ºC for18- 24 h and on ADA-V medium (USEPA, 2001)and incubated at 37ºCfor 24 h .

Identification

The Typical colonies on ADA-V medium (yellow) were subjected to biochemical tests including: Gram stain, oxidase, catalase, indol, nitrate reduction, motility, gelatin liquefication, ornithine and lysine decarboxylase, growth with 6% and without NaCl, DNA hydrolysis, Phenylalanine deaminase, methyl red, Vogas Proskauer, H_2S production and haemolysin production on blood agar using 5% sheep blood for the presence of alpha or beta hemolysis. Also standardized HI media index has been used by isolating the organism on Brain Heart Infusion Agar (Himedia /India) for 24h at 37 ºC, then a single isolated colony was picked in 5 ml Brain –Heart broth and incubated for 4-6h at 37 ºC until the inoculum turbidity is ≥ 0.5 OD at 620nm. A loopful of inoculum was placed in each well of the kit and incubated for 24h at 35 ºC. The results were interpreted as per the results interpretation chart.

Antibiotic susceptibility tests

The antibiotic susceptibilities of *Aeromonas* isolates were tested *in vitro* according to National Committee for Clinical Laboratory Standards (NCCLS, 2001). The following antibiotics were tested (Bioanalyze /Turkey): ampicillin, kanamycin, erythromycin, gentamicin, tetracycline, ceftriaxone, streptomycin, chloramphenicol, vancomycin, neomycin, ciprofloxacin, and cefixime.

RESULTS

Stations water samples

One hundred and twenty two isolates were isolated on ADA-V medium, of which only 55 isolates as *Aeromonas* represented by *A. hydrophila* (22), *A. caviae* (21), *A. schubertii* (8) and *A. eucrenophila* (4). Aero monads showed average log. Numbers of 2.89 for raw water and 0.93 for water coming out of station. While for FC an average of 3.24 and 1.58 for raw water and water coming out of station respectively (Table 1).

Tap water samples

Of 127 tap water samples 6 *Aeromonas* spp. were identified including: *A. hydrophila* (35), *A. caviae* (32), *A. schubertii* (26), and *A. eucrenophila* (21) while the total number of isolates of *A. encheleia* and *A. veronii* bv. *veronii* was 17 and 15 respectively. The distribution of these types differed according to different districts (Table 2). Quantitative data are presented in table 2 which show presence of FC and aero monads irrespective of high chlorine concentration. Presence of aero monads and FC was recorded in all the 18 districts.

Reverse Osmosis (RO) water samples

41 samples were collected from tankers which supplying RO in 19 districts in Basrah governorate, in fact only seven districts showed occurrence of Aeromonas and in very low density despite the presence of FC in 17 out of 19 districts. While all the five RO water supplying plants in the governorate were negative for Aeromonadstestes. The total number of isolates on ADA-V were 584 isolates of which, 298 isolates were diagnosed according to Holt *et al.* (1994) and Martin-Carnahan & Joseph (2005). The number that belong to the genus *Aeromonas* were 208 isolates depending on their morphological and biochemical characteristics. The percentage of *Aeromonas* spp was 35.70% of the total isolates' number and 69.79% of the diagnosed isolates. The six of *Aeromonas* spp. that have been isolated appeared in the following decreasing order *A. hydrophila*, *A. caviae*, *A. schubertii*, *A. eucrenophila*, *A. encheleia* and, *A. veroniibv. veronii*. Antibiotic susceptibility test was done for 19 *Aeromonas* spp. from water samples as 6 *A. hydrophila* 3 *A. caviae* 2 *A. eucrenophila* 3 *A. schubertii* and 2 *A. veronii* bv.*veronii*. All examined species displayed multiple drug resistance, particularly to ampicillin, vancomycin and cefixime. Overall resistance to antibiotics was significantly higher in *A. eucrenophila* which was resistant to 7 antibiotics, on the other hand, all species were sensitive to ciprofloxacin (Table 3).

DISCUSSION

The current study has shown that precipitation and filtration processes resulted in a reduction in the size of bacterial population in most of the plants included in the study. The remaining chlorine concentration was variable ranging (0-1.32 mg/L) while the remaining chlorine concentration in samples of drinking (tap) water varied among different regions and from area to area in the same region and it ranged from (0-2mg/L). It was noted that chlorine was absent in more than half of the samples (55.9%) and the remaining chlorine concentration varied in RO plants and ranged from (0.15-2 mg/L). By comparing these results with Iraqi standards for remaining chlorine concentration which ranged from (0.3-2.0 mg/L) (The Central Bureau for Quality and Control Measurement, 1984) and with international standards for remaining chlorine concentration which ranges from (0.2-0.5

mg/L)(WHO, 1996) it is below the lower limit. The reduction and absence of chlorine concentration in the last parts of distribution network are due to a number of factors like chlorine volatilization in the distribution network or during the process of water transportation from RO plants to storage tankers (Uhl *et al*., 2003 and Vrouwenwelder *et al*., 2000) and may be due to decreased water flow or its stagnation inside the pipes because the plant system works in an interrupted manner beside the temperature, pH, organic and inorganic materials (Vrouwenwelder and Kooij, 2001). In this study, ADA-V medium was used as a selective medium for isolating this bacteria according to EPA (USEPA, 2001) on which most of the samples were positive for the growth of *Aeromonas* in case of crude water entering the plants. Their numbers were uncountable in some plants and this is because it is a natural part of minute plants and may be due to scarcity of sewage water processing systems before dumping it into the rivers. Human beings contribute directly or indirectly in increasing pollutants in drinking water sources (Carr *et al*., 2004). These results are compatible with what El-Taweel and Shaban (2001) have reached when examining eight processing plants in Egypt.

Aeromonas bacteria were isolated from precipitation tanks and it was noted that the number of this bacteria has increased noticeably in some plants as compared to their number in the crude water. This may be related to the badness of the primary treating stage of the crude water entering the plants and by so, the precipitation tanks may work as a reservoir for the growth of these bacteria as a result of the presence of the suitable conditions such as precipitants and growth of algae and also because some of the precipitation tankers are exposed to the environment. It was also noted that performances of filtration unit has decreased in some of water treating plants in Basrah and this may be because some of the plants are old or the filters used in this unit are old and there is no maintenance or periodic cleaning or changing for these filters. Despite the presence of *Aeromonas* in all the samples of filtration water, it was noted that the number of this bacteria has decreased in this stage of treatment. For the samples of water coming out of plants, it was noted that the number of this bacteria varied between plants and their number were uncountable in Al-Jubaila plant and they were absent in Al-Rabat, Al-Abbas, Al-Maaqal and Abu Al-Khaseeb plants. This may be due to the differences in the remaining chlorine concentration in the water coming out from plant to plant, but despite that, the growth of these bacteria was noted in other plants where remaining chlorine concentration was 2 mg/L as chlorine activity depends on many factors like: temperature, pH which considered as effective factors on chlorine activity (Ozbas and Aytac, 1994).

Aeromonas bacteria were isolated from drinking (tap) water in different percentages from region to region and from area to area in the same region as it is part of biological membranes formed in the distribution pipes network (Flemming, 2002) and several studies have indicated to the type of the materials that these pipes are made of that affect the formation of the biological membranes like rubber, stainless steel and polyvinyl chloride (PVC) pipes (Kilb *et al*., 2003).The decrement or absence of remaining chlorine concentration in the extreme ends of the distribution system increases the percentages of this bacteria relatively. It is well known that many of the middle east countries suffer from chronic water deficiency as a result of operating the plants on an interrupted manner (Tokajian and Hashwa, 2003) which leads to creation of conditions that contribute to deterioration of water quality mainly because of the precipitation and re-growth through network pipes. Also most of the networks undergo continuously breaks and corrosions that facilitate the entry of the pollutants from rain water or infiltration of sewage water to the inside of networks. In the current study six types of *Aeromonas* spp. were isolated among these *A. hydrophila* was the highest percentage. These results are similar to the data reported in the literature, where *A. hydrophila* is the predominant species in freshwater and municipal drinking water supplies (Ghenghesh *et al*., 2001 and Koksal *et al*., 2007).

Regarding samples of water treated by Reverse Osmosis (RO water),it was found that 15 samples were positive for *Aeromonas* bacterial tests and results have shown that all samples belong to *A. hydrophila* and this is compatible Ghenghesh *et al.* (2007) finding that *A. hydrophila* was the dominant type. This may be due to the feasibility of these bacteria to address the problem of lack of nutrients or their limitations by entering into a state of starvation for survival i.e. viable but not culturable (Rowan, 2004 and Croci *et al*., 2001). Additionally there is a possibility of pollution of this water during transportation and the fact that this process occurs in basin-wheels which may contribute to increase the density of microbes as a result of failure of following the health conditions of cleaning and drying transporters or storage tankers. An association between *Aeromonas* spp. occurrence and presence of fecal coliforms was not observed as *Aeromonas* spp. were detected in water samples where these indicators were absent. The opportunistic pathogen was commonly isolated in the absence of fecal contamination (Simmons *et al*., 2001 and Hirotani *et al*., 1999).

Aeromonas species exhibit differences in their susceptibility to antibiotics. Antibiotic-resistant strains of *Aeromonas* have been isolated from aquatic environments and this resistance is principally plasmid mediated (Borrego *et al*., 1991). Multiple antibiotic-resistances was common among the isolated aero monads; all were resistant to (ampicillin, vancomycin and cefixime). Antibiotic resistance has been reported in isolates from chlorinated water distribution system in Lebanon (Tokajian and Hashwa, 2004). Because resistance is plasmid mediated, it is easily transferred among strains, and has been demonstrated in isolates from Norway, Scotland, England, and Germany (Rhodes *et al*., 2000).While all strains of *Aeromonas* have been isolated from aquatic environments were sensitive to Ciprofloxacin, These results are compatible with Castro *et al.* (2007). Findings of this study suggest that *A. eucrenophila* isolates are more resistant to antibiotics than the otherisolates isolated from aquatic environments. The results confirm that the presence of *Aeromonas* bacteria in sources of the crude water and in general drinking water and tankers of drinking water and most of the types were capable of production of virulence markers like the hemolytic activity of red blood cells with a percent of 83.65% in the diagnosed samples which belong to the *Aeromonas* spp. isolated from drinking water. Production of exotoxin (α and β-hemolysin) is associated with enteric disturbances, as reported by Macedo *et al*.

(1997).Residual disinfectant, dimensions of the network, residence time, water temperature, and concentration and type of compounds serving as food sources for bacteria are major factors determining the rate and extent of bacterial multiplication in drinking water distribution systems (after growth). In the current study, it was noticed that the species *A. encheleia* was isolated from drinking water for the first time in Basrah, Iraq since no studies were found referring to these isolates in the drinking water. The appearance of these virulence factors in drinking water represents a source of worry for the public health and should be considered seriously when evaluating the quality of drinking water. In addition, ciprofloxacin is suitable drugs that can be used in the treatment of *Aeromonas*-associated infections, resulting from contact with water.

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