Risk Assessment of Heterotrophic Bacteria from Bottled Mineral Water Consumed in Syria

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ABSTRACT

Four hundred and thirty samples of bottled mineral water belonging to ten different local and imported brands collected from the Syrian market have been analyzed bacteriologically. Heterotrophic plate count (HPC) was determined using R2A agar culture medium and incubating at $22^{o}C$ for 5–7 days. Pseudomonads and Aeromonads members were selectively investigated and identified biochemically.

Around 70 % of the imported water samples exhibited HPC counts more than 100 cfu/ml versus 13 % for local ones. Risk assessment of the heterotrophic bacteria revealed that 53.49% of the strains showed resistance to one or two of the twenty antibiotics tested and the highest resistance was found against nalidixic acid, novobiocin, ampicillin, streptomycin, imipenem and ampicillin/sulbactam. The majority of *Pseudomonas* spp. and *Aeromonas* spp. strains were found to be resistant to nalidixic acid, trimethoprim/ sulfamethoxazole, ampicillin, and novobiocin. *Pseudomonas* spp. showed also high resistance to tetracycline, imipenem, ceftazidime, amikacin, erythromycin and carbencillin. The most effective antibiotics against *Pseudomonas* spp. were ciprofloxacin, gentamicin, aztreonam, colistin, kanamycin and ceftriaxone. However, the following antibiotics showed complete activity against *Aeromonas* spp. amikacin, ceftazidime, ceftriaxone, ciprofloxacin and gentamicin. The complete activity was also seen for aztreonam, chloramphenicol and gentamicin against other HPC bacteria. Strains with multiple antibiotic resistance (MAR) represented 60.18% of all isolates and the most resistant organism belonged to the genus *Pseudomonas* followed by *Aeromonas* strains. The high load of heterotrophic bacteria with relatively high counts of opportunists such as Pseudomonads and Aeromonads in the studied mineral bottled water represents hygienic and quality challenge for this ready-to-consume commodity. Moreover, multiple antibiotic resistance detected among these bacteria might pose health significances, at least to some defined sensitive individuals, and should be considered properly.

Keywords: Bottled mineral water; Risk assessment; Heterotrophic bacteria; *Pseudomonas*; *Aeromonas*; Antibiotic resistance.

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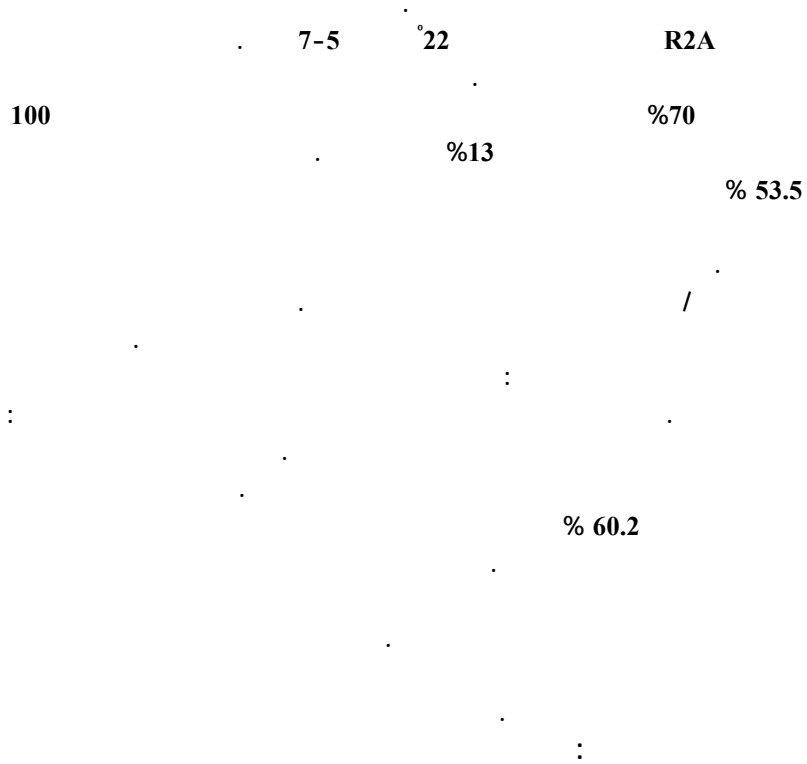
I- Introduction

There has been a dramatic increase in the consumption of bottled drinking water, especially, natural mineral water in Syria. Although the growing of tourism industry in Syria can be considered as a main cause, the increase in demand worldwide has been attributed to many factors. The public's concern over increased water pollution and the so common belief among people that bottled water is superior to municipal water in that it contains no microorganisms (Papapetropoulou *et al.*, 1997; Hunter, 1993), and fashioned trends towards the consumption of designer and trademarked water (Hunter, 1994). In addition, bottled water is often recommended for patients with immune-system deficiencies as well as marketed as ideal for infant nutrition and reconstitution of foods (Warburton, 1993). Objection to the local water supplies for offensive taste and odor as well as fluoride, chlorine and other additives might be considered as additional factors (Warburton *et al.*, 1992; Tamagnini and Gonzalez., 1997; Bharath *et al.*, 2003). Because the emergent mineral water is an oligotrophic environment, their content of viable bacterial cell is as low as 10 cfu ml^{-1} (Ferreira *et al.*, 1994; Leclerc, 1994). This low count of autochthonous organisms are of little concern to the healthy consumer. However, as contamination may be induced during bottling process, potential pathogens may persist.

Therefore, because there is no permitted disinfection or sterilization process of commercially available mineral waters for removal of microorganisms, the presence of such pathogens may be of great public health concern.

Because Pseudomonads constitute the main part of these both naturally occurring and contaminating bacteria (Rosenberg and Hernandez- Duquino, 1988; Guillot and Leclerc, 1993) as well as their role in opportunistic infections (Rusin *et al.*, 1997; Kudinha *et al.*, 2000), the assessment of the health risk from these organisms after bottling continues to be of high interest for both of microbiologists and health workers.

Aeromonas spp. are widespread in surface waters. Clinically, they are incriminated as a causes of diarrhea, peritonitis, endocarditis, meningitis, septicemia, urinary tract and wound infections mostly associated with consumption of contaminated drinking water (Pin *et al.*, 1997; Rusin *et al.*, 1997; Kudinha *et al.*, 2000). Therefore, as for *Psd.aeruginosa*, their occurrence in mineral water is considered as quality indicator. Drinking water has been suggested as an important

source of human infections caused by the members of *Aeromonas* (Burke *et al.*, 1984; Havelaar *et al.*, 1990).

From health risk point of view, the great distribution of *Pseudomonas* spp. and *Aeromonas* spp. as the main components of heterotrophic bacteria in bottled mineral water and their increased resistance to clinically available antimicrobial agents has been considered as health concern particularly to immunocompromised patient and because there is the risk of transferring the resistance to other bacteria present in the human body and some pathogenic ones (Rosenberg and Hernandez- Duquino, 1988; Massa *et al.*, 1995; Mary *et al.*, 2000).

By taking into account that there are many local and imported brands of bottled mineral water in the Syrian market, and there is no documented and reliable information on the microbiological quality of these water products and consequently their hygienic safety for consumptions, the increased consumption of such waters raises the question as to whether they are hygienically safe. Therefore, the aim of the study is to provide an adequate information on the microbiological quality of these water products and an assessment of their health risks through investigation of the antibiotic resistance among *Pseudomonads* spp., *Aeromonads* spp. and other heterotrophic bacteria isolated from it.

II- Materials and methods

Collection of samples. A total of 160 bottles of natural Syrian mineral water as well as 270 bottles of imported bottled natural mineral water were directly collected from the retail outlets in Syria throughout the years 2006 and 2007 . The studied local water are Syrian in origin and produced (bottled) locally. Mineral water bottles were stored at room temperature (20 to 22°C) prior to investigation. Bottles were vigorously agitated before analysis. All analysis tests were performed in laboratories of Tishreen University.

Bacteriological analysis: The samples were analyzed to investigate the members of Psuodomonads and Aeromonads as well as total heterotrophic test. For heterotrophic plate counts (HPC), different volumes of undiluted water samples were plated on Tryptic Soy Agar TSA (Oxoide *ltd*) and R2A medium (Reasoner and Geldreich, 1985) and incubated at 22° C for 5–7 days. For *Pseudomons* spp. and *Aeromonas* spp., membrane filtration technique was applied.

Volumes up to 1L of sample were filtrated using cellulose membrane filter of 0.45 μm pores (Millipore *Ltd*) and filters were

transported onto selective culture media and incubated up to 72 hr at 30°C. Pseudomonas Agar Base (Oxoide *Ltd*) supplemented with 200 mg/l cetrimide and 15 mg/l nalidixic acid was used for detecting of *Pseudomons aeruginosa*. Other Psuodomonads members were presumptively isolated by using pseudomonas isolation agar (Hi media *Ltd*) as selective culture medium.

Selective isolation of *Aeromonas hydrophila* was performed by using Pril-ampicillin-dextrin-ethanol [PADE] agar containing 15-30 μg ampicillin (Imziln *et al*., 1997) .

For isolation of the other presumptive members of *Aeromonas* spp., filters were transferred to Glutamate Starch Phenol Red (GSP) agar plates (Merck *Ltd*) supplemented with 100 000 U l^{-1} of penicillin G (Sigma Chemical). Yellow colonies were counted and submitted to further identification tests as mentioned below.

Identification and classification. For identification purposes, all suspected colonies either from heterotrophic plate count (HPC) or membrane filters were subcultured on Tryptic Soy Agar for 24 hr at 30° C. Each strain was tested by Gram stain, for oxidase production and glucose assimilation; further identification was carried out with the API 20 NE identification system for nonfermenters (Bio Mereux *ltd*). All tests were carried out at 22°C. Further confirmation tests were carried out to identify the isolates of *Aeromonas* spp. and *Pseudomonas* spp. ; these tests are: the oxidation/fermentation (O/F), sensitivity to vibriostatic agent O/129, gas production from glucose, H$_2$S production from cysteine, esculin hydrolysis, motility, growth at 41.5° C , characteristic growth on King's A' agar, growth on acetamide broth, denitrification, arginine dihydrolase, and gelatin liquefaction (Popoff, 1984; Carnahan *et al*., 1991; Murray *et al*., 1999).

Antibacterial susceptibility testing. For all strains isolated, antibiotic sensitivity was tested by the agar disk diffusion method (Bauer *et al*., 1966). Bacterial strains were suspended in sterile 0.85% saline and the cell density was adjusted to match the turbidity of McFarland No. 2 standard, diluted 1:20, and swabbed on Mueller-Hinton agar (Hi media *Ltd*) using sterile cotton swabs. The antibiotics and the concentrations used were as follows: amikacin (AMK: 30 μg), ampicillin (AMP: 10 μg), ampicillin-sulbactam (SAM: 10/10 μg), aztreonam (AZT: 30 μg), carbencillin (CAB: 100 μg), ceftazidime (CFZ: 30 μg), ceftriaxone (CRO: 30 μg), chloramphenicol (CHL: 30 μg), ciprofloxacin (CIP: 5 mg), colistin (COL: 10 μg), erythromycin (ERY: 15 μg), gentamicin (GEN: 10 μg), imipenem (IMP: 10 μg),

kanamycin (KAN: 30 μg), nalidixic acid (NAL: 30 μg), novobiocin (NOV: 30 μg), streptomycin (STR: 10 μg), tetracycline (TET: 30 μg), tobramycin (TOB: 10 μg), trimethoprim/sulfamethoxazole (SXT: 1.25/23.75 μg). Plates were incubated at 30 ºC for 24–48 h. Results of susceptibility were reported as sensitive, intermediate or resistant strain upon the size of the inhibition zone around each disk and information supplied by the antibiotics manufacturing company (Hi Media *ltd*) and referenced criteria (NCCLS, 1999).

Multiple antibiotic resistance (MAR) indexing of each isolate was carried out by dividing the number of antibiotics to which the isolate was resistant to the total number of antibiotics to which the isolate was exposed.

III- Results

A total of 216 bacterial isolates were obtained and subjected to susceptibility tests. These isolates can be divided into three categories: Pseudomonads, Aeromonads, and other heterotrophic bacteria (HPC) as shown in Table 1. The count of viable heterotrophic bacteria as measured on R2A ranged from 10 to $5.6.10^5$ cfu ml^{-1} for imported water and from 0 to $8.7.10^2$ cfu ml^{-1} with only two samples exceeded 10^3 cfu ml^{-1} for local water. It is clear that the level of occurrence of *Pseudomonas* spp. is somewhat related to HPC counts while this is not the case for *Aeromonas* spp. any way. So, samples with more than 10000 cfu ml^{-1} were found to be crowded with *Pseudomonas* members. As shown in Table 1, there were 22 (13.75%) positive local samples for occurrence of *Pseudomonas* spp. Of these, *Pseudomonas* spp. were recovered in only 10 (6.25%) samples with tested volumes less than 250 ml. In contrast, the imported samples showed as high as 81 (30%) positive samples and 59 (21.85%) of them were less than 250 ml. On the other hand, *Aeromonas* spp. showed high frequency of isolation and occurred in 42 (26.25%) and 118 (43.70%) samples of local and imported waters respectively.

Table 1. Percentages of positive samples of bottled mineral waters for presence of *Pseudomonas* spp. and *Aeromonas* spp. as related to HPC counts.

Mineral bottled water	No. of samples	Bacteria	No and (%) of samples positives when HPC as cfu[*]/ml is:			
			$0-10^2$	10^2-10^3	10^3-10^4	$>10^4$
Local	160	*Pseudomonas* spp.	5 (3.12)	15 (9.37)	2 (1.25)	-
		Aeromonas spp.	19 (11.87)	21 (13.12)	2 (1.25)	-
Imported	270	*Pseudomonas* spp.	8 (2.96)	18 (6.67)	24 (8.89)	31 (11.48)
		Aeromonas spp.	28 (10.37)	30 (11.11)	27 (10)	33 (12.22)

* cfu: colony forming unite

Of 87 strains isolated of Pseudomonads, 23 (26.44%) were tentatively identified as *Psd.aeruginosa* followed by *Pseudomonas fluorescens* (16.09%), and the rest i.e. (58.61%) of strains distributed to other *Pseudomonas* spp. as illustrated in Table 2.

Table 2. Total number and (%) of the identified species of Pseudomonads and Aeromonads isolated from bottled mineral waters.

Bacteria	Number and (%) of strains identified		
	Mineral water		Total
	Local	Imported	
Pseudomonas spp.	**27**	**60**	**87**
Psd. aeruginosa	6 (22.22)	17 (28.33)	23(26.44)
Psd. fluorescens	5 (18.52)	9 (15)	14 (16.09)
Psd. alcaligenes	4 (14.81)	7 (11.67)	11 (12.64)
Psd. putida	3 (11.11)	7 (11.67)	10 (11.49)
Psd. vesiculares	3 (11.11)	6 (10)	9 (10.34)
Psd. stutzeri	1 (3.70)	6 (10)	7 (8.04)
Psd. picketti	1 (3.70)	4 (6.67)	5 (5.75)
Psd. diminuta	0 (-)	5 (8.33)	5 (5.75)
Psd. paucimobilis	0 (-)	4 (6.670	4 (4.60)
Other Pseudomonas	4 (14.81)	9 (15)	13 (14.94)
Aeromonas spp.	**22**	**38**	**60**
Aer hydrophila	7 (31.82)	12 (31.58)	19 (31.66)
Aer .caviae	5 (22.73)	11 (28.95)	16(26.67)
Aer .sorbia	5 (22.73)	8 (21.05)	13 (21.67)
Other Aeromonas	5 (22.73)	7 (18.42)	12 (20)
HPC bacteria	**43**	**43**	**86**
Enumeration	**92**	**141**	**233**

Aeromonas hydrophila has been found to be predominant species among the isolated strains of *Aeromonas* spp.; So, of 60 strains, 19(31.66%) were identified as *Aer.hydrophila* followed by other species (Table 2).

In addition, there were 86 isolates of heterotrophic bacteria other than Pseudomonads and Aeromonads. This separation was tentatively achieved by subculturing each HPC colony on Pseudomonads and Aeromonads selective culture media mentioned above. Furthermore, each colony was subjected to the main biochemical tests for identification of Pseudomonads and Aeromonads. Consequently, any colony failed to meet these criteria was considered as HPC isolate.

As shown in Table 2, the identified strains belonged to genus Pseudomonads were represented by nine species. However, there were difficulties to classify some of the suspected strains of *Pseudomonas* spp. and hence cited as other Pseudomonads. As it can be seen from

Table 2, the obvious difference between the two sources of water in term of species distribution was significant. Notably, the imported water has showed higher counts of *Pseudomonas* spp. and subsequently *Psd.aeruginosa* compared with local one. However, this is not the status of *Aeromonas* spp. distribution since *Aeromonas hydrophila* were observed with almost similar percentages of occurrence for local and imported water.

Results of the antibiotic sensitivity testing are presented in table 3 and figure 1. At the level of species isolated, and as related to antibiotic sensitivity profile, no significant differences were found between the two sources of water.
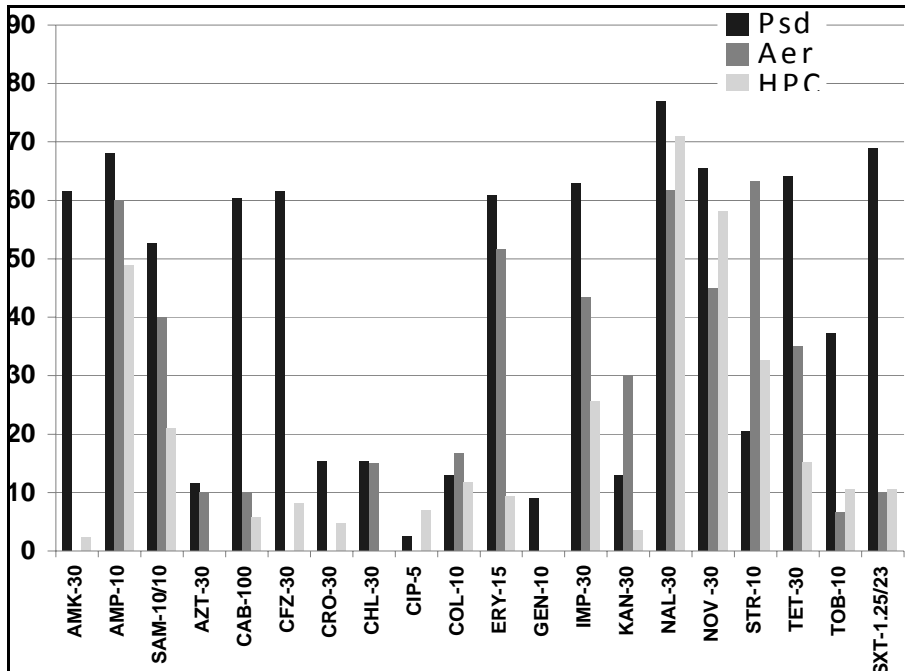

Figure 1. Percentage of antibiotic resistance among *Pseudomonas* spp., *Aeromonas* spp., and other heterotrophic bacteria isolated from bottled mineral water.

AMK: amikacin 30 µg, AMP: ampicillin 10 µg, SAM: ampicillin-sulbactam 10/10 µg, AZT: aztreonam 30 µg, CAB: carbencillin 100 µg, CFZ: ceftazidime 30 µg, CRO: ceftriaxone 30 µg, CHL: chloramphenicol 30 µg, CIP: ciprofloxacin 5 mg, COL: colistin 10 µg, ERY: erythromycin 15 µg, GEN: gentamicin 10 µg, IMP: imipenem 10 µg, KAN: kanamycin 30 µg, NAL: nalidixic acid 30 µg, NOV: novobiocin 30 µg, STR: streptomycin 10 µg, TET: tetracycline 30 µg, TOB: tobramycin 10 µg, SXT: trimethoprim/sulfamethoxazole 1.25/23.75 µg.

Most of the heterotrophic bacteria (HPC) isolated from the bottled mineral water were resistant to nalidixic acid, novobiocin, and ampicillin. Complete sensitivity were recorded towards aztreonam, chloramphenicol, and gentamicin. Intermediate resistances were more often observed against the following antibiotics: streptomycin, nalidixic acid, chloramphenicol, tetracycline, ampicillin and novobiocin. (Table 3, figure 1)

For *Pseudomonas* spp., the highest frequency of resistance was to nalidixic acid (76.92) followed by trimethoprim/sulfamethoxazol (68.96%) and ampicillin (67.95%). (Table 3).

Table 3. Percentage of antibiotic resistance among *Pseudomonas* spp., *Aeromonas* spp., and other heterotrophic bacteria isolated from bottled mineral water.

Antibiotic	Percentage of sensitive, intermediate resistant and resistant strains of :								
	Pseudomonas spp. (n[*] = 78)			*Aeromonas* spp. (n= 60)			Other HPC bacteria (n= 86)		
	S[#]	I[##]	R[###]	S	I	R	S	I	R
Amikacin	38.46	0	61.54	100	0	0	94.19	3.49	2.32
Ampicillin	32.05	0	67.95	40	0	60	41.86	9.30	48.84
Amp/sulbactam	47.44	0	52.56	60	0	40	79.07	0	20.93
Aztreonam	88.46	0	11.54	90	0	10	100	0	0
Carbencillin	39.75	0	60.25	90	0	10	94.19	0	5.81
Ceftazidime	38.46	0	61.54	100	0	0	91.86	0	8.14
Ceftriaxone	84.62	0	15.38	100	0	0	95.35	0	4.65
Chloramphenicol	62.83	21.79	15.38	71.67	13.33	15	83.72	16.28	0
Ciprofloxacin	97.44	0	2.56	100	0	0	93.02	0	6.98
Colistin	87.18	0	12.82	83.33	0	16.67	88.37	0	11.63
Erythromycin	39.08	0	60.92	48.33	0	51.67	90.70	0	9.30
Gentamicin	91.03	0	8.97	100	0	0	100	0	0
Imipenem	37.18	0	62.82	56.67	0	43.33	74.42	0	25.58
Kanamycin	87.18	0	12.82	70	0	30	96.51	0	3.49
Nalidixic acid	5.13	17.95	76.92	26.67	11.66	61.67	10.47	18.60	70.93
Novobiocin	34.48	0	65.52	50	5	45	36.05	5.81	58.14
Streptomycin	43.59	35.90	20.51	26.67	10	63.33	47.67	19.77	32.56
Tetracycline	21.80	14.10	64.10	56.67	8.33	35	70.94	13.95	15.11
Tobramycin	62.82	0	37.18	93.33	0	6.67	89.53	0	10.46
Trim/sulfameth	31.04	0	68.96	81.67	8.33	10	89.53	0	10.46

n[*]= number of strains isolated. [#]: S= sensitive, [##]:I= intermediate resistant, [###]:R= resistant.

Six antibiotics have showed noticeable activity against *Pseudomonas* spp. These antibiotics and their sensitivity percentages are ciprofloxacin (97.44%), gentamicin (91.03%), aztreonam (88.46%), colistin (87.18%), kanamycin (87.18%) and ceftriaxone (84.62%). The most effective antibiotics against *Pseudomonas* spp. were ciprofloxacin, gentamicin, aztreonam, colistin, kanamycin and

ceftriaxone with sensitivity levels ranging from 97.44% for ciprofloxacin to 84.62% for ceftriaxone. Intermediate resistance were exclusively found with the antibiotics streptomycin, chloramphenicol, nalidixic acid and tetracycline.

Overall, although there were some exceptions, all strains of *Pseudomonas* spp. have showed relatively high level of resistance to antibiotic tested since a 77.14% of strains were resistant to three or more of twenty antibiotics tested (Table 4).

All isolates of *Aeromonas* spp. were found to be susceptible to amikacin, ceftazidime, ceftriaxone, ciprofloxacin and gentamicin. Sensitivity was almost complete to aztreonam, carbencillin, and tobramycin since resistance rate did not exceed 10% of the tested strains. Highest resistance level were detected for streptomycin, nalidixic acid, and ampicillin.

In addition to high resistance to ampicillin and ampicillin/ sulbactam, *Aer.hydrophila* strains have showed high resistance levels towards erythromycin, imipenem, streptomycin and novobiocin.

Strains resistant to more than two antibiotics were considered as multiple antibiotic resistant (MAR). Accordingly, as high as 77.14%, 60%, and 46.51% of the isolates of *Pseudomonas* spp., *Aeromonas* spp. and HPC respectively in our investigation were in that category.

As indicated previously, multiple antibiotic resistance (MAR) indexing gives some information about the size of resistance for individual isolate. In this study, a value greater than 0.15 indicates that the isolate is multiple antibiotic resistant. The MAR index values and the percentages of MAR strains of all bacterial strains studied are shown in Table 4.

Single resistance (i.e.: resistance to one antibiotic) occurred in only 8.57 % of *pseudomonas* spp. strains, and 14.28 % were also resistant to two drugs. Single resistance was found in 18.33% of the *Aeromonas* spp. strains, 21.67% had double resistance. However, single and double resistance among HPC bacteria were observably increased to 23.25 and 30.23 respectively. Thereby, MAR strains were less common among other HPC than that of *Pseudomonas* spp. and *Aeromonas* spp.

Accordingly, the MAR strains of *Pseudomonas* spp. had resistance indices ranging from 0.15 to 0.85 as compared with 0.15 to 0.65 for *Aeromonas* spp. strains. However, HPC strains had low MAR indices and the range was 0.15 - 0.45.

The most occurred profiles of resistance among *Pseudomonas* spp. strains were the combined resistance to nalidixic acid, with tetracycline, ampicillin,amikacin and erythromycin; nalidixic acid, with carbencillin, ampicillin, ceftazidime, imipenem and trim/sulfamethoxazol.

In case of *Aeromonas* spp., the resistance profile most recorded was the combined resistance to Streptomycin with nalidixic acid, ampicillin/ sulbactam and erythromycin or novobiocin.

Table 4. Multiple antibiotic resistance (MAR) index and the percentages of MAR strains of *Pseudomonas* spp., *Aeromonas* spp., and heterotrophic bacteria (HPC) from bottled mineral water.

MAR index	No of antibiotics	Number and (%) of resistant and MAR strains.			Total, n= 216
		Pseudomonas spp., n= 70	Aeromonas, spp., n= 60	HPC, n= 86	
0.05	1	6 (8.57)	11 (18.33)	20 (23.25)	37 (17.13)
0.1	2	10 (14.28)	13 (21.67)	26 (30.23)	49 (22.68)
0.15	3	3 (4.28)	3 (5)	11 (12.79)	17 (7.87)
0.2	4	3 (4.28)	4 (6.67)	8 (9.30)	15 (6.94)
0.25	5	3 (4.28)	5 (8.33)	7 (8.14)	15 (6.94)
0.3	6	3 (4.28)	5 (8.33)	6 (6.98)	14 (6.48)
0.35	7	4 (5.71)	4 (6.67)	5 (5.81)	13 (6.02)
0.4	8	4 (5.71)	4 (6.67)	2 (2.32)	10 (4.63)
0.45	9	6 (8.57)	3 (5)	1 (1.16)	10 (4.63)
0,5	10	6 (8.57)	3 (5)	-	9 (4.17)
0.55	11	5 (7.14)	2 (3.33)	-	7 (3.24)
0.6	12	5 (7.14)	2 (3.33)	-	7 (3.24)
0.65	13	4 (5.71)	1 (1.67)	-	5 (2.31)
0.7	14	2 (2.86)	-	-	2 (0.92)
0.75	15	2 (2.86)	-	-	2 (0.92)
0.8	16	2 (2.86)	-	-	2 (0.92)
0.85	17	2 (2.86)	-	-	2 (0.92)

IV- Discussion

In general, the viable counts of heterotrophic bacteria has been suggested as satisfactory indicator of the overall quality of bottled mineral water production (Ferreira *et al*., 1994). However, in many countries, and as quality parameters demanded of bottled mineral waters, mineral water must be free of *Pseudomonas aeruginosa* in any 250-ml sample (European Community, 1980).This because the opportunistic pathogen *Psd. aeruginosa* is currently considered a

primary infectious agent implicated in foodborne and waterborne diseases (Warburton, 1993; Morais *et al.*, 1997). The significance of *Pseudomonas* spp. and related species in bottled mineral waters is related to their capability of multiplying abundantly in such low-nutrient water (Gonzalez *et al.*, 1987; Mavridou *et al.*, 1994; Tsai and Yu, 1997) and surviving with somewhat constant numbers for at least 6 months after bottling (Hunter, 1993; Leclerc and Moreau, 2002; Daood, 2008). It is also worthy of noting that, *Psd. aeruginosa* has been previously suggested as a surrogate indicator for the presence of other opportunistic pathogens (Geldreich, 1992).

Accordingly, and since *Pseudomonas* spp. were discovered in high numbers in volumes of 250 ml, this puts as total as 59 (21.85%) samples of imported studied waters as unsatisfactory. Fortunately, this rate decreased to 10 (6.25%) samples of local waters.

As *Pseudomonas* spp. have occurred in only 13.75% and 30% of domestic and imported water samples respectively (Table 1), our results about the predomination of *Pseudomonas* species are not fully in agreement with those obtained by many authors. In 1988, Rosenberg and Hemandez- Duquino showed that various *Pseudomonas* spp. presented in 45% of the samples from 87 different carbonated and noncarbonated mineral waters purchased in Germany (Rosenberg and Hemandez- Duquino, 1988). This proportion was decreased to 30% in a study performed by (Mavridou, 1992). Likewise, Guillot and Leclerc reported that 40% of the isolates originated from mineral water were identified as *Pseudomonas* species (Guillot and Leclerc, 1993). *Pseudomonas* spp. are also abundantly isolated from most of the mineral water samples analyzed by (Manaia *et al.*, 1990).

All *Pseudomonal* species identified in this study (Table 2) and our previous study (Daood, 2008) have been frequently isolated by others. *Psd. aeruginosa , Psd. Stutzeri. Psd fluorescens* and *Psd. putida* have been commonly isolated from drinking bottled water (Hernandez-Duquino and Rosenberg, 1987) and bottled mineral water (Rosenberg and Hernandez-Duquino, 1988; Venieri *et al.*, 2006)

In their study on five brands of French mineral water, Mary *et al.*, (2000) have identified the following pseudomonal species: *Psd. maltophilia, Psd. fluorescens,*and *Psd. alcaligenes*. More recently, the species *Psd. aeruginosa, Psd. testoalcaligenes, Psd. maltophilia, Psd. diminuto, Psd. fluorescens*, and *Psd. vesicularis* were isolated from domestic bottled water in Greece with being *Psd. aeruginosa* is the most isolated microorganisms (Venieri *et al.,* 2006).

Although there are numerous reports that have documented, at least under some circumstances, the incidence of diarrheal disease after ingestion of drinking water contaminated with *Aeromonas* spp., the health significance of *Aeromonas* spp. in water is not fully understood (Borchardt *et al*., 2003; WHO, 2003). However, there is a worldwide distribution of *Aeromonas* spp. in bottled drinking water (Slade and Falah, 1986; Manaia *et al*., 1990; Villari *et al*., 2002) and approved ability of growth in it (WHO, 2003).

It is common to isolate *Aeromonas* spp. from both drinking and mineral water as well as food and environmental samples (Araujo *et al*., 1991; Hanninen and Siitonen, 1995; Sen and Rodgers, 2004; Daood, 2008). Pavlov et al (2004) have reported that *Aeromonas* spp. constituted 18.10% of the isolated bacteria. Contrary, *Aeromonas* spp. have predominantly found in 26.25% and 43.70% in samples of local and imported waters. (Table 1) *Aeromonas hydrophila* has been frequently isolated; for examples, it constituted 0.3%, 3.3% and 3.11% of isolates from bottled water (Venieri *et al.,* 2006), municipally treated tap water (Emekdas *et al*., 2006) and bottled mineral water (Daood, 2008) respectively. In this study, this proportion was notably increased to 8.15% of all bacterial isolates.

Because it is not possible to recognize the potential pathogenity of its components, HPC test is still somewhat ambiguous term and consequently it is not recommended to rely on to assess the health significance and risks associated with drinking water (Edberg and Allen, 2004; Allen *et al*., 2004). Furthermore, there is no clear-cut evidence to link the gastroenteritis with heterotrophic bacteria ingested by healthy people (Rusin *et al.,* 1997; Colford *et al.,* 2002). Under these circumstances, the antimicrobial sensitivity profile of the heterotrophic bacteria constituted the HPC and particularly the members of *Pseudomonas* and *Aeromonas* seems to be, at least currently, reasonable measurement in this situation.

Collectively, the incidence of antibiotic resistance among aquatic bacteria is a growing area of global public health concern (Pavlov *et al*., 2004; Shrivastava *et al.,* 2004; Hernandez-Duquino and Rosenberg, 1987; Schwartz *et al*., 2003; Venieri *et al*., 2006). The problem will become worse with increased levels of multiresistances. The antibiotic resistance among heterotrophic bacteria isolated from the bottled drinking water has been well studied. Collectively, high resistance levels have been reported among the majority of HPC towards penicillin and ampicillen (Pavlov *et al* 2004), ampicillin, nalidixic acid and novobiocin (Jeena *et al*., 2006), ampicillin and

nalidixic (Mary *et al*., 2000; Papandreou *et al*., 2000), and ampicillin, colistin, and chloramphenicol (Messi *et al*., 2005). Resistance to antibiotics such tetracycline, streptomycin, were less common with percentages ranged from 20% to 40% (Jeena *et al*., 2006). On the other hand, the majority of such studies have showed the somewhat complete susceptibility of HPC to ciprofloxacin and to less extent to gentamicin (Pavlov *et al*., 2004; Jeena *et al*., 2006; Mary *et al*., 2000; Papandreou *et al*., 2000). However, in mineral water, one study has recorded high resistance rates among HPC to quinolones and fluoroquinolones represented by nalidixic acid and ciprofloxacin respectively (Guyard *et al*., 1999).

Although there is some agreement, the resistance levels of HPC reported above were higher than those encountered in the present investigation. High sensitivity levels were recorded most of the tested twenty antibiotics. For instance, in addition to absolute activity showed by two antibiotics: azterionam and gentamicin, the sensitivity levels ranged between 83.72% to 96.51% for eleven antibiotics. (Table 3). As such, this result might be considered as a unique in such studies.

Clinical strains of *Pseudomonas aeruginosa* have been frequently found to be resistant to the following antimicrobials: ceftazidime, piperacillin/tazobactam, imipenem, aztreonam, amikacin, tobramycin, gentamicin, and ciprofloxacin (Pitten *et al*., 2001; Wang *et al*., 2006). Fortunately, in the present study, some of the above antibiotics have showed considerable activity against isolates of *Pseudomonas*. Namely, ciprofloxacin, gentamicin, and aztreonam were the most effective antibiotics against *Pseudomonas* spp. and tobramycin has also appeared to be relatively effective. However, of the twenty antibiotics tested in the present study, ten had no significant inhibition of *Pseudomonas* spp. Thus, as much as 60% to 76% of the tested isolates were resistant towards the following antibiotics: carbencillin, erythromycin, amikacin, ceftazidime, imipenem, tetracycline, novobiocin, ampicillin, trimethoprim/sulfamethoxazole, and nalidixic acid.

It is noticeable in the present study, a part of their innate resistance to primary antibiotics such as penicillin, tetracycline, and erythromycin, to find some of such that non-clinical *Pseudomonas aeruginosa* isolates being resistant to what so called anti-pseudomonal antibiotics (i.e.: imipenem, amikacin, ceftazidime, and carbencillin).

The obvious exception is colistin that has anti-pseudomonal activity and has been used previously for treatment of pneumonia caused by multidrug resistant *Pseudomonas aeruginosa* (Levin *et al*.,

1999). In the present study, this antibiotic has showed observable activity toward both *Pseudomonas* spp. and *Aeromonas* spp.

The results of complete or high susceptibility rates of *Aeromonas* spp. isolates towards amikacin, aztreonam, ceftriaxone, gentamicin and ciprofloxacin are in agreement with those obtained by other studies on drinking water and other sources (Emekdas *et al.*, 2006; Motyl *et al.*, 1985; Reinhard and George,1985; Bizani and Brandelli, 2001). Comparatively, the high level of resistance towards ampicillin/sulbactam showed by our *Aeromonas* spp. isolates (40% resistant) were lower than that found among food and clinical ones recorded by Palu *et al.*, (2006) who reported that 73.5% of isolates were resistant to that antibiotic. However, the high resistance to this β-lactamic antibiotics (ampicillin 60% and ampicillin/sulbactam 40%) might be explained by natural ability of *Aeromonas* spp. to produce β-lactamase (Fosse *et al.*, 2003).

High susceptibility to trimethoprim/sulfamethoxazole is in agreement with that has been recorded among freshwater *Aeromonas* spp. (Miranda and Castillo, 1998). However, resistance to tetracyclines and trimethoprim/sulfamethoxazole has been described for some isolates of *Aeromonas* spp. (Ko *et al.*, 1996; Vila *et al.*, 2003). In recent study, food strains of *Aeromonas* spp. have showed resistance to ampicillin/sulbactam, cefoxitin and tetracycline while clinical strains were found resistance to ampicillin/sulbactam, cefotaxime, ceftazidime, cefoxitin, sulfamethoxazole/trimethoprim, chloramphenicol and tetracycline (Palu *et al.*, 2006).

Our *Aer.hydrophila* strains were found highly resistant to ampicillin and ampicillin/sulbactam, erythromycin, imipenem, streptomycin and novobiocin. These results are partially in agreement with that recorded by Imziln *et al.*, (1996) on wastewater strains and by Ilhan *et al.*, (2006) on veterinary strains of *Aer. hydrophila*. In the latter study, high resistance were recorded to penicillin G, erythromycin and gentamicin while these strains were found susceptible to the other antibiotics with amoxicillin and enrofloxacin being the most effective.

Multiple resistance to antibiotics have been frequently recorded among both of environmental and non-environmental isolates of *Pseudomonas* spp. and *Aeromonas* spp. Multiresistant *Aeromonas* spp. have been isolated from clinical and food samples (Radu *et al.*, 2003; Palu *et al.*, 2006). Furthermore, the resistance to chloramphenicol, sulfamethoxazole/trimethoprim and tetracycline has been recently suggested to be mediated by plasmids (Schmidt *et al.*, 2001; Casas *et al.*, 2005; Palu *et al.*, 2006).

It is not uncommon to find multiresistant HPC strains of mineral water origin, for instances, Messi *et al*., (2005) have found that 55% of HPC strains were MAR whereas as high as 80% of these isolates were resistant to one or more antibiotics. More recently, Jeena *et al*., (2006) have reported that 45% of HPC strains showed multiple resistance. The very related result recorded in the present study (i.e.: 46.5% of HPC strains were MAR) suggests to the relative uniformity of the HPC components and their response to antibiotics in different studies on mineral waters. However, the high values of MAR for *Pseudomonas* spp. (77.14%) and *Aeromonas* spp. (60%) recorded in the current study could have two significant perspectives; first is the obvious contribution of such opportunists in elevation of antibiotic resistance of HPC when they are involved in it. Second is a health consideration resulted from the presence of these MAR opportunists in bottled mineral water that should be considered whenever the sanitary quality of mineral water is assessed. Given increased findings about the possibility of conjugational and transformational transfer of resistance elements to indigenous flora of aquatic, terrestrial environments and biofilms (Witte, 2000; Lilley and Bailey, 2002), the concern is coming from the potential possibility of transfer the resistance factors namely plasmids to the normal human susceptible microflora. This will create real hazards in patients on antibiotic as well as the possibility of emerging more virulent pathogens. Given that, as reported by Boronin, (1992), plasmids are ubiquitous in *Pseudomonas* spp., where in one study for example, plasmids were detected in 46% of Pseudomonads isolates (Messi *et al*., 2005) as well as the ability of such bacteria to transfer them to other ones, their occurrence in drinking water might be considered with particular health significance.

So, although there is no clear cut off evidence of responsibility of these plasmids in huge prevalence of more resistant bacteria, the presence of such plasmid-harboring bacteria in drinking water must be taken seriously.

V- Conclusion

In conclusion, the increases in antibiotic resistance in bottled water heterotrophic bacteria (HPC) and particularly among the members of *Pseudomonas* and somewhat *Aeromonas* is of clinical concern, both because this kind of water is ready to consume commodity, and because some of these bacteria can act as real pathogens and induce infections in humans. Fortunately, in this study, many antibiotics are still active against opportunists such as *Aeromonas* spp. and *Pseudomonas* spp. Overall, irrespective of some exceptions, the other heterotrophic bacteria (HPC) and *Aeromonas* spp. have showed more

susceptibility to available antibiotics. Moreover, some of the antibiotics have maintain their complete activity in these situations.

It has been observed that might be somewhat potential relationship between the load of heterotrophic bacteria and the level of antibiotic resistance. So, in our study, increased HPCs had mostly led to increased resistance to antibiotics among more virulent bacteria like *Pseudomona* spp. and *Aeromonas* spp. It is recommended, upon the results obtained, to consider the risk of presence of such resistant bacteria in bottled mineral water in the Syrian market. Partially, this may be achieved by routine monitoring and applying the international criteria for bottling and importing of these goods.

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