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Original Article

Pseudomonas aeruginosa and *Heterotrophic* Bacteria Count in Bottled Waters in Iran

*Matin MOHAMMADI KOUCHESFAHANI [1], *Mahmood ALIMOHAMMADI [1], Ramin NABIZADEH NODEHI [1] ,Hassan ASLANI [1], Sassan REZAIE [2,3], Samieh ASADIAN [3]*

1. Dept. of Environmental Health Engineering, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
2. Dept. of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
3. Dept. of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

***Corresponding Author:** Email: m_alimohammadi@sina.tums.ac.ir

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Abstract

Background: Nowadays, due to increased public awareness about water pollution and water borne diseases as well as water network deficiencies, bottled water consumers have increased dramatically worldwide, including Iran. *Pseudomonas aeruginosa* is an opportunistic human pathogen capable of causing widespread infections in burn and immune-compromised patients. The aim of this study was to investigate, *P. aeruginosa* in bottled waters selling in Iranian markets.

Methods: One hundred and twenty samples of five unknown (not famous) domestic bottled water brands were purchased from Tehran retailers during 2013. The samples were evaluated for the presence of *P. aeruginosa*. In addition, heterotrophic plate counts were determined by incubation at 37 °C for 24 h.

Results: *P. aeruginosa* was detected in 36.7% (44 samples) of all samples examined. In addition, heterotrophic bacteria in 32.5% (39 samples) of the samples were higher than 100 CFU/mL, while in 7.5% (9 samples) of the samples HPC relied between 20 and 100 CFU/ml.

Conclusion: In contrast to public believe, bottled waters are not free of microorganisms, and it is suggested that authorities should provide stricter monitoring and control plan for water resources and plants. Concerning HPC and *P. aeruginosa* brands B and D were not suitable for drinking.

Keywords: Heterotrophic plate count, *Pseudomonas aeruginosa*, Bottled water

Introduction

Nowadays, there is a tremendous increase in bottled water consumption worldwide. Consumer demand for bottled water In North America has shown an annual growth rate of 25% (1, 2), also bottled water consumption In European Union during 2003, was reported 45,000 ml (expect bottles >10) per capita (2). With the significant increase in bottled water consumption in recent years, microbiological quality of the products has become as a major concern in regard with consumer's health. Autochthonous and allochthonous bacteria are the two common groups of bacteria, which are found in bottled waters (3).

Bottled water can be classified as drinking waters, natural mineral waters and spring waters. Drinking water is often disinfected, while according to the EU direction in case of mineral and spring waters no treatment is required (4, 5). Natural mineral water is originated from an underground water table, protected from chemical and organic contamination and microbiologically is pure water (5). Spring water is reserved in its natural state and comes from underground water tables or deposited and emerging from a spring tapped at one or more natural or bore exits (3). Because of the public awareness over water pollution, deficiencies in water treatment plant, odours, tastes, fluoride, chlorine, and as well as due to success of marketing companies, in recent years most consumers have decided to replace their drinking water resources with non-carbonated bottled water (2).

In contrast to public believe, bottled waters are not always completely safe and free of contaminations. Some kind of microbial contamination such as bacteria may be indigenous in water resources or enter the water during bottling process. The bacteria could be proliferate during transport and storage of filled bottles, and attain infective doses. *Echerchia coli*, *Pseudomonas* spp. and *Salmonella* spp. are able to survive and proliferate in bottled water (6, 7). The pathogens can cause outbreaks in consumers (8). Heterotrophic bacteria consist of species in the genera *Pseudomonas*, *Aeromonas*, *Alcaligenese*, *Acinetobacter*, *Klebsiella*, *Flavobacterium*, and use organic compounds for their carbon requirements (3). Microorganisms isolated from the bottled water tested by Venieri et al. were identified as species of *Pseudomonas*, *Aeromonas*, *Pasteurella*, *Citrobacter*, *Flavobacterium*, *Providencia* and *Enterococcus*. The most frequent isolated microorganism during the period of the study was *P. aeruginosa* (7).

Pseudomonads are highly versatile and can adapt to a wide range of habitats, even are able to grow in distilled water. *Pseudomonas aeruginosa* is a gram-negative, non-sporulating, rod-shaped bacterium, which can produce the blue-green pigment pyocyanin or the fluorescent pigment fluorescein or both (9). It is also an opportunistic human pathogen capable of causing urinary tract infection, respiratory system infection, deramatits, soft tissue infection, bacteremia and a variety of systemic infection particularly in burn patient and immunocompromised individuals (3, 10, 11). Besides, *P. aeruginosa* is a major cause of hospital-acquired infections with a high mortality rate (12), and one of the special characteristic of *P. aeruginosa* is the ability to multiplying in low-nutrient water (10, 13, 14). Being a primary cause of disease, *P. aeruginosa* is often monitored as an indicator of other bacterial contaminants of fecal origin (10, 15). It usually is an indicator for contamination during the bottling process (3, 16).

The aim of the present study was to determine *P. aeruginosa and* heterotrophic bacteria in some unknown bottled waters.

Materials and Methods

Water samples

A total of 120 samples of bottled water of 5 different other famous domestic brands were randomly purchased from retailer located all over Tehran, Iran from June through October, 2013. Polyethylene terephthalate bottled water (250 ml) were taken and transported to the laboratory. The samples were stored at refrigeration temperature, and all samples were analyzed within 24 h after collection. Chemical parameters were analyzed using ion chromatograph according to the standard methods for the examination of water and wastewater (17).

Bacteriological analysis

Membrane filtration technique was used for enumeration of heterotrophic bacteria at 37 °C and incubation for 24 h (18). All colonies were counted and the results were reported as colony-forming units (CFU) per milliliter of the water sample.

Detection of *P. aeruginosa* was carried out using membrane filtration method. After enrichment on malachite broth, agar supplemented with cetrimide and nalidixic acid was used for bacteria isolation (19), and the confirmation step was performed using oxidase test (3). The membrane filtration method is considered the most flexible method for qualitative and quantitative studies of bottled water (7, 8).

Statistical analysis

The data were statistically analyzed by analysis of variance (ANOVA) and *t*-test using SPSS 20 software (Chicago, IL, USA). The post-hoc test of Tukey was used at a significance level of 0.05. For relationships between numbers of HPC and the presence of *P. aeruginosa* crosstabs and chi-square tests was used.

Results

Physico-chemical characteristics of examined samples are listed in Table 1. Results of *P. aeruginosa* and heterotrophic bacteria count at 37 °C are presented in Table 2.

Either heterotrophic bacteria count or positive samples of *P. aeruginosa* were significantly (P<0.025) different between various brands. Brand D with maximum HPC of more than 9700 CFU/ml was more contaminated than others were, and brand B with maximum 2700 CFU/ml was in the second order, whereas the HPC contamination was below the promulgated standards in other three brands.

Table 1: Chemical properties of bottled waters examined

Composition	Unit	Brands					Standard
		A	B	C	D	E	
pH		6.99	6.89	7.76	7.32	7.86	6.5-9
Bicarbonate	mg/L	97.6	73.2	292.8	61	189.1	na*
Cl	mg/L	24.43	87.12	63.75	2.248	2.04	250
SO_4	mg/L	7.57	104.53	126.05	21.583	26.62	250
Na	mg/L	15.2	125.78	101.12	8.278	3.1	200
Ca	mg/L	31.35	8.83	58.37	19.445	51.48	300
K	mg/L	0.32	0.19	1.48	0.409	0.43	na
F	mg/L	0.09	0.13	0.23	0.086	0.09	1.5
NO3	mg/L	2.33	25	12.1	4.75	3.79	50
Mg	mg/L	8.41	2.29	29.86	5.02	17.58	30
TDS	mg/L	120.9	305	411	67.7	164.9	1000
EC	µS/cm	243	609	821	135.7	331	na
Turbidity	NTU	0.14	0.18	0.45	0.07	0.08	1
Alkanity		80	60	240	50	155	na

*not available

Table 2: Heterotrophic plate counts (HPC) separately brands

HPC(cfu/ml)	Number (%) of different brand samples					
	Brand A	Brand B	Brand C	Brand D	Brand E	Total
HPC <20	24 (100)	0	24 (100)	0	24 (100)	72 (60)
20 ≤ HPC <100	0	1 (4.2)	0	8 (33.3)	0	9 (7.5)
HPC ≥ 100	0	23 (95.8)	0	16 (66.7)	0	39 (32.5)
Min	0	90	0	57	0	
Max	1	2700	1	9760	2	
STD	0.3	594	0.28	194	0.59	
Total	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	120 (100)

To investigate the correlation between the HPC and the presence of *P. aeruginosa,* the results were classified in three groups including samples containing HPC less than 20 CFU/ml, samples with HPC between 20 and 100 CFU/ml, and those with higher HPC than 100 CFU/ml. A significant correlation (P<0.05) was found between HPC above 100 CFU/ml and the presence of *P. aeruginosa*. The mean amount of HPC for all examined brands is presented in Fig. 1.

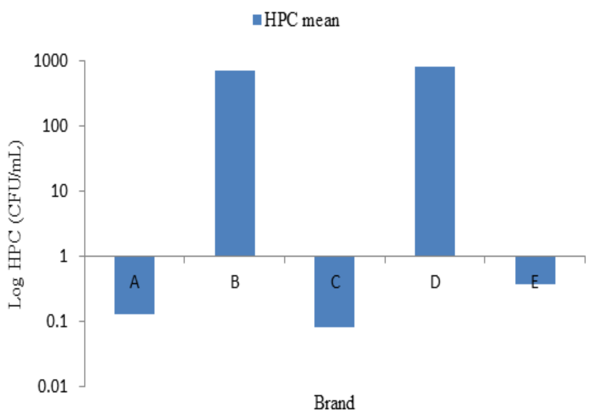

Fig. 1: The mean amount of HPC any brands

In the two out of the five brands tested, heterotrophic bacteria count was higher than the standards. Among the 120 samples analyzed 60% (72 samples) had HPC content less than 20 CFU/ml, whereas in 7.5% (9 samples) and 32.5% (39 samples) of the samples HPC content ranged between 20 and 100 CFU/ml, and higher than 100 CFU/ml, respectively.

Table 3 shows *P. aeruginosa* results in the samples tested. It is clear that 36.7% of the 120 samples tested were positive for *P. aeruginosa*. The presence of *P. aeruginosa* was demonstrated in two brands, B and D. The brands showed higher percentage of positive results, so that 95.8% and 87.5% of the samples were contaminated, respectively.

Table 3: Distributions of *P. aeruginosa* in the bottled waters tested in this study

	Number (%) of positive samples					
	Brand A	**Brand B**	**Brand C**	**Brand D**	**Brand E**	**Total**
P. aeruginosa	0	23 (95.8)	0	21 (87.5)	0	44 (36.7)
Total	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	120 (100)

Discussion

One major goal of this study was to demonstrate that bottled waters were not always perfectly healthy. In comparison to the standards established by International Bottled Water Association (IBWA), all measured parameters were acceptable. According to the results, the HPC contamination in samples of brands A and B was higher than the standards set by EU Direction for the HPC levels in mineral bottled water (Table 2), in other words, 40% of all samples examined were not safe for drinking. EU legislation and Iranian regulation for the HPC levels in bottled mineral waters, limits heterotrophic bacteria to lower than 20 CFU/ml (20).

The HPC was not measured within 12 h after bottling, therefore it could not be claimed that these samples did not comply with the EU Directive 2009/54/EC. The result, also, indicated a significant correlation between HPC above 100 CFU/ml and the presence of *P. aeruginosa*. However, due to the lack of direct correlation between the presence of heterotrophic bacteria, *P. aeruginosa* and disease, risk assessment in terms of importance to public health is difficult. Several studies, as well as the present study have shown the microbial contamination in bottled water. Zeenat et al. evaluated three domestic brands in Fiji, and found that in 28% of the samples HPC levels were above the standards (22). Varga (3) examined bacteriological quality of 492 samples of domestic and imported brands of carbonated and non-carbonated mineral waters and reported that in 1.6% of non-carbonated and 1.2% of carbonated samples the heterotrophic bacteria count was between 20 and 100 CFU/ml. Vantarakis et al. indicated 298 out of 1860 bottled water samples had HPC greater than 20 CFU/ml (23). Any sample in the studies conducted earlier, (3, 23) did not comply with EC legislation, because the analyses were not carried out within 12 h after bottling. According to Directive 2009/54/EC, HPC should not exceed 20 CFU/ml12 h after bottling. Among all 32 brands of bottled waters (24), in 9.4% of them HPC was lied between 20 and 200 CFU/ml, and in the 12.5% of the samples the HPC ranged between 200 and 500 CFU/ml, whereas for the 37.5% of the samples HPC was higher than 500 CFU/ml. 18.8% of samples revealed positive signs for total coliforms, but all samples showed negative growth results for fecal coliforms (24). Rapid growth of microbial count after bottling is due to increase in oxygen content during processing, amount of nutrients, the increase in temperature, and surface area provided by the bottles (25).

Our findings showed that almost 37% of the samples were positive when examined for *P. aeruginosa*. This is a high rate of contamination in comparison to other studies. Out of 1860 samples examined 6.1% showed positive results when tested for the presence of *P. aeruginosa* (23). Whereas, according to EU legislation and Iranian water regulations *P. aeruginosa* should not be detected in 250 ml bottled water samples. Bharath et al. examined microbial quality of 344 (262 domestic brands and 82 imported brands) samples of bottled water in Trinidad and found that 7.6% of samples contained *Pseudomonas* Spp. (1). Furthermore, the presence of *P. aeruginosa* indicated in 1.4% of the 492 bottled water samples sold in Hungary. However, our findings are well compared with previous studies (4, 10). *P. aeruginosa* was detected in 43% of the samples (58.4% of the 20-L bottles, 50% of the new 20-L bottles and 29.1% of tap water samples) (10). Marzano et al. evaluated 120 samples from spring-bottled water and found that 57.5% of samples were positive for *P. aeruginosa* (4).

P. aeruginosa is a common indicator of water contamination during bottling process (16). Infection risk for public if *P. aeruginosa* is present in bottled water is not as high as other pathogens such as cryptosporidium cysts, for example, the infection risk is 1:10000 if 5.0×10^3 to 1.0×10^4 CFU/ml is ingested by 2L bottled water daily (26). So far no outbreaks or infection cases have been reported due to the bottled water contamination with *p. aeruginosa* (27). However, presence of *P. aueroginosa* is not accepted because it is an opportunistic microorganism, which is able to cause infection in some sensitive groups like children, elderly, and people with immune-compromised system.

Conclusion

Control of HPC (<100 CFU/ml) in bottled water could assure its microbiological quality during storage. In addition, including *P. aeruginosa* in mineral water monitoring program is because it is considered as an opportunistic pathogen as well as an index for vulnerability or weak control environment packaging.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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