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Evaluation of microbiological quality of bottled water to *Pseudomonas aeruginosa* by membrane filter, culture and polymerase chain reaction method

Fatemeh Sadati Khadar, Ali Mohamadi Sani*

Department of Food Science and Technology, Quchan Branch, Islamic Azad University, Quchan, Iran

Key words: Bottled water, membrane filter culture (MFC), polymerase chain reaction (PCR), *P. aeruginosa*.

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Abstract

Over the recent years the consumption of bottled water has increased tremendously, and this trend is expected to continue, demanding the continuous surveillance of public health service. The propose of this study was to determine the microbial quality to *Pseudomonas aeruginosa* by membrane filter (MFC), enrichment culture and polymerase chain reaction (PCR) method of commonly available brands of bottled water in Iran. In this cross sectional study, 52 sample from 17 bottled water of available brands of domestically produced were examined by membrane filter (MFC) culture and polymerase chain reaction (PCR) methods for detection of *P. aeruginosa*. Results showed that none of the 52 samples tested were positive for total coli forms and *E. coli*. The MFC method showed that 14 (26.9%) of all 52 water samples were positive, the culture method showed that 8 (15.3%) were positive and by PCR as gold standard 5 (10.4%) of all samples were positive for *P. aeruginosa*. This study showed that PCR method can be an extremely high specificity and sensitivity, safety and can be served as a gold standard for monitoring of drinking waters.

* **Corresponding Author:** Ali Mohamadi Sani ✉ mohamadisani@yahoo.com

Introduction

Bottled water is considered a global billion dollar business (Bharath, Mosodeen *et al.* 2003). Over the past decade the consumption of bottled water in Iran has increased tremendously, and this trend is expected to continue, demanding the continuous surveillance of public health service. The dramatic increase in the consumption of bottled water worldwide has been attributed to the consumers' concern over water pollution, storage, offensive taste, odor, fluoride and chlorine as well as a lack of regulations and limited understanding and awareness among the population (Levesque, Simard *et al.* 1994, Armas and Sutherland 1999, Bharath, Mosodeen *et al.* 2003). Despite the perceived purity, the microbiological quality of bottled water has been questioned over the years.

Several studies have documented the detection of coliforms and heterotrophic bacteria in bottled water with levels exceeding drinking water guidelines (Bharath, Mosodeen *et al.* 2003, Bartram, Cotruvo *et al.* 2004). Potential pathogens such as Aeromonas spp. (Venieri, Vantarakis *et al.* 2006), *Staphylococcus aureus* (Leclerc, Mossel *et al.* 1982), Pseudomonas spp.(Svagzdiene, Lau *et al.* 2010) Shigella spp. (Khan, Saha *et al.* 1992), *Vibrio cholera* (Blake, Rosenberg *et al.* 1977) has been detected and caused concerns about its safety. Bacteria belonging to the genus Pseudomonas are widespread in the environment and are often responsible, as opportunist bacteria, for very serious episodes of infection (Ringen and Drake 1952). *P. aeruginosa* is the species most frequently involved in infections ranging widely such as, pneumonia, Skin and soft-tissue infections, folliculitis or pyodermitis, external otitis, kidney and a variety of systemic infections (Legnani, Leoni *et al.* 1999). Several authors have studied the development of P. aeruginosa in natural water environments, in tap water, and in specially prepared saline solutions (Botzenhart and Kufferath 1976).

The presence of *P. aeruginosa* indicates either that the source has become polluted by organic material or contamination during the bottling process. During the period of storage, after growth may lead to high levels of *P. aeruginosa* in the bottled water, thus posing a risk for consumers, especially those who are weak, very young or elderly. Among all diagnostic techniques such as culture, serology, and molecular methods, the last one is the fastest .Molecular method having advantage such as velocity, safety, specificity and sensitivity. Nonetheless, culture methods remain popular because of their ease and simplicity. Polymerase Chain Reaction (PCR) is one of the most widely used molecular methods for detection of a wide variety of microorganism P.aeruginosa in bottled sample water (Momtaz, Dehkordi *et al.* 2013). The purpose of this study was to determine the microbial quality to *Pseudomonas aeruginosa* by MFC, enrichment culture and PCR method of commonly available brands of bottled water in Iran.

Materials and methods

Water sample

For this study, 17 bottled water from available brands of domestically produced were purchased from sealed container.

Detection and enumeration of Pseudomonas aeruginosa

The presence of *P. aeruginosa* was determined by 3 methods.

Membrane filter technique

The membrane filter (MF) technique is fully accepted and approved as a procedure for monitoring drinking water microbial quality in many countries. The mineral waters were put into glass bottles cleaned with acid and autoclaved before use. To detect *P. aeruginosa*, membranes were placed on to cetrimide and incubated at 37Ć for 48 h for observing colonies of *P. aeruginosa* demonstrating pyocyanin production (green coloration). We examined the filter under UV lamp (Figure1) and counted all fluorescent colonies. These colonies, pigmented or without pigment, considered as presumptive *P. aeruginosa*.

Enrichment cultures

For all water samples, 10 ml of malachite green broth as the enrichment culture for *P. aeruginosa* added and incubated at 37Ć for 48 hours. Suspected cases in membrane filter culture were isolated and cultured in Cetrimide agar (CET) and Tryptic Soy Agar (TSA) incubated at 42 °C for 24-48 hours. For Confirmatory tests, *P. aeruginosa* colonies grown on TSA agar placed in contact with the Oxidase strip. If the color was purple the sample regard as positive (Figure2).

PCR method

DNA extraction

Purification of DNA (deoxyribonucleic acid) directly from filtered water samples was achieved using a genomic DNA purification kit (HIMEDIA, India) according to the manufacturer's instructions. Primers used in this study were selected from a paper by Purohit (Purohit, Raje *et al.* 2003) and accuracy of them checked at internet gen bank such as NBCI. And gyrB gens selected from NCBI bank by blasting operations and specificity of them was confirmed. Also, the location of the primers on the gyrB gens confirmed by alignment operations.

Preparation of PCR products

At first 14 ml of sterile deionized water is poured into the micro-tube dry kit. One microliter of each primer was added by reciprocating and finally 4 ml of DNA was added to the micro-tube. Prepared micro-tubes for PCR test transfered to the machine by following schedule. The temperature program included: denaturation at 95°C for 4 minute, annealing at 62°C for 30 second, extension at 72°C for 45 second and the final extension at 12°C.

Electrophoresis

After PCR test the product was loaded into a 1% agarose gel. After electrophoresis, samples were loaded at 80 voltage. All operations were performed with the size marker electrophoresis (ladder) 100 bp and with appropriate positive and negative control samples (Figure 3). After that, the electrophoresis was examined with a trans laminar Ultra-violet.

Statistical analysis

Data were tabulated and analyzed using Statistical Package for Social Sciences (SPSS) version 11.5 computer software package. To determine whether statistically significant differences existed in the prevalence of total coli forms, *E. coli* and *P. aeruginosa* chi-square test was used. In all data analysis, a value of $P<0.05$ and was considered statistically significant.

Results and discussion

In total, 52 samples of still mineral water in polyvinyl chloride bottles were analyzed for the presence and enumeration of total coliforms, *E. coli* and *P. aeruginosa*. All of the 17 brands of bottled water were domestic and date of consumption was not expired. Overall, none of the 52 samples tested were positive for total coli forms and *E. coli*.

Table 1. Contamination rate to *P. aeruginosa* in bottled water samples by different methods.

Brand Code	No. of samples tested	Percent of positive samples		
		MFC method*	Enrichment cultures	PCR method**
A	6	2 (33)	1 (17)	1 (17)
B	4	3(75)	2 (50)	1 (25)
C	3	2 (66)	1 (33)	0(0)
D	1	1 (100)	1 (100)	0 (0)
E	3	0 (0)	0 (0)	0 (0)
F	5	0 (0)	0 (0)	0 (0)
G	4	2 (50)	1 (25)	1(25)
H	2	0 (0)	0 (0)	0 (0)
I	3	2 (66)	2 (66)	2 (66)

J	1	1 (100)	0 (0)	0 (0)
K	1	1 (100)	0 (0)	0 (0)
L	1	0 (0)	0 (0)	0 (0)
M	5	0 (0)	0 (0)	0 (0)
N	2	0 (0)	0 (0)	0 (0)
O	1	0 (0)	0 (0)	0 (0)
P	5	0 (0)	0 (0)	0 (0)
Q	5	0 (0)	0 (0)	0 (0)
total	52	14(26.9)	8(15.3)	5(10.4)

*MFC method: Membrane filter technique

**PCR method: polymerase chain reaction.

The samples from each brand for presence of *P.aeruginosa* analyzed by three methods and the results are shown in Table 1. The contamination percent of positive samples for *P.aeruginosa* by MFC method is shown in Figure 4. Some brands had relatively high percentages of positive samples for *P. aeruginosa*. Brands of D, J and K were the worst where their percentage of unacceptable samples was 100. For brands B, C, I G and I the contamination rate was respectively 75, 66, 66, 50 and 33% by MFC method. The contamination rate of positive samples by Enrichment cultures method is shown in Figure 5 which is about 15.3%. The result of contamination rate by PCR (as a valid test) decrease even to 10.4%. Yet, the differences were not statistically significant for *P. aeruginosa* in 3 mentioned methods (p=0 .15).

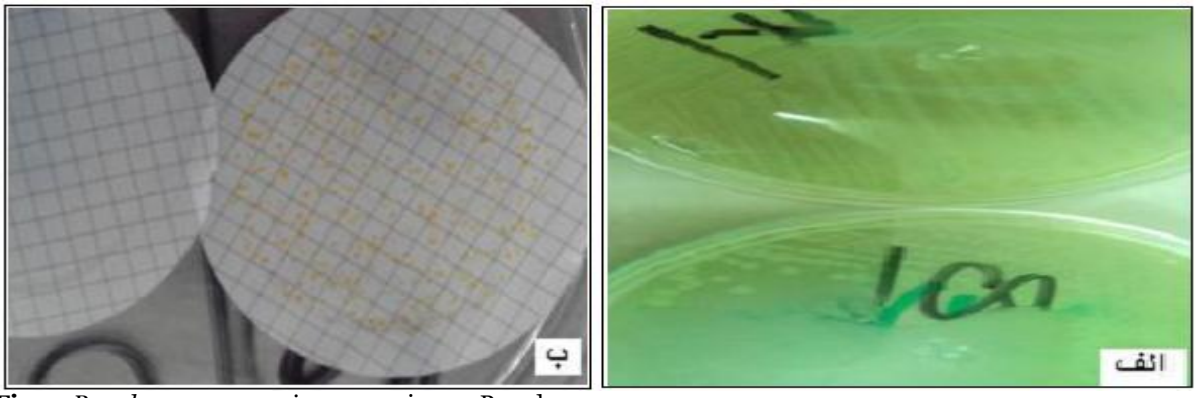

Fig. 1. *Pseudomonas aeruginosa* growing on Pseudomonas agar.

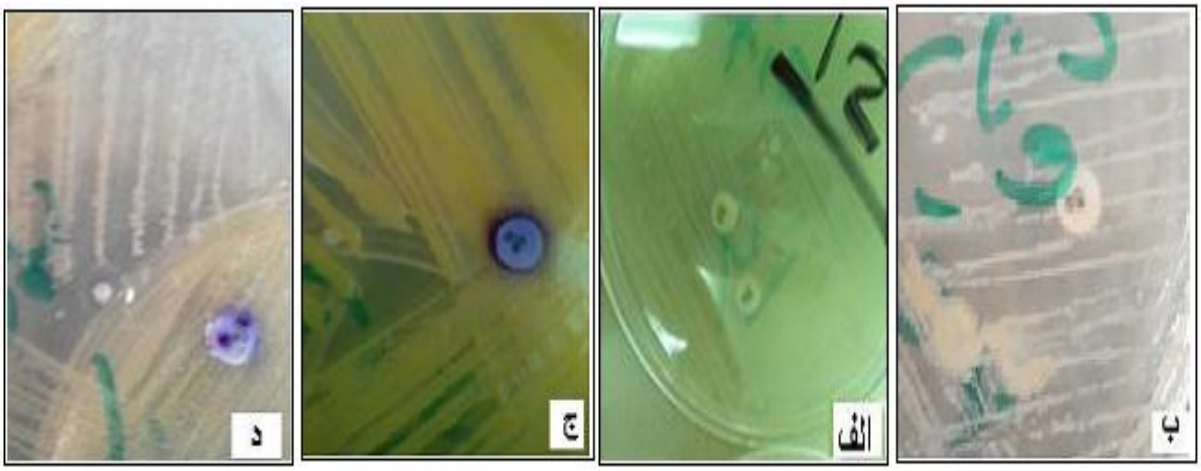

Fig. 2. Oxidase test.

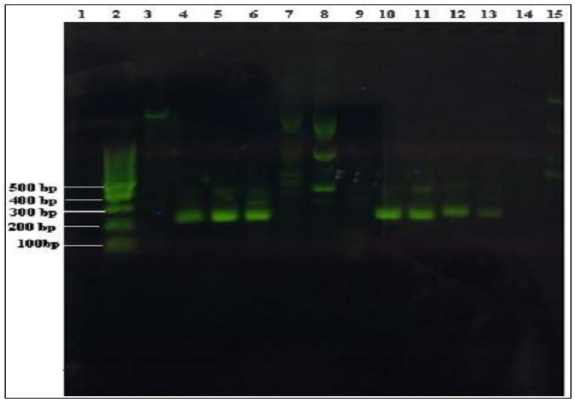

Fig. 3. PCR Test.

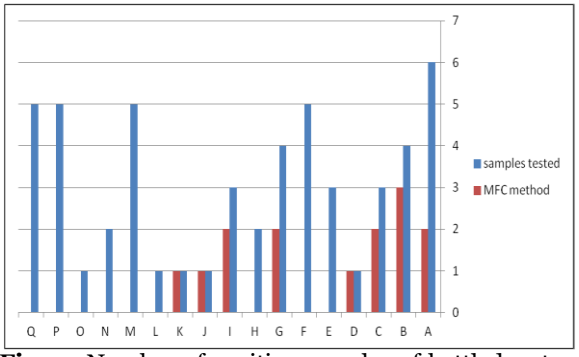

Fig. 4. Number of positive samples of bottled water to *P. aeruginosa* in different brands by MFC method.

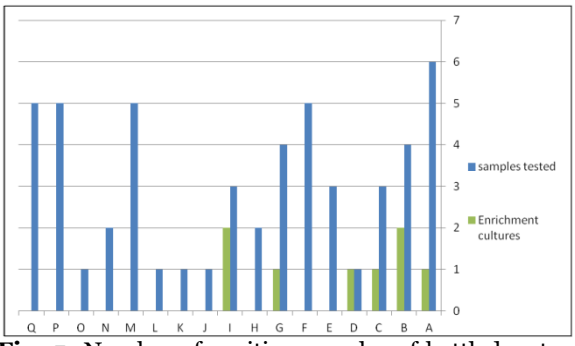

Fig. 5. Number of positive samples of bottled water to *P. aeruginosa* in different brands by Enrichment cultures method.

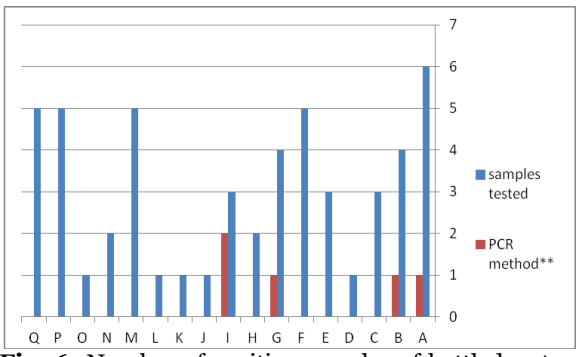

Fig. 6. Number of positive samples of bottled water to *P. aeruginosa* in different brands by PCR method.

Abbreviation

Membrane filter culture (MFC), Polymerase chain reaction (PCR).

Conclusion

Bottled water is widely available in both developed and developing countries. In the recent years huge Urbanization and general belief that it is safe and free of all impurities is a major reason for the increase in consumption of bottled waters in Iran. In the present study the bacteriological examination of 17 different bottled mineral water samples was carried out. In the current study, all proportion of the samples tested were suitable for drinking as they were not contaminated with coli forms and *E. coli*. The presence of colifirms in bottled water considered an indicator of faecal pollution and it requires an improved surveillance system for the bottled water industry (Schindler, Vogel *et al.* 1995). According to this study, no total coliforms and *E. coli* bacteria were found among all brands. In a study by Jahed Khaniki (Jahed Khaniki, Zarei *et al.* 2010) 14.2% of the sample were positive for total coliforms. In a study done by Zamberlan da Silva (Zamberlan da Silva, Santana *et al.* 2008) in Brazil, 6.4% of the mineral water samples was positive this bacteria. Abd El-Salam indicated to presence of coliforms in 28.6 % of the examined bottled water samples in Egypt (Abd El Salam, Ghitany *et al.* 2008), but *E. coli* was not found. Selka (Selka 1988) and Warburton *et al.* (Warburton, Harrison *et al.* 1998) showed the presence of E. Coli in 2-3 and 3.7% of the samples respectively and Richards *et al.* (Richards, Stokely *et al.* 1992) and Abdel Aziz *et al.* (Abdel Aziz, Shoeb *et al.* 1989) observed no coliform in their research on mineral water analysis. *P. aeruginosa* is an opportunistic pathogen that is known to cause urinary tract infections, respiratory tract infections, skin and soft-tissue infection, kidney and a variety of systemic infection, particularly those who are debilitated or immune compromised. *P. aeruginosa* is a common cause of infection in ICUs. HIV patients, particularly those in advanced stages, are at risk of acquired *P. aeruginosa* infections. Outbreaks caused by this organism have been reported in various settings. The

strain responsible for the outbreak may be spread via the hands of health care workers or by environmental sources of transmission such as contaminated water (Kolmos, Thuesen *et al.* 1993). In the current study, 10.4% of all samples were contaminated by *P. aeruginosa*. Similar results were reported by Hernandez-Duquino and Rosenbeg (Hernandez-Duquino and Rosenberg 1987), Papapetropoulou (apapetropoulou, Iliopoulou *et al.* 1994) and Hunter (Hunter 1993) observed lower contamination rates. The findings of the current study demonstrate that the safety status in bottled water industry in Iran has good situation but to maintain the quality of the final product in the manufacturing companies, we must control and monitor the safety risks continually.

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