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Original Article

PCR detection of *Vibrio cholerae*, *Escherichia coli,* and *Salmonella sp*. from bottled drinking water in Iran

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Abstract

Introduction: The quality of drinking water has an important role in human health. This study was aimed to detect *Escherichia*. *coli*, *Salmonella sp.* and *Vibrio cholerae* from bottled drinking waters produced in Iran.

Methodology: A total of 240 samples of bottled water of different brands were collected for testing between March 2015 to December 2015 in Shahrekord-Iran. Samples were examined by polymerase chain reaction (PCR) combined with culture methods for the detection of *E. coli*, *Salmonella sp.*, and *V. cholerae*.

Results: The results of PCR revealed that the *uidA* gene from *E. coli*, *IpaB* gene from *Salmonella sp*, and *epsM* gene from *V. cholerae* were detected in 6 (2.5%), 1 (0.4 %), 0 (0%) of the samples, respectively. But in culture methods, only *E. coli* 5 (2.1%) were isolated from the samples. The contamination with *E. coli* was significantly higher ($P < 0.05$) in water produced during the hot seasons than the cold seasons.

Conclusions: This study confirmed the presence of *Escherichia coli* as the main microorganism in bottle drinking water in Iran. Also, our study showed that PCR can be used as a screening method for monitoring the enteric pathogens in drinking water.

Key words: *E. coli*; *Salmonella sp*.; *V. cholerae*; bottled water; Iran.

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Introduction

Due to an increase in world population and popularity of drinking bottle water, the microbiological safety of bottled drinking water is becoming a worldwide concern. Furthermore, population aging in developed countries and increasing use of immunosuppressive drugs have led to decreased immunity to waterborne pathogens. The clinical impact of drinking unsafe bottled water is higher in developing countries who drink bottled water to avoid drinking unsafe drinking water from local resources which is subject to poor sanitation. Unfortunately, children are the main victims of infectious and parasitic diseases which can result in a high morbidity rate. In all urban areas in developed countries, reliance is placed on the supply of adequately treated water by municipal authorities. In developing countries, however, there is little or no access to such treated water and so, portable water is usually difficult or even impossible to get. One of the means of satisfying the need for portable water especially in urban communities is to consume packaged water which in Iran is sold in plastic bottles. Bottled water is drinking water which has been packaged in plastic bottles ranging in size from small single serving polyethylene terephthalate bottles of 500 mL −1.5 L capacity to large carboys (20 L) for watercoolers [1]. Apart from microbiological considerations, the upsurge in the demand for bottled water has prompted the interest of many manufacturers in the production of bottled water.

Close to two decades ago, bottled water was a product of a few multinational and large scale food processing and beverage producing companies in Iran. These water bottling companies use various water purification methods which may be one of or a combination of two of filtration, Ozonisation, ultraviolet irradiation, and chlorination. Studies showed that ingestion of water contaminated with *Escherichia coli*, *Salmonella sp*, and *Vibrio cholerae* can create serious complications including diarrhea, enteritis, and even death, leading to high economic losses [2,3]. These bacteria are found almost everywhere. Humans, animals, and sewage can be sources of these bacteria. Contaminated water plays an important role in the transmission of bacteria to humans. Studies on the quality of bottled water in many parts of the world including Canada, South Africa, Iran, Egypt, and Nigeria have shown that bottled water samples are not

always of the required microbiological quality [4,5] and bottled water has been reported to be associated with waterborne outbreaks in the last few years. In 2006, *Salmonella enterica* serovar Kottbus from bottled water was significantly associated with 41 cases in an outbreak in infants in Gran Canaria [6]. Eckmanns *et al.* (2008) have described an outbreak of hospital-acquired *Pseudomonas aeruginosa* infection caused by contaminated bottled water in intensive care units in a hospital in Germany [7]. International drinking water-quality monitoring programs have been established in order to prevent or to reduce the risk of contracting water-related infections.In Iran, the water for human consumption, including the water coming from dispensers is required to be free from any pathogenic microorganism which may be hazardous to the human health [8,9]. Previous studies showed that the drinking waters were contaminated with some pathogenic bacteria in some parts of Iran [10]. Therefore, the objective of the present study was to assess the current quality of bottled drinking water produced in Iran by the detection of main pathogenic bacteria including *E. coli*, *Salmonella sp*. and *V. cholerae*.

Methodology

Study Setting

This study was conducted in the laboratory of food quality control, Shahrekord University, Shahrekord-Iran. This laboratory is a reference laboratory which receives samples of food and water from industries and health services for testing.

Study Design

A cross-sectional descriptive study assessing different brands of bottled water samples produced in Iran, collected between March 2015 to December 2015 was undertaken. Microbiological and molecular studies were performed on samples to identify the targeted microorganisms.

Sampling

A total of 240 samples of six brands (A-F) of 500 mL bottled water within a period of 12 months were tested for the presence of *E. coli*, *Salmonella sp*. and *V. cholerae* using PCR and culture methods. The samples were inspected and ascertained to be in good condition with the caps and protective seal intact before purchase. The dates of production, as well as the batch numbers, were documented. The sampling was repeated with an interval of 3 months (March, June, September, December). Ten samples of each brand were chosen.

DNA extraction and PCR conditions

Purification of DNA directly from water samples filtered was achieved using a Genomic DNA purification kit (Fermentas, Sankt Leon-Rot, Germany) according to the manufacturer's instructions. Oligonucleotide primers specific for the *uidA* gene of *E. coli* encoding for Beta-D-Glucuronidase [11], the *ipaB* gene of *Salmonella sp*. encoding the invasion plasmid antigen B [11] and the *epsM* gene of *V. cholerae* encoding the enterotoxin extracellular secretion protein of toxigenic *V. cholerae* [12] were used, as they have been reported to be specific for the respective bacteria (Table 1).The PCR reactions were performed in a total volume of 25 µL, including 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µM dNTPs each (Fermentas, Sankt Leon-Rot, Germany), 25 pmoL of each *V. cholera* specific primer or 50 pmoL of the *E. coli* or *Salmonella sp*. specific primers, 1.5 U of Taq DNA polymerase (Fermentas, Sankt Leon-Rot, Germany), and 3 µL (40-260 ng/µL)of DNA. Amplification was carried out using a DNA thermo-cycler (Eppendorf Mastercycler 5330, Eppendorf-Nethel-HinzGmbH, Hamburg, Germany) as follows: for *V. cholerae* and *E. coli*: heat denaturation at 94°C for 2 minutes followed by 25 cycles of heat denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute and DNA extension at72°C for 1 minute. After the last cycle, the samples were kept at 72°C for 2 minutes to complete the synthesis of all strands [13]. The PCR amplification for *Salmonella sp*. was performed as described by Kong *et al*. (2002): heat denaturation at 94°C for 2 minutes followed by 35 cycles of heat denaturation at 94°C for 1 minute, primer annealing at 62°C for 1 minute and DNA extension at 72°C for 2.5 minutes, followed by incubation at 72°C for 10 minutes and cooling at 4°C [12]. Amplified

Table 1. Primers using for detection of *E. coli*, *Salmonella sp*. and *V. cholerae.*

Gene	Target organism	Primer	Annealing T	Size bp
uidA	*E. coli*	F: 5′- AAAACGGCAAGAAAAAGCAG-3′ R:5′- ACGCGTGGTTAACAGTCTTGCG-3′	58°C	358
ipaB	*Salmonella sp.*	F: 5′GGACTTTTTAAAAGCGGC GG-3′ R: 5′-GCCTCTCCCAGAGCCGTC TGG-3′	62°C	314
epsM	*V. cholerae*	F: 5′- GAATTATTGGCTC CTGTGCAGG-3′ R: 5′-ATCGCTTGGCGCA TCACTGCCC-3′	58°C	247

samples were analyzed by electrophoresis (120 V/208 mA) in 1.5% agarose gel and stained with ethidium bromide. A molecular weight marker with 100 bp increments (100 bp ladder, Fermentas, Sankt Leon-Rot, Germany) was used as size standard.

Sequencing

PCR products of positive samples were purified with a High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany). Single DNA strands were sequenced with an ABI 3730 XL device and Sanger sequencing method (Macrogen, Seoul, South Korea). The sequence of each gene was aligned with the gene sequences recorded in the Gen Bank database on the NCBI.

Isolation of E. coli, Salmonella sp., and V. cholerae

All samples were culture tested for the isolation of target bacteria. For isolating *E. coli* from water samples, each sample was labeled to show the serial number, place of water, type of water as well as time and date of collection. Examination of the water samples was completed within 24 hours after collection using Standard Total Coliform Multiple-Tube (MPN) Fermentation Techniques. After determination of MPN, the tubes showing growth were inoculated onto MacConkey, and EMB agar plates (Merck, Darmstadt, Germany). After a 24 hours' incubation at 35°, C ± 0.5°Cfor 24 hours ± 2 hours, Gram-negative microorganisms were isolated from MacConkey agar and EMB agar and identified at the species level using cytochrome oxidase, urea, and indole tests as putatively *E. coli* [14]. For *Salmonella sp*. isolation, water was filtered through each Sterivex unit until the 0.22 µm pore-size membrane became occluded. Three filters were prepared at each site as follows. After the desired 1.5 L of water was filtered, water remaining inside the filter housing was forced out with a 50 mL syringe, and 2 mL of buffered peptone water BPW was added into the housing with a 20 mL syringe equipped with a 25 gauge, 5/8-in 1.6 cm needle. The entry and exit ports of the filter unit were capped, and the filter unit was incubated at25°C for 4 hours. After pre-enrichment, 1 mL of the BPW was drawn off each filter and replaced with 1 mL of either 2 × Rappaport Vassilia (RV broth), 2 × Rappaport-Vassiliadis Novobiocin (RVN) broth, or 2 × Dulcitol Selenite (DS) broth (Merck, Darmstadt, Germany).After incubation at 43°C for 24 hours, a loopful of each enrichment broth was streaked onto Xylose Lysine Decarboxylase- Novobiocin (XN) agar, Tryptic Soy Brilliant Green (TSBG) agar, and Tryptic Soy Brilliant Green- Sucrose (TSBG-S) agar (Merck, Darmstadt, Germany). Plates were incubated at 43°C, and *salmonella*-like colonies (Pink colonies with black centers) [14] were picked at 24 and 48 hours and inoculated into Triple Sugar Iron (TSI) agar slants (Merck, Darmstadt, Germany). The slants were incubated at 43°C for 24 hours, and isolates which fermented only glucose were inoculated onto API 20E test strips (Analytab Products, Plainview, N.Y., USA) for identification to the *Salmonella sp.* [14]. For *Vibrio cholerae* isolation, two liters of water were filtered through 0.22 µm membranes, using 12 to 15 membranes per sample. Membranes were subsequently incubated in 100 mL of alkaline peptone water, pH 8.6, for 6-8 hours at 35°C. Two loops of broth were streaked on Thiosulfate Citrate Bile agar (TCBS agar, Difco, Trinity, USA) and incubated for 18 hours at 37°C. Six to 12 typical colonies (yellow and 1 to 3 mm diameter) were transferred to nutritive soft agar (T1N1, 0.75% agar) and incubated for24 hours at 37°C. All colonies were stored at room temperature for further testing [14].

Statistical Analysis

Chi-square test was performed for the difference between seasons and also different brands for the occurrence of bacteria in water by SPSS/20 software and differences were considered significant at values of P-value < 0.05. Also sensitivity and specificity of PCR was evaluated regarding to the culture based as the gold standard.

Table 2. Detection of *E. coli*, *Salmonella sp*. and *V. cholerae* from different brands (A-F) of bottle drinking water.

Brands	PCR			Culture		
	***uidA* (E.coli)**	***IpaB* (Salmonella sp.)**	***epsM* (V. cholerae)**	***E.coli***	***Salmonella sp.***	***V. cholerae***
A	0	0	0	0	0	0
B	3	1	0	3	0	0
C	0	0	0	0	0	0
D	2	0	0	1	0	0
E	1	0	0	1	0	0
F	0	0	0	0	0	0
Total	**6 (2.5%)**	**1 (0.4%)**	**0 (0%)**	**5(2.1%)**	**0(0%)**	**0(0%)**

Table 3. Sensitivity and specificity of PCR for identification of tested bacteria in the water.

PCR	Culture		Total
	Positive	Negative	
Positive	5	2	7
Negative	0	233	233
Total	**5**	**235**	**240**

Sensitivity= 100%; Specificity = 99%.

Results

The results of the PCR techniques showed that the specific *uidA*, *IpaB*, and *epsM* gene targets of *E. coli*, *Salmonella sp*., and *V. cholera*e were detected in 6 (2.5%), 1(0.4%), and 0 (0%) of 240 bottle drinking water samples, respectively. The results of the culture of the samples revealed that five (2.1 %) samples were contaminated with *E. coli*, but *Salmonella sp.* and *V. cholerae* were not culture positive from the samples (Table 2). As the results show, PCR is an accurate and more sensitive method for screening of water for the target bacteria (sensitivity = 100% and specificity = 99%), (Table 3). Culture based results indicated that the majority of positive samples related to the samples that were produced in summer so there was the significant difference between the samples of the hotseason and other seasons. ($P < 0.05$). Also, there was a significant difference between the number of positive samples of bottled drinking water from brand B and other brands. The results revealed that bottle water of 3 brands (A, C and F) were not contaminated with tested microorganisms (Table 4).

Discussion

There is a limit to the number and types of organisms permissible in drinking water with the World Health Organization stipulating that the bacteria present in bottled water should not exceed 50 cfu mL−1 and that there should be no coliform present per 100 mL of water [15]. In this study, 6 brands of bottled water produced by 6 bottling companies were investigated. From the results of this study, the samples of three companies were of acceptable quality. While the water samples from the other 3companies were found not to be acceptable. The results of present study showed that some bottles of water were contaminated with *E. coli*, *Salmonella sp*. The presence of *E. coli*, *Salmonella sp*. in drinking water is a threat to human health. Some serotype of these bacteria can cause hemorrhagic colitis and enteritis [16], diarrhea, nausea, abdominal cramps, fever, and vomiting [17], respectively. The previous study in south Africa indicated that water-borne diseases are responsible for about 20% of all deaths in children fewer than five years of age [18]. Several studies have been reported a statistically significant increase in gastrointestinal illness in populations that drink contaminated water with different types of enteric bacteria [19]. Therefore, regularly checking for contaminants in water supplies and using appropriate techniques for water disinfection is very important. With the positive results of target organisms in this study, the main source of contamination of the samples could be the source of water, used by these companies (B, D and E). Most of the companies in Iran use the water from wells and ponds as the source of water where contamination of these sources with enteric pathogens is more prevalent. According to recommendations from the WHO (World Health Organization), CAWST (Center for Affordable Water and Sanitation Technology), drinking water must be free from *E. coli*, Salmonella spp., and *V. cholera*e [15,20]. The results of our study found that this was not the case for bottle water in Iran and was comparable with multiple studies including a recent report in Esfahan-Iran, Momtaz *et al*. [10] which reported that 2.63% of bottled water samples were positive for *E. coli*, *V. cholera*e, and *Salmonella sp*. Knight *et al.* detected *Salmonella sp.* in 12 (75%) of 16 study sites producing drinking water in New York (USA) [21] and another study in Sudan showed that 0.25% of water

Table 4. Isolation of *E. coli* from bottle drinking water in Iran.

Brand	Spring (n = 60)	Summer (n = 60)	Autumn (n = 60)	Winter (n = 60)	Total (n = 240)
A (n = 40)	0	0	0	0	0 (0%)
B (n = 40)	0	3	0	0	3 (7.5%)
C (n = 40)	0	0	0	0	0 (0%)
D (n = 40)	0	1	0	0	1 (2.5%)
E (n = 40)	1	0	0	0	1 (2.5%)
F (n = 40)	0	0	0	0	0 (0%)
Total (n = 240)	**1 (1.66%)**	**4 (6.66%)**	**0 (0%)**	**0 (0%)**	**5 (2.1%)**

samples were positive for *V. cholerae* [22]. In addition, Heijnen *et al*. reported that 7.4% of water samples in Netherland were contaminated with *E. coli* [23] showing that contamination of drinking water with our target organism in this study is happening on a global scale.

Other studies have detected different microorganisms in drinking water, Oluwatoyin *et al*. reported that coliforms present in 100 mL of water were detected in 16.3% of the samples and 26.7% of the bottled water brands. The coliforms detected included *Enterobacter agglomerans* from two brands and *Citrobacter freundi* from another two brands of the samples. Other indicator organisms detected included Staphylococci isolated from 27.9% of the samples (33.3% of the brands) and specifically *Staphylococcus aureus* found in four brands constituting 14% of the samples. Pseudomonas strains were consistently detected in consecutive batches of three brands of the water samples [24]. In a study carried out in Egypt [25] coliforms were detected in 28.6% of 84 bottled water samples examined and they were not of fecal origin as *E. coli* was absent in the samples. The results of this study shows that the contamination of bottle waters with *E. coli* and *Salmonella sp.* is significantly higher ($P< 0.05$) in hot seasons (summer and spring). This result is close to the results of previous studies in Iran, Italy, and Nigeria [10,24,26] that showed the contamination of bottle water with coliform bacteria and also *E. coli* specially in summer. The results of this present study revealed that PCR is an accurate and more sensitive method for screening of water samples in comparable to culture based methods to monitor the bacteria in drinking water. Our finding was comparable to previous study which concluded that application of PCR techniques provided an accurate, safe, sensitive, specific, and fast results that could be used to monitor the microbial load of drinking water. According to the results of our study, the sensitivity and specificity of the PCR were more than 99%. It is very close to the results of the Momtaz *et al.*, study on the water samples in Esfahan province [10].

Conclusion

According to the results of PCR bottle water from Iran were contaminated with *E. coli* and *Salmonella sp.* it is possible that the source of these samples (B, D and E) was contaminated. The deficiencies observed in these samples suggest that some of the producers of the bottled water samples examined in this study do not follow stipulated guidelines in their production processes.

This is however avoidable if there is adequate control or monitoring of the practices of the bottling companies. The results from this study indicate that there may be a lack of required infrastructure within the manufacturing process. Requirements such as clean rooms, automatic bottle filling machines, clean water source, adequately trained personnel and the need for staff supervision by personnel who are knowledgeable in the science of water purification and dispensing must be met by companies (B, D and E) which are involved in the production of water packaged for human consumption. There is a need for a rigorous inspection and follow-up of water bottling facilities so that only those companies with adequate purification procedures and consistently produce bottle water of acceptable bacteriological quality is allowed to produce water for public consumption.

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