Contamination of Tap Water with *Pseudomonas aeruginosa, Legionella pneumophila,* and *Escherichia coli* in Guilan, Iran

Masoumeh Ahmadi Jalali Moghadam [1], *Hamidreza Honarmand* [2*], *Sajad Asfaram Meshginshahr* [3]

[1] *Cellular and Molecular Research Center, Guilan University of Medical Sciences, Rasht, Iran.*

[2] *Department of Microbiology, Guilan University of Medical Sciences, Rasht, Iran.*

[3] *Department of Microbiology, Lahijan Branch, Islamic Azad University, Lahijan, Iran.*

ARTICLE INFO

Article type:
Original Article

Article history:
Received: 17 Dec 2015
Revised: 19 Jan 2016
Accepted: 11 May 2016
Published: 15 Aug 2016

Keywords:
Tap water, Pseudomonas aeruginosa, Legionella pneumophila Escherichia coli, PCR

ABSTRACT

***Background*:** River and underground waters are main sources of tap water in Guilan, Iran. Overland wastes move into rivers during periods of heavy or extended rain that is very common in the area. *Pseudomonas aeruginosa*, *Legionella pneumophila*, and *Escherichia coli* are main human pathogens with water source. This study is designed to determine the load of these bacteria in main water supplies of the area.

***Methods*:** Samples were collected directly into sterile containers, concentrated by centrifuge, inoculated in enrichment medium and incubated for 3-4 days. DNA was extracted by using commercial kit. Several rounds of PCR was performed to search *P. aeroginosa*, integron I, Metallo-β-lactamases gene, *L. pneumophila*, mip gene, and *E. coli*.

***Results*:** About 92.0% of the samples showed bacterial contamination as revealed by PCR with primers of 16S rRNAgene, 9.5% of the samples had *L. pneumophila, and* 11,1% had *Pseudomonas aeruginosa*, but *Escherichia coli* was not detected. We found the *mip* gene in 66.6% of the samples with *L. pneumophila*. Metallo-β-lactamasesgene was found in 11.1% of all samples. We also found Integrin 1 in 28.5% of the samples with *P. aeruginosa.*

***Conclusion*:** This study indicates that in spite of chlorination, total bacterial contamination of pot waters in the area is high and contamination with *L. pneumophila* and *P. aeroginosa* is considerable. It might be related to the biofilm formation and the growth of water microflora. It seems that free residual chlorine is ineffective. We suggest a more effective decontamination procedure based on modern technology.

- ***Please cite this paper as:*** Ahmadi Jalali Moghadam M, Honarmand H, Asfaram Meshginshahr S. Contamination of Tap Water with *Pseudomonas aeruginosa*, *Legionella pneumophila*, and *Escherichia coli* in Guilan, Iran. *J Med Bacterial.* 2016; **5** (1, 2): pp.21-28.

*Corresponding Author: Hamidreza Honarmand, Department of Microbiology, Guilan University of Medical Sciences, Rasht, Iran.

Tel: +98-1342226121, *E-mail*: honarmand.3@gmail.com

Introduction

Water is considered as the essence of life and access to safe drinking water is a basic human right. Water is necessary for the welfare of humankind and for sustainable development. Safe drinking water should not represent any significant risk to health over a lifetime of consumption. Sources of drinking water include piped water, rivers, reservoirs, springs, streams, wells, ponds and rain. It should be noted that the way water is collected, handled after collection and stored at home cause quality deterioration to such an extent that the water poses potential risks of infection to consumers (1, 2).

The lack of microbiologically safe drinking water and adequate sanitation measures leads to a number of diseases including cholera, dysentery, salmonellosis, typhoid, and every year millions of lives are lost in developing countries (3). Diarrhea is a major cause of death of more than 2 million people per year worldwide, mostly children under the age of five and it is the symptom of infection or the result of a combination of a variety of enteric pathogens (4).

There are bacterial pathogens, including enteric and aquatic bacteria, which are strongly resistant not only to the water environment, but also to most of the disinfectants (5). Some early bacterial agents may contaminate surface waters through wildlife or domestic animal feces (5). A number of pathogens including environmental bacteria are capable of surviving and proliferating in water distribution systems and the general population is refractory to infection with them (5). The presence of *E. coli* in the water samples collected from the ground and surface water sources emphasizes that there has been fecal contamination of the drinking water sources and its presence in the water indicates recent fecal contamination of the water (5). *Legionella* and *Pseudomonas* are environmental pathogens that have found an ecologic niche in drinking and hot water supplies (6). Now, P. aeruginosa is considered as an opportunistic pathogen in patients with low resistance to infections and its presence in water and food is unacceptable because it has been implicated in waterborne and foodborne diseases (6). It is now considered to be a primary infectious agent (7). The presence of *P. aeruginosa* as a mean of assessing the hygienic quality of drinking water has been advocated, and levels of this organism provide an indication of the general safety of the water distribution system (8).

The aim of the present study was to determine the contamination degree of tap waters with *P. aeruginosa*, *L. pneumophila*, and *E. coli* in all cities in Guilan province, Iran.

Material and method

Sampling, Processing, and Enrichment

During April-June 2014, sixty three pot water samples (about 50 ml for each) were aseptically collected from 47 cities after the water had run for 5 min. Potable water is supplied to the cities by the municipal system. The water has detectable free residual chlorine during sampling. All water samples were concentrated 20-fold (by dividing with 10 ml in 5 tubes and centrifugation with 12000 rpm for 30 min in 4 °C). After discarding supernatant, the precipitates were resolved in 1 ml of the remained solution and then this solution was divided into two parts. One part was treated in 50 °C for 30 min to reduce other contaminants for effective recovering of *Legionella*. After heat treatment, each sample was inoculated in 5 ml liquid medium containing yeast extract 10 g/l, L-cystein 0.04%, ferric pyrophosphate 0.25 g/l, glycin 0.3% and vancomycin 5 µg/ml with final pH 6.9, and incubated in 35 °C for 3 days. The selectivity of the medium was subsequently improved by the incorporation of vancomycin and glycine. This selective medium was assumed to facilitate the enrichment of the members of the family Legionellaceae from environmental sources (9). The second part of each sample was inoculated in Tripticase Soy Bean broth and incubated in 35 °C for 3 days for recovering *p. aeruginosa* and *E. coli*.

DNA Extraction and Primers

All cultured samples were divided over 1 ml in volume and centrifuged in a Sigma model 3k30 centrifuge at 12,000 rpm in 4 °C for 20 min. The supernatant was discarded, and the pellet was suspended in 1 mL of TE buffer before DNA extraction. DNA was extracted by using commercial procedure (Genomic DNA Purification Kit, Thermoscientific, Lot: 00155557, Fermentas, Lithuania). The extracted DNA was either used immediately for PCR or stored at – 20 °C until analysis.

Several rounds of PCR was performed for detecting *Pseudomonas aeroginosa*, integron1, Metallo-β-lactamases gene, *Legionella pneumoohila*, *mip* gene, and *E.coli* as below: 1) PCR by using specific primers for *L. pneumophila* and *mip* gene separately. 2) Multiplex PCR by using specific primers for *pseudomonas aeruginosa*, Integron1, and Metallo-β-lactamases gene. 3) Conventional PCR for detecting *E. coli* using specific primers, and 4) final round of PCR to investigate presence of other bacteria by using universal primers. All primers which were used in this study are characterized in Table 1.

We used genomic DNA of *L. pneumophila* strain NCTC 11192 as the positive control for the first round of PCR. A standard strain of *P. aeroginosa* (ATCC 27853) was used as the positive control for second round of PCR and *E.coli* (ATCC 44338)was used as the positive control for third and fourth rounds of PCR. Pure water was used as the negative control for all PCR amolification reactions.

PCR Conditions

Two micro liters of the extracted template DNA were used in a 20 µl reaction mixture that included 10 µl of PCR premix [Prime Taq Premix (2x), Chorea Lot No 201208], 0.5 µl of each primer, and 7 µl of ddH_2O. Cycling conditions for amplification of *L. pneumophila* - species specific fragment began with an initial denaturation at 94 °C for 5 min, and then 35 cycles including 94 °C for 60 s, 45 °C for 50 s, and 72 °C for 60s that were followed by a final extension at 72 °C for 10 min. Cycling program for the second round PCR was the same but the annealing temperature was 50 °C. Annealing temperature for third round of PCR was 58 °C and for 16S rRNA amplification with the universal primers was 47 °C with the same cycling program. Electrophoresis of amplified product was performed on agarose gel (2% w/v) by 125 volt for 45 minutes and analyzed by GelDoc Transluminator system (VilberLourmat model).

Table 1. Features of the primers which were used in this study.

Primer	Sequence (5' ? 3')	Used for detection of:	Reference
Lpms1F Lpms1R	CAGGGAAATGCTCTAGCACAC TCGCTTCGGACTGAATTTCT	*L. pneumophila*	10
Lmip F LmipR	GGCCAATAGGTCCGCCAACG GGTGACTGCGGCTGTTATGG	*mip* gene	11
gyrB-F gyrB-R	CCTGACCATCCGTCGCCACAAC CGCAGCAGGATGCCGACGCC	*P. aeroginosa*	12
Vim1-1A Vim1-1B	TTATGGAGCAGCAACGATGT CAAAAGTCCCGCTCCAACGA	blaVIM	13
Int1-1A Int1-1B	TACCTCTCACTAGTGAGGGG ATGAAAACCGCCACTGCGCC	int1	14
EPE-F EPE-R	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	*E. coli*	15
16srRNA F 16srRNA R	GGATTAGATACCCTGGTAGTCC TCGTTGCGGGACTTAACCCAAC	whole bacterial load	16

Table 2. Summary of amplification results.

P.aeroginosa	Metalobetalactamase gene in *P.aeroginosa*	Integron1 in *P.aeroginosa*	*E.coli*	*L.pneumophila*	*mip* gene in L.pneumophila	total bacterial presence
7(63) 11.1%	3(7) 42.8%	2(7) 28.5%	0 0	6(63) 9.5%	4(6) 66.6%	58(63) 92.0%

Results

Sixty tree samples were collected from water outlets of 48 cities. We found *L. pneumophila* in 6 samples (9.5%) but only 4 samples (66.6%) had *mip* gene (Table 2). Totally, 7 samples were positive for *P. aeroginosa* (11.1%) in which 3 samples (42.8%) had Metallo-β-lactamases gene

and also one sample (14.3%) had integrin 1 (table 2 and figure 1). In general, 4.8% of samples had Metallo-β-lactamases gene and 1.6% of them had integrin 1 (table 2). *E. coli* was not found in any sample, but 58 samples (92.0%) were positive in amplification assay with 16S rRNA universal primer (table 2) indicating whole presence of bacteria (mainly water normal flora).

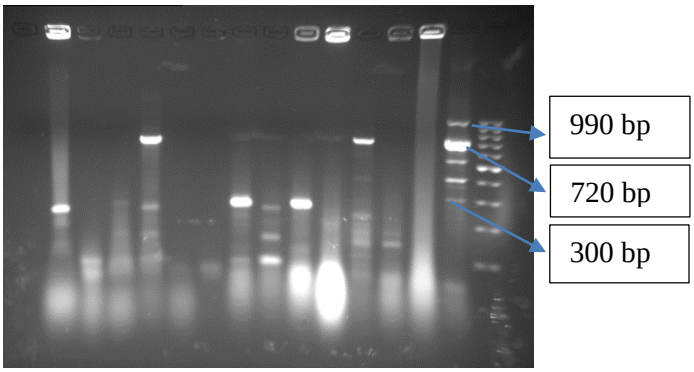

Figure 1. Results of multiplex PCR with *P. aeroginosa* species specific, Integron1, and Metallo-β-lactamases gene primers for 13 samples. Lines 16 is the 100-1000 bp ladder. Lines 15 is positive control (*p. aeroginosa* type strain ATCC 27853). Lines 5, 6, 9, and 12 are positive samples for *P. aeroginosa* (720 bp specific bands), Lines 2, 5, 8, 9, and 10 are positive samples for Metallo-β-lactamases gene (300 bp). All samples are negative for integrin 1. Lines 3, 4, 6, 7, 11, 12, 13, and 14 are negative samples and line 1 is negative control (water).

Discussion

Presence of *L. pneumophila* is associated with biofilms in warm water plumbing systems (17). This bacterium can also survive at lower temperatures in drinking water environments (18, 19). Coliform bacteria are occasional contaminants of drinking water distribution systems and have been found in the biofilms on pipe walls and rubber-coated valves in these systems (20). *E. coli* is a member of the fecal coliform group and is a more specific indicator of fecal pollution than other fecal coliforms, so, at present, *E. coli* appears to provide the best bacterial indication of fecal contamination in the drinking water (21). It should be noted that a negative coliform no longer guarantees that water is free from all pathogens and low levels of pathogen occurrence may be responsible for the endemic transmission of enteric disease and also there is no direct correlation between numbers of any indicator and entire pathogens (21). In the present study we did not find *E. coli* in the studied samples.

The detection of opportunistic bacteria in drinking water biofilms has usually been performed using cultivation method. However, bacteria may enter a viable but non-cultivable (VBNC) state as a response to some form of environmental stress (22). In the VBNC state, the bacteria fail to grow on routine bacteriological media, but they are still alive and typically demonstrate low levels of metabolic activity. Some non-pathogenic coliforms are also viable but will not grow in the media prescribed for them; and coliforms found in the environment are often stressed thereby making recovery very difficult despite the growth media used (21). The detectable bias in the culture-based methods in detecting *E. coli*, as an indicator organism is why it has been substituted by PCR-based methods (23). For all above mentioned reasons we used molecular approach in the present study. PCR analysis for screening drinking water and environmental samples has been used to detect *E. coli* in primary water specimens, stool specimens and in outbreaks by other pathogens as well (23, 24).

Liguori et al. found *P. aeruginosa* in only 1 of 38 (2.6%) of tap water samples (25) whereas we found this microbe more often 11.1%. Besides, similar to our results they also did not detect *E.coli* in the water samples tested. The findings of the present study and in Liguori *et al* were in accordance with similar studies recently conducted (26, 27).

Shamabadi performed a similar investigation in Qom, Iran, and showed that contamination rate of pot water with *E. coli* was zero but 16.7% of their

samples were shown to be contaminated with *P. aeruginosa* (28). Comparatively, such a finding is very close to ours. Although coliforms are known as the best index for monitoring water microbial quality, in some cases this index is not very efficient. Some believe that the excess growth of heterotrophic bacteria cause in suppression of the coliforms. Therefore, it is recommended to use other bacteria as an alternative index in microbial quality control of the water samples and some species of *Pseudomonas* can be among these indicators. Interestingly, results of our study are compatible with this idea. In this study, the average of the detection of bacteria was 92.0%, contamination rate with *E. coli* was zero whereas contamination rate with *P. aeroginosa* was 11.1%. It seems that an excess number of bacteria can suppress *E.coli* but not *Pseudomonas aeruginosa*.

Study of Yassin *et al*. in Gaza Strip showed that the contamination level of total and fecal coliforms exceeded that of the World Health Organization (WHO) limit for water wells and networks and it is attributed to the intermittent water supply, sewage flooding and age of water, and wastewater networks (29).

AlOtaibi detected fecal coliforms in desalinated, surface, and well water, with percentages of 3.23, 60.0 and 87.88, respectively (30). Abu-Zeid et al. studied 201 tap water samples and found 26.4% contamination with coliforms (31) but Ahmad et al. did not find any contamination in pot water supplies of the industrial city of Yanbu in Saudi Arabia (32).

Legionella is a part of microflora in some aquatic environments. The cooling tower and the air condition system have also been reported to be sources of several outbreaks (33). Manmade water systems especially hot water systems are the main sources of *Legionella* (34). These bacteria can survive in biofilms and aslo can resist against chlorine and other disinfectants (35). Study of Borella *et al.* (2005) on 119 hot water samples of hotels in Italy showed a high rate of contamination with *L. pneumophila* (45.8%) (44). This study showed that there is adverse association between bacterial count in the water samples and the free residual chlorine. The study of Tison *et al.* (1983) showed that *Legionella* count in water decreases up to 1000 fold by chlorination (36). Palmer *et al.* (1995) also reported that viable count of *Legionella* decreased by chlorination (37).

Chlorine-based disinfectants are the most commonly used disinfectants and are cheap and easy to use. Free chlorine is an effective disinfectant for bacteria and viruses. Main limitation of using chlorination is that it produces some potential harmful disinfection by-products. However, in the latest edition of the WHO Guidelines for Drinking Water Quality, it is recommended that the risks of human illness and death from pathogens in drinking water are much greater than the risks from exposure to disinfectants and disinfection by-products (38).

Based on efficiency, ozone is the most efficient disinfectant for inactivating bacteria, viruses, and protozoa. In contrast, chloramines are the least efficient and are not recommended for use as primary disinfectants. Chloramines are favored for secondary water disinfection, because they react more slowly than chlorine and are more persistent in distribution systems. In addition, chloramines produce lower disinfectant by product levels than does chlorine, although microbial activity in the distribution system may produce nitrate from monochloramine. The failure of conventional treatment processes to eliminate critical waterborne pathogens in drinking water demand that improved and/or new disinfection technologies be developed such as using nanotechnology to solve the problem , through the use of nanosorbents, nanocatalysts, bioactive nanoparticles, nanostructured catalytic membranes, and nanoparticle-enhanced filtration.

Conclusion

The data of the present study raise concern about the microbiological quality of the tap water in the area and highlights the importance of adopting appropriate monitoring system. This study indicates that in spite of chlorination, total bacterial presence in potable water in the area is high and contamination with *L. pneumophila* and *P. aeroginosa* is considerable. It is shown that it may be related to the biofilm formation and the growth of water microflora. It seems that the free residual chlorine is not effective, so we suggest a more effective decontamination procedure based on new technology such as using nanosorbents, nanocatalysts, bioactive nanoparticles, nanostructured catalytic membranes, and nanoparticle-enhanced filtration.

Acknowledgements

Special thanks to Cellular and Molecular Research Center Guilan University of Medical Sciences.

Conflict of interests

No conflict of interests is declared.

Financial disclosure

This study is supported by Guilan University of Medical Sciences.

References

1. Pinfold JV. Fecal contamination of water and fingertiprinses as a method for evaluating the effect of low-cost water supply and sanitation activities on fecal-oral disease transmission. II. A hygiene intervention study in rural north-east Thailand. *Epidemiol Infect* 1990; **105**(2): 377–389.
2. Karikari AY, Ampofo JA. Chlorine treatment effectiveness and physico-chemical and bacteriological characteristics of treated water supplies in distribution networks of Accra-Tema Metropolis, Ghana. *Appl Water Sci* 2013; **3**: 535-543.
3. Clasen T, Haller L, Walker D, Bartram J, Cairncross S. Cost-effectiveness of water quality interventions for preventing diarrheal disease in developing countries. *J Water Health* 2007; **5**(4): 599-608.
4. Durmishi BH, Ismaili M, Shabani A, Abduli Sh. Drinking Water Quality Assessment in Tetova Region. *Am J Environ Sci* 2012; **8**(2): 162-169.
5. Leclerc H, Schwartzbrod L, Dei-Cas E. Microbial agents associated with waterborne diseases. *Crit Rev Microbiol* 2002; **28**(4): 371-409.
6. Palleroni NJ. Human- and animal-pathogenic pseudomonads. In The Prokaryotes, 2nd ed.; Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH. Eds.; Springer-Verlag: New York, USA; 1992; pp 3086-3102.
7. Morais PV, Mesquita C, Andrade J, DaCosta M. Investigation of persistent colonization by *Pseudomonas aeruginosa* -like strains in a spring water bottling plant. *Appl Environ Microbiol* 1997; **63**: 851-856.
8. Al-Qadiri HM, Al-Holy MA, Lin M, Alami NI, Cavinato AG, Rasco BA. Rapid detection and identification of *Pseudomonas aeruginosa* and *Escherichia coli* as pure and mixed cultures in bottled drinking water using fourier transform infrared spectroscopy and multivariate analysis. *J Agric Food Chem* 2006; **54**(16): 5749-5754.
9. Villari P, Motti E, Farullo C, Torre I. Comparison of conventional culture and PCR methods for detection of *Legionella pneumophila* in water. *Lett Appl Microbiol* 1998; **27**(2): 106-110.
10. Pourcel C, Vidgop Y, Ramisse F, Vergnaud

G, Tram C. Characterization of a tandem repeat polymorphism in *Legionella pneumophila* and its use for genotyping . *J Clin Microbiol* 2003; **41**(5): 1819-1826.
11. Lindsay DSJ, Abraham WH, RonalJ. Fallon RJ, Detection of mip Gene by PCR for diagnosis of Legionnaires' disease. *J Clin Microbiol* 1994; **32**(12): 3068-3069.
12. Lavenir R, Jocktane D, Laurent F, Nazaret S, Cournoyer B. Improved reliability of *Pseudomonas aeruginosa* PCR detection by the use of the species-specific ecfX gene target. *J Microbiol Methods* 2007; **70**(1):20-9.
13. Yan JJ, Hsueh PR, Ko WC, Luh KT, Tsai SH, Wu HM, Wu JJ. Metallo-b-lactamases in clinical *Pseudomonas* isolates in Taiwan and identification of VIM-3, a novel variant of the VIM-2 enzyme. *Antimicrob Agents Chemother* 2001; **45**: 2224-8.
14. Yan JJ, Hsueh PR, Lu JJ, Chang FY, Ko WC, Wu JJ. Characterization of acquired b-lactamases and their genetic support in multidrug-resistant *pseudomonas aeruginosa* isolates in Taiwan: the prevalence of unusual integrons. *Journal of Antimicrobial Chemotherapy* 2006; **58**: 530-536.
15. Oswald E, Schmidt H, Morabito S, Karch H, Marches O, Caprioli A. Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. *Infect Immun* 2000; **68**: 64-71.
16. Kariyama R, Mitsuhata R, Chow JW, Clewell DB, Kumon H. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycine-resistant Enterococci. *J Clin Micriobiol* 2000; **38**(8): 3092-3095.
17. Lau HY, Ashbolt NJ. The role of biofilms and protozoa in *Legionella* pathogenesis: implications for drinking water. *J Appl Microbiol* 2009; **107**: 368-378.
18. Gião MS, Wilks SA, Azevedo NF, Vieira MJ, Keevil CW. Comparison between standard cultures and peptide nucleic acid 16S rRNA hybridization quantification to study the influence of physico-chemical parameters on *Legionella pneumophila* survival in drinking water biofilms. *Biofouling* 2009: **25**: 335-343.
19. Lehtola MJ, Torvinen E, Kusnetzov J, Pitkanen T, Maunula L, von Bonsdorff CH, et al. Survival of *Mycobacterium avium*, *Legionella pneumophila*, *Escherichia coli* and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. *Appl Environ Microbiol* 2007; **73**: 2854-2859.
20. Wingender J, Flemming HC. Contamination potential of drinking water distribution network biofilms. *Water Sci Technol*. 2004; **49** (11–12): 277-286.
21. Odonkor ST, Ampofo JK. Escherichia coli as an indicator of bacteriological quality of water: an overview. *Microbiol Res* 2013; **4**:e2: 5-11.
22. Oliver JD. The viable but nonculturable state in bacteria. *J Microbiol*. 2005; **43**: 93-100.
23. Feldsine PT, Falbo-Nelson MT, Hustead DL. Colicomplete substrate-supporting disc method for confirmed detection of total coliforms and *Escherichia coli* in all foods: comparative study. *J AOAC Int* 1993; **76**: 988-1005.
24. Gevaert K, Van Damme J, Goethals M, Thomas GR, Hoorelbeke B, Demol H, et al. Chromatographic isolation of methionine-containing peptides for gel-free proteome analysis: identification of more than 800 *Escherichia coli* proteins. *Mol Cell Proteomics* 2002; **1:** 896-903.
25. Liguori G, Cavallotti I, Arnese A, Amiranda

C, Anastasi d, Angelillo IF. Microbiological quality of drinking water from dispensers in Italy. *BMC Microbiol* 2010, **10**:19.
26. Baumgartner A, Grand M. Bacteriological quality of drinking water from dispenser (coolers) and possible control measures. *J Food Prot* 2006; **69**: 3043-3046.
27. Zanetti F, De Luca G, Sacchetti R. Control of bacterial contamination in microfiltered water dispensers (MWDs) by disinfection.*Int J Food Microbiol* 2009; **128**: 446-452.
28. Shamabadi N, Ebrahimi M. Use of bacterial indicators for contamination in drinking water of Qom, Iran. *Journal of Applied Sciences* 2007; **7**(17): 2456-2461.
29. Yassin MM, Amr SS, Al-Najar HM. Assessment of microbiological water quality and its relation to human health in Gaza Governorate, Gaza Strip. *Public Health* 2006; **120**(12): 1177-87.
30. AlOtaibi EL. Bacteriological assessment of urban water sources in Khamis Mushait Governorate, southwestern Saudi Arabia. *Int J Health Geogr* 2009 **21**; 8:16.
31. Abu-Zeid HA, Aziz MA, Abolfotouh M, Moneim MA. Bacteriologic pot ability of the drinking water in a diarrhea hyper endemic area in southwestern Saudi Arabia. *J Egypt Public Health Assoc* 1995; **70**(3–4):279-91.
32. Ahmad M, Bajahlan AS. Quality comparison of tap water vs. bottled water in the industrial city of Yanbu (Saudi Arabia). *Environ Monit Assess* 2009; **159**(1–4):1–14.
33. Delgado-Viscogliosi P, Simonart T, Parent V, Marchand G, Dobbelaere M, Pierot E, et al. Rapid method for enumeration of viable *Legionella pneumophila* and other *Legionella* spp in water. *Appl Environ Microbiol* 2005; **71**(7): 408-4096.
34. Borella P, Montagna MT, Stampi S, et al. *Legionella* contamination in hot water of Italian hotels. *Appl Environ Microbiol* 2005; **71**(10): 5805-5813.
35. Holmberg RE. Pavia AT, Montgomery D, Clark JM, Eggert LD. Nosocomial *Legionella pneumophila* in the neonate. *Pediatrics* 1993; **92**(3): 450-453.
36. Meenhorst PL, Reingold AL, Groothuis DG, et al. Water-related nosocomial pneumonia caused by *Legionella pneumophila* serogroup 1 and 10. *J Infect Dis* 1985; **152**(2):356-364.
37. Tison DL, Seidler RJ. *Legionella* incidence and density in potable drinking water supplies. *Appl Environ Microbiol* 1983; **45**(1): 337-339.
38. Ngwenya N, Ncube EJ, Parsons J. Recent advances in drinking water disinfection: successes and challenges. *Rev Environ Contam Toxicol* 2013; **222**:111-70.