Iranian Journal of Microbiology
Volume 8 Number 4 (August 2016) 219-225

ORIGINAL ARTICLE

Microbiological qualification of air, water and dialysate in a haemodialysis centre: a new focus on *Legionella* spp.

Saeid Nazemi[1], Mehdi Mirzaii[2*], Somayeh Yaslianifard[3], Davood Darban-Sarokhalil[4], Seyyed Sajjad Khoramrooz[5], Pirasteh Norozi[2], Fatemeh Davardoost[6]

[1]Department of Environmental Health Engineering, School of Public Health, Shahroud University of Medical Sciences, Shahroud, Iran
[2]School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran
[3]Department of Microbiology, School of Medicine, Alborz University of Medical Sciences, karaj, Iran
[4]Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[5]Medicinal Plant Research Center, Yasuj University of Medical Sciences, Yasuj, Iran
[6]School of Public Health, Shahroud University of Medical Sciences, Shahroud, Iran

Received: November 2015**, Accepted:** June 2016

ABSTRACT

Background and Objectives: The microbiological monitoring of the water used for haemodialysis is important especially for *Legionella* and non-fermentative bacteria since patients with end stage renal disease (ESRD) are suffering from deteriorated function of immune system.

Materials and Methods: A total 50 water and dialysate samples were weekly collected over a period of 10 weeks from 5 sites. Total and faecal coliforms were determined by utilizing the most probable number (MPN) method. For isolation of *Legionella,* water samples were inoculated on a BCYE medium. DNA extraction was performed and was used to amplify *16S rRNA* gene of *Legionella* species. Airborne bacteria were sampled using a single stage Andersen air sampler.

Results: Out of total 50 water samples, 24 samples had bacterial contamination. The highest rate of *Legionella* contamination was observed in the storage tank (67 cfu/ml). *Legionella* was not isolated from the dialysate effluent samples. The highest rate of total bacterial count was related to the dialysate effluent and the maximum total count of coliforms was related to the reverse osmosis. The isolated bacteria were Gram-negative bacilli (mostly *Pseudomonas* isolates), Gram-positive cocci (mostly *Micrococcus spp.*) and Gram-positive bacilli (mostly *Bacillus spp.*). Six samples were contaminated with coliforms. No faecal coliform was isolated from the samples.

Conclusion: These results indicated that dialysis machine is an important source of contaminations such as *Staphylococcus, Pseudomonas* and *Legionella.* Therefore an efficient prevention program is needed to eliminate bacterial contamination of dialysis water system. Moreover, in haemodialysis centres, periodic surveillance programs for microbiological qualification can lead to a better planning for disinfection of haemodialysis water systems.

Keywords: Microbiological qualification, Haemodialysis, *Legionella*

*Corresponding author: Mehdi Mirzaii PhD, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran.
Phone/Fax: +98 23 32394031
E-mail: mirzaii1386@gmail.com

INTRODUCTION

Haemodialysis is the most effective modality in treatment of end stage renal disease (ESRD) (1, 2). Patients undergoing the haemodialysis treatment are at a greater risk of acquiring systemic infections (3). Standard haemodialysis treatment sessions last 4-6 hours and patients are exposed to 15,000-20,000 litres of dialysis fluid, annually. In haemodialysis procedure, dialysis water is directly administered in the form of dialysate and infusate up to 3,400-6,800 litres through two or three dialysis water monitor ultrafilters (4). The trend of using online haemodialysis procedures increased dialysate contamination which in turn may lead to the infections caused by many antibiotic resistant bacteria (5). Inefficient systems in treating water (filtration and reverse osmosis) and ineffective methods of disinfection are responsible for the majority of bacteraemia and sepsis among patients in haemodialysis (6). Therefore water systems in haemodialysis should be under constant microbiological monitoring (6, 7). On the other hand, haemodialysis services in hospitals have the same water distribution networks. Occasionally, these networks encounter problems such as low flow of water to meet the required tap water for the procedure. Therefore, haemodialysis machines are frequently connected to a storage water tank to ensure adequate volume and pressure of water especially in times of peak demand (8). However, with any water distribution network, those in the hospitals are subjected to biofilm formation (9). A number of pathogens, such as *Legionella, Pseudomonas,* and Mycobacteria grow well in biofilms and may be more resistant to disinfectant utilized in the haemodialysis water than their planktonic forms (9). *Legionella* spp. are thin, Gram-negative, obligate aerobic and non-spore forming rods with complexed nutritional requirements which contaminate the water distribution networks particularly dialysis water and can be transmitted from water to the air via hospital water supply. Inhalation of *Legionella* contaminated aerosols is a common source of human infections (10). In addition, some species of *Legionella* such as *Legionella pneumophila* are strongly associated with asymptomatic infections (Legionnaires' disease) or produce mild cough, sore throat and pontiac fever (11). Several comprehensive epidemiological studies have linked the exposure of individuals to the contaminated water distribution systems of hospitals to the acquisition of nosocomial Legionnaires' disease (12, 13). However, few studies have been conducted on detection of *Legionella* infection through haemodialysis water systems. The objective of the present study was to investigate the prevalence of *Legionella* spp. and other bacterial infections in air, water and dialysate utilized in a haemodialysis centre in an educational hospital in Iran.

MATERIALS AND METHODS

General conditions. This study was conducted in a public haemodialysis centre having 17 dialysis machines. One separated machine was especially assigned for haemodialysis treatment of patients infected with hepatitis B virus. The centre performs approximately 1100 haemodialysis sessions monthly in three shifts a day for 95 patients suffering from ESRD and approximately 45 sessions of haemodialysis treatment for acute cases of renal failure. Water treatment system is a built-in unit which includes tap water pre-treated with filter system, a water softener, and an activated carbon filter followed by a final purification with reverse osmosis (RO) process. Treated water is stored in a reservoir tank where it will be distributed to the haemodialysis unit and dialysis machines. After each session of haemodialysis, the machines are rinsed and disinfected according to the protocols enforced by the Ministry of Health.

Water sampling procedure. A total of 50 water and dialysate samples were weekly collected from November 2013 to January 2014. The water samples (500 ml) were collected according to the water sampling guidelines (14) from 5 sites as follows: distribution loop (n=6), raw water (n=4), reverse osmosis (RO) (n=11), water storage tank (n=11) and dialysate effluent (n=18).

Determination of total and faecal coliforms. The total and faecal coliforms were determined by utilizing the most probable number (MPN) method (15). MPN test was carried out according to the standard method (10-tube form). MPN was calculated for positive tubes using MPN standard table. Moreover, samples were plated on MacConkey (MC) agar (Conda, Spain) to determine the presence of faecal coliforms. After incubation at 37 °C for 24 h, the grown colonies were identified by standard biochemical tests. The tubes showing gas formation and the

presence of *Escherichia coli* were tested for determination of MPN for faecal coliforms. The tubes that only showed turbidity were also plated on MC agar plates to be tested for non-fermentative bacteria.

Examination of water samples for the presence of *Legionella* spp. Isolation of *Legionella* spp. was done as follows: Sterile water samples were used as negative control whereas sterile water samples inoculated with *Legionella* ATCC33152 (10^4-10^5 cfu/ml) were used as positive controls. Water samples of 1000 ml volume were passed through the cellulose nitrate membrane filters (Sartorius AG, Germany) with a pore size of 0.45 µm. Membranes were fragmented into small pieces while they were poured into 100 ml sterile plastic beaker with 50 ml original filtered water. This beaker was placed on a shaker at 37 °C for 30 min for releasing bacteria from the filter into the water. One ml was taken from each container and heat-treated at 50 °C for 30 min to inactivate the microorganisms other than *Legionella* spp. (16). Afterwards, 100 µl of each water sample was inoculated on a BCYE medium (Difco, USA) supplemented with glycine, vancomycin, cycloheximide and polymyxin B (GVPC). The plates were incubated under microaerophillic condition at 35 °C (90% humidity, 3% CO_2) for 7 days (16).

DNA Extraction from *Legionella* spp. for PCR amplification. DNA extraction was performed as previously explained (17). Conditions of PCR and size of the amplified fragments were as described by Hosseini et al. (18). DNA of *Legionella pneumophila* ATCC 33152 was used as the positive control. PCR products were analyzed by agarose gel electrophoresis using 1.5% agarose gel.

Air sampling. Two air samples from haemodialysis centre were weekly collected for 10 weeks. Airborne bacteria were sampled using a single stage Andersen air sampler (model Quick take 30 SKC, Scientific Co) which impacted air at a rate of 28.3 l/min for 10 min/day (30 min for *Legionella*) through a narrow slit. The air sampler was located approximately 100 cm away from the patient's bed (at the height of 91 cm). The air samples were collected for a period of 10 minutes. After each sampling, the culture plates were immediately transferred to the laboratory and incubated at 37 °C for 24-48 h. Trypticase soy agar (Conda, Spain) plates and cellulose nitrate membrane filters with a pore size of 0.45 µm were used to isolate the other bacteria and *Legionella* spp. After sampling, the filters were treated and cultured on BCYE agar plates. The number of grown colonies on each plate was recorded and the concentration of the airborne bacteria and *Legionella* spp. were calculated following the process of culturing in certain air volume (m^3). The results were expressed as colony-forming units per volume of sampled air. Finally, the mean levels of airborne bacteria in the haemodialysis centre were compared with the European Union Good Manufacturing Practices Guidelines ($\leq$ 1 cfu/m^3 in class A rooms and $\leq$ 100 cfu/m^3 in class C rooms) (19).

Statistical analysis. Statistical analysis was done using SPSS software (v. 16). The P value of < 0.05 was deemed as statistically significant.

RESULTS

A total of 50 water samples were taken from dialysis systems among which 24 samples (48%) showed bacterial contamination. *Legionella* spp. were counted based on colony forming units (cfu) in ml. The highest rate of *Legionella* contamination was observed in the storage tank (67 cfu/ml) whereas they were not isolated from the dialysate effluents. The total count of bacteria was obtained through MPN method. The most total bacterial count was related to the dialysate effluent (785 ± 185.3 MPN/ml, PV$\leq$ 0.05) and the maximum total count of coliforms was related to the reverse osmosis (48 ± 31.7 MPN/100ml, PV$\leq$ 0.05). In contrast, the minimum number of total bacteria and total coliforms was observed in the distribution loop (353 ± 107.9 and 0, respectively, PV$\leq$ 0.05) (Table 1).

Table 2 illustrated other bacteria isolated from water samples of dialysis systems. A great number of isolated bacteria were Gram-negative bacilli (mostly *Pseudomonas* spp.) which accounted for 48.3% of culture positive samples. Six samples were contaminated with coliforms. No faecal coliform was isolated from the samples. Frequency of Gram-positive cocci (mostly *Micrococcus* spp.) and Gram-positive bacilli (mostly *Bacillus* spp.) was 24.1 and 16.7%, respectively. The maximum and minimum bacterial counts were reported from dialysate effluents as well as distribution loops.

Legionella spp. were isolated from 4 cases (8%) using BCYE medium from reverse osmosis outlets (two cases), distribution loop outlet (one case) and storage tank (one case). No *Legionella* strain was isolated from the dialysate effluent. The most contamination rate of *Legionella* was observed in the water storage tank. Tracking of *16S rRNA* gene via PCR revealed five positive cases were the source of contamination which was confirmed by culture method as well. Merely one additional case in the storage tank found to be positive using PCR. Overall, 20 air samples were taken from the haemodialysis centre (Table 3). In these systems, the mean (± SD) of airborne bacterial colony count was 169.52 (± 29.09). A majority of the isolated bacteria were Gram-positive cocci which were mostly non-pathogenic. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the most important pathogenic bacteria isolated from the air samples. No *Legionella* was isolated from these samples. The mean (± SD) of airborne fungal colony count among the investigated samples was 30.7 (± 9.8) which were saprophytic molds mostly belonging to the *Aspergillus* genus.

DISCUSSION

The hospitalized patients undergoing haemodialysis treatment are exposed to large volumes of water in each haemodialysis session. Innovative procedures are being implemented to improve efficacy of haemodialysis treatment for patients suffer from end stage renal disease. However, they are prone to infections caused by various pathogenic agents (20). In the present study, most of the Gram-negative bacteria isolated from haemodialysis systems were non-fermentative bacteria. This finding is in consistent with other published studies (21, 22). These bacteria can easily grow in sterile distilled water and dialysate which may be due to the presence of bicarbonate and glucose in these samples (21). Two cases (8.3%) of *Burkholderia cepacia* were isolated from the dialysate effluents and the storage tanks. *Burkholderia* is an opportunistic respiratory bacterium with high rate of antibiotic

Table 1. Mean value of detected bacteria in the haemodialysis water system (P≤ 0.05)

	Sampling sites			
Detected bacteria	**Distribution loop**	**Reverse osmosis**	**Storage tank**	**Dialysate effluent**
Total bacteria (MPN/ml)	353 ± 107.9	431 ± 129.8	580 ± 146.2	785 ± 185.3
Total coliforms (MPN/100 ml)	-	48 ± 31.7	23 ± 11.5	21 ± 33.2
Legionella. spp. (CFU/ml)	53	47	67	-

Table 2. Isolated bacteria from water of haemodialysis centre

	No. (%)					
Isolated bacteria	**Raw water**	**Distribution loop**	**Reverse osmosis**	**Storage tank**	**Dialysate effluent**	**Total**
Gram positive cocci	2 (8.3)	-	1 (4.2)	1 (4.2)	2 (8.3)	6 (25)
S. aureus	-	-	1 (4.2)	1 (4.2)	1 (4.2)	3 (12.6)
Bacillus spp.	1 (4.2)	1 (4.2)	1 (4.2)	1 (4.2)	-	4 (16.7)
E. coli	-	-	1 (4.2)	1 (4.2)	1 (8.3)	3 (12.5)
P. aeruginosa	-	1 (4.2)	1 (4.2)	2 (4.2)	2 (8.3)	6 (25)
B. cepacia	-	-	-	1 (4.2)	1 (4.2)	2 (8.3)

Table 3. Bacterial and fungal air contamination in the haemodialysis centre

	S. aureus	*S. epidermidis*	*Micrococci spp.*	*S. viridans*	*P. aeruginosa*	*Aspergillus*	Mucor spp.	Penicillium spp.	Total
CFU/m^3	8.9 ± 4.47	47.5 ± 14.53	86.9 ± 21.7	13.1 ± 5.23	13.12± 4.45	12.9 ± 4.15	8.9 ± 3.12	8.9 ± 2.88	169.52 ± 29.09

resistance (23). Gram-positive cocci were the second important group of isolated bacteria. *Staphylococcus aureus* was the most common Gram-positive coccus. The coliforms isolated from the dialysis system were not from a faecal origin which was probably due to the environmental contamination of the salt and resins used in the dialysis water system. Diverse forms of bacteria isolated from dialysis water system in different studies may be related to different times of sampling, different methods of sterilization, inadequate disinfection of dialysis machines, burnout of dialysis water system and availability of various types of dialysis machines (21, 23, 24).

In the present investigation on dialysis water system, 4 *Legionella* isolates were grown on plates but 5 isolates were detected by PCR among which the fifth PCR-detected isolate was originated from storage tank. Negative culture result might be likely due to the difficulties in culturing this bacterium. All *Legionella* positive samples were isolated from water cycle before dialysis machine. When the dialysis machine is operating, the temperature increases up to 60-70 °C. According to the studies, most cases of *Legionella* have been isolated at lower temperatures (45-60 °C) (25). In the present study, a case of *Legionella* was also isolated from raw water. Given that the dialysis water system is connected to the hospital pipelines it is likely that *Legionella* strains enter into the haemodialysis water system through the hospital pipelines and colonize in different areas and form biofilms. It is believed that some *Legionella* strains are able to tolerate continuous treatments of disinfection. This tolerance is related to the biofilm production by *Legionella* in the dialysis water systems.

According to the European Union Good Manufacturing Practices guidelines, Haemodialysis centres are placed in the class C of air surveillance standards (≤ 100 CFU/m^3) (19). In the present study, the total mean (±SD) of airborne bacterial colony count in the haemodialysis centre was more than the standard value which indicates lack of suitable air conditions in the haemodialysis centre. The presence of *S. aureus* and *P. aeruginosa* in the air and dialysis water systems raises the possibility of bacterial recirculation in the physical milieu of the haemodialysis centre. The results of the total count of bacteria in the air and the pattern of isolated bacteria were consistent with other studies conducted in health centres and other wards such as Intensive Care Units (26-28).

Haemodialysis centres usually consume urban public water supply (8). Current conventional techniques applied in water treatment process in civic areas cannot considerably reduce the bacterial endotoxin. Therefore, pyrogenic reactions may occur in patients undergoing the dialysis treatment. Although urban public water supplies are chlorinated, they may possibly contain small amounts of Gram-negative bacteria. Dialysis water filtration systems effectively remove chlorine in the water. Water containing Gram-negative bacteria with the dialysate together can enhance overgrowth of bacteria in the absence of chlorine. Therefore, the reverse osmosis (RO) is being used for complete treatment of water used for dialysis. It is able to remove bacteria and bacterial endotoxin from the sources of water. However, small amounts of Gram-negative bacteria and non-tuberculosis mycobacteria in the water may pass through the filters or will colonize in the reverse osmosis unit (8).

The high viable agents detected in this study indicated that the microbiological quality of the haemodialysis water was lower than the limits recommended by the AAMI (29). After each period of disinfection, increased contamination was observed which was probably due to the bacterial biofilms generated in the water pipes. The worrisome finding in the present study was the presence of *Legionella* isolates along with non-fermentative bacteria in the haemodialysis water system which are capable of forming biofilms in the haemodialysis water system pipes. The presence of biofilm in the pipes makes bacteria to grow recurrently few hours after each disinfection period. It is recommended that the storage tank and the reverse osmosis membranes should be weekly disinfected rather than once a month to remove biofilms adequately. The accumulation of water in the reservoir holding tank is a good source for growth of bacteria.

CONCLUSION

Dialysis machine is an important source of contamination such as *Staphylococcus, Pseudomonas* and *Legionella.* Therefore an efficient prevention program is needed to eliminate bacterial contamination from the dialysis water system. Periodic surveillance programs for microbiological qualification in haemodialysis centres can also lead to a better planning for disinfection of haemodialysis water acknowledgment.

This study was supported by shahroud University of

Medical Sciences (grant No: 9012).

REFERENCES

1. Dadgari A, Dadvar L, Eslam-Panah H. Multidimensional fatigue syndrome and dialysis adequacy among elderly patients under hemodialysis treatment. *IJHS* 2015; 1:5-8.
2. Ebrahimi H, Sadeghi M, Amanpour F, Dadgari A. Influence of nutritional education on hemodialysis patients' knowledge and quality of life. *Saudi J Kidney Dis Transpl* 2016; 27:250-255.
3. Abbott KC, Agodoa LY. Etiology of bacterial septicemia in chronic dialysis patients in the United States. *Clin Nephrol* 2001; 56:124-131.
4. Locatelli F, Altieri P, Andrulli S, Bolasco P, Sau G, Pedrini LA, et al. Hemofiltration and hemodiafiltration reduce intradialytic hypotension in ESRD. *J Am Soc Nephrol* 2010; 21:1798-1807.
5. Berns JS, Tokars JI. Preventing bacterial infections and antimicrobial resistance in dialysis patients. *Am J Kidney Dis* 2002; 40:886-898.
6. Jackson BM, Beck-Sague CM, Bland LA, Arduino MJ, Meyer L, Jarvis WR. Outbreak of pyrogenic reactions and gram-negative bacteremia in a haemodialysis center. *Am J Nephrol* 1994; 14:85-89.
7. International Organization for Standardization (IOS). Water for haemodialysis and related therapies; ISO 13959. IOS; Geneva, Switzerland Apr 15, 2009.
8. Ward RA. Worldwide guidelines for the preparation and quality management of dialysis fluid and their implementation. *Blood Purif* 2009; 27:2-4.
9. O'Neill E, Humphreys H. Surveillance of hospital water and primary prevention of nosocomial legionellosis: what is the evidence? *J Hosp Infect* 2005; 59:273-279.
10. Colbourne JS, Dennis PJ, Trew RM, Berry C, Vesey G. *Legionella* and public water supplies. *Water Sci Technol* 1998; 20:5-10.
11. Palusińska-Szysz M, Cendrowska-Pinkosz M. Pathogenicity of the family Legionellaceae. *Arch Immunol Ther Exp* (Warsz) 2009; 57:279-290.
12. Yaslianifard S, Mobarez AM, Fatolahzadeh B, Feizabadi MM. Colonization of hospital water systems by *Legionella pneumophila, Pseudomonas aeroginosa,* and Acinetobacter in ICU wards of Tehran hospitals. *Indian J Pathol Microbiol* 2012; 55:352-356.
13. Kusnetsov J, Torvinen E, Perola O, Nousiainen T, Katila ML. Colonization of hospital water systems by legionellae, mycobacteria and other heterotrophic bacteria potentially hazardous to risk group patients. *APMIS* 2003; 111:546-556.
14. Department of environment and heritage protection, Queensland government (2009). Monitoring and sampling manual. Environmental protection (Water) policy. Version 2, July 2013 format edits.
15. Sutton S. The most probable number method and its use in QC microbiology. *J GXP Compliance* 2010; 14:28-33.
16. Veronesi L, Capobianco E, Affanni P, Pizzi S, Vitali P, Tanzi ML. *Legionella* contamination in the water system of hospital dental settings. *Acta Biomed* 2007; 78:117-122.
17. Miyamoto H, Yamamoto H, Arima K, Fujii J, Maruta K, Izu K, et al. Development of a new seminested PCR method for detection of *Legionella* species and its application to surveillance of legionellae in hospital cooling tower water. *Appl Environ Microbiol* 1997; 63:2489-2494.
18. Hosseini Doust R, Mohabati Mobarez A, Esmailli D. Detection of *Legionella* in hospital water supply using *Mip* based primers. *J Biol Sci* 2008; 8:930-934.
19. EudraLex (2010). "The rules governing medicinal products in the European Union (EU)" (Vol 4), Guidelines of Good Manufacturing Practices for medicinal products for human and veterinary use (http://ec.europa.eu/health/documents/eudralex/vol-4/index_en.htm).
20. Hoenich NA, Levin R. The implications of water quality in hemodialysis. *Semin Dial* 2003; 16:492-497.
21. Pisani B, Simöes M, Prandi MAG, Rocha MMM, Gonçalves CR, Vaz TMI, et al. Outbreak of *Pseudomonas aeruginosa* bacteremia in a hemodialysis center in Campinas, Säo Paulo, Brazil. *Rev Inst Adolfo Lutz* 2000; 59:51-56.
22. Zunino P, Beltrán L, Zunino L, Méndez H, Percovich V, Rocca R, et al. Microbiological quality of hemodialysis water in a three-year multicenter study in Uruguay. *J Nephrol* 2002; 15:374-379.
23. Magalhães M, Doherty C, Govan JR, Vandamme P. Polyclonal outbreak of *Burkholderia cepacia* complex bacteraemia in haemodialysis patients. *J Hosp Infect* 2003; 54:120-123.
24. Arvanitidou M, Vayona A, Spanakis N, Tsakris A. Occurrence and antimicrobial resistance of Gram-negative bacteria isolated in haemodialysis water and dialysate of renal units: results of a Greek multicentre study. *J Appl Microbiol* 2003; 95:180-185.
25. Hrubá L. The colonization of hot water systems by *Legionella*. *Ann Agric Environ Med* 2009; 16:115-119.
26. Nunes ZG, Martins AS, Altoe AL, Nishikawa MM, Leite MO, Aguiar PF, et al. Indoor air microbiological evaluation of offices, hospitals, industries and shopping centers. *Mem Inst Oswaldo Cruz* 2005; 100:351-357.
27. Huang PY, Shi ZY, Chen CH, Den W, Huang HM, Tsai JJ. Airborne and surface-bound microbial contamination in two intensive care units of a medical center in central Taiwan. *Aerosol Air Qual Res* 2013; 13:1060-

1069.
28. Mirzaii M, Emaneini M, Maleknejad P, Jonaidi N, Fooladi AA, Aligholi M, et al. Distribution of bacterial contamination in a teaching hospital in Tehran-a special focus on *Staphylococcus aureus*. *Acta Microbiol Immunol Hung* 2012; 59:1-11.
29. Association for the advancement of medical instrumentation (AAMI). Water treatment equipment for haemodialysis applications and related therapies. ANSI-AAMI project RD62: 2006.