JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov 2015. Vol. **9**(Spl. Edn. 2), p. 437-441

Inhibition of T4ss Gene *Legionella pneumophila* with Catalytical Ozonization

Masomeh Gholinezhad[1], Davoud Esmaeli[2]* and Ghader Ghanizadeh[3]

[1]Department of Biology, Urmia Branch, Islamic Azad University,Urmia, Iran.
[2]Department of Microbiology, Faculty of Medicine,
Baqiyatallah University Medical of Sciences, Tehran, Iran.
[3]Health Research Center, and Department of Environmental Health, Health School,
Baqiyatallah University of Medical Sciences, Tehran, Iran.

(Received: 10 May 2015; accepted: 20 July 2015)

Legionella **bacteria are aerobic gram-negative rods associated with respiratory infections. Of the 52 known species legionella , 20 have been linked to pneumonia infections in humans. The species *L.pneumophila* (particularly serogroups 1-6) has been accepted as the principal cause of human outbreaks of legionellosis, which includes both legionnaires, disease and Pontiac fever. Legionella are ubiquitous in natural aquatic environments, capable of existing in waters with varied temperature, PH levels, and nutrient and oxygen contents. Contamination by legionella has occurred in the distribution systems of many hospitals. Their widespread survival in nature can be attribute to their relationships with other microorganisms in the environment. Symbiotic existence with algae and other bacteria , particularly in biofilms, increases the availability of nutrients. They also are able to infect protozoans and subsequently reproduce within these organisms. These relationships provide protection against adverse environmental conditions, including standard water disinfection techniques. Consequently, legionella are also prevent in anthropogenic waters such as potable water, cooling tower reservoirs, water distribution systems and whirlpools. Aerosol- generating systems such as fauceta, shower heads, cooling towers, and nebolizers aid in the transmission of legionella from water to air. Human inhalation of contaminated aerosols leads to legionella infections and disease outbreaks. Collection of legionella was done from hospital water .These samples are typically concentrated by filtration, treated with an acid buffer and temperature , and isolated on a BCYE agar culture medium. Legionella pneumophila was treated with concentration of MIC catalytical ozonation then evaluated t4ss gene expression by RT-PCR technique. The results indicated that catalytical ozonation have inhibitory effects on virulence genes *Legionella pneumophila*. So we can use it as an active disinfectant in hospital distribution systems.**

Key word: *Legionella pneumophila*, RT-PCR, Catalytical Ozonization, T4ss gene silencing.

The genus *Legionella* is a pathogenic group of Legionellaceae family, that includes the 52 species Legionella *and L. pneumophila* is cause of 70% Legionellosis disease.Legionella may be readily visualized with a Dietherl silver stain. *Legionella* is ubiquitic aquatic microorganism in many environments, including soil and aquatic systems, with at least 50 species and 70 serogroups identified[1, 2].

Legionella pneumophila in the water distribution systems have been epidemiologically related to nosocomial Legionellosis disease[3]. Many of hospitals in world have been implemented disinfection procedures for their water distribution systems. The two methods most widely are used contain of Ozonation and Copper&Silver ionization. Both methods have proven effective in eradicating

* To whom all correspondence should be addressed.
E-mail: esm114@gmail.com

L. pneumophila[4]. Ozone is a very potent oxidizing disinfectant with appropriate biocidal activity against *Legionella*[4]. Ozone is widely used in many countries for water supply and swimming pool disinfection at concentrations of 0.1 to 0.5 mg/liter[4].

Copper-silver ionization is used by control systems for *Legionella* eradication and prevention. For disinfection, copper and silver ion concentrations must be maintained at optimal levels in order to eradication *Legionella*. The function of disinfection within all of a facilities water distribution networks will occur within 30 to 45 days. Engineering evaluation such as 10 amps per ion chamber cell and automated variable voltage outputs having no less than 0–100 VDC are but only a few of the required features for proper *Legionella* control and prevention, using a specific, CuAg system. Swimming pool ion generators are not designed for potable water treatment. Facility or public water system using CuAg ionization for disinfection should monitor their copper and silver ion concentrations to ensure that they are within intended levels - both minimum and maximum. Further, there are no recent standards for silver in the EU and other regions utilizing this technology. CuAg ionization is an effective process to control *Legionella* in potable water distribution systems. CuAg ionization is not proper for cooling towers because of pH levels over 8.6 that cause ionic copper to precipitate[5, 6, 7].

Because hospital drinking water systems is a reservoir of infectious outbreaks occurring via microbial pathogens, the CDC recommended the disinfected water for prevention of these potential risks in critical activities including intensive care units (ICUs) and transplant recipient ward for drinking and oral hygiene[8,9].

Increase Legionella as an opportunistic pathogen in hospital water line systems (>30%) lead to increasing rate of community- acquired pneumonia (CAP) related to inhalation of treated drinking water aerosols containing *Legionella*[10].

In Iran over than 30% of hospital's water systems contaminated with *legionella*, the increasing risk of health care-acquired LD should be considered[11].

Several chemical or physical processes such as chlorination, ozonation, ultraviolet (UV) , thermal disinfection and CU^{2+}/Ag^{+} ionization have been used for the eradication of Legionella but any method have some drawbacks therefore is essential study appropriate disinfection techniques[12].

The main objective of this study was to evaluation of the catalytical ozonation process with copper as a promising technique for the gene silencing of *L.pneumophila* from hospital water lines in order to improve prevention measures. In this experimental research the efficacy of catalytical ozonation process was verified via stude gene expression T4SS gene and comparison it with housekeeping mip gene by RT-PCR technique.

MATERIALS AND METHODS

Ozone Process

The experimental set up which consisted of a Pollexy glass reactor, which was Ø3 cm× 80 cm. A quantity of 1000 mL *Legionella* contaminated water with a different initial density was used for each run, and small liquid samples were taken online periodically by sterile bottles from the bottom of the reactor to determine the viable residual bacteria. Temperature control was done via immersion the reactor onto an external thermostat batch controlled jacket. Ozone was produced from dry pure oxygen which passed from the silicagel bed and using an ARDA ozone generator. This was rated at 5 g O3/ h as the maximum generation capacity[4].

Disinfectant Susceptibility Test

Susceptibility to catalytical ozonation as applied disinfection procedure was determined by broth dilution method in accordance with Clinical Laboratory Standard Institute (CLSI) guidelines[14].

Determination of Minimum Inhibitory Concentration (MIC)

MIC values were determined by broth macro dilution assay recommended by the CLSI 2014.To determine the MIC for the catalytical ozonation and ozonation processes $1_{*}10^{5}$ CFU *L. pneumophila* were inoculated into system then varied ozon concentrations were injected. Then, in order to growth survey, *L. pneumophila* was cultured in BCYE agar and incubated at 37°C up to 5 days within 2.5 % of CO_2[14].

Design of Primers

Specific primers for t4ss, ss and mip genes were designed using Genscript software (GenScript Real-time PCR (TaqMan) Primer Design) (Table 1).

Table 1. The sequence of used primers

Primer	Sequence	Amplicon
F_{mip}	GTCAACAG CAATGGCTGCAA	242bp
R_{mip}	CAGCAGTACGCTTTGCCATC	
F_{t4ss}	GTGTGGTGTAGGCTGGTTTG	84 bp
R_{t4ss}	CTAACCCAGAAGTGCCGATT	

RT-PCR

When MIC was determined the bacterium was candidate for gene expression. Then, $mRNA_s$ were isolated from none exposed and exposed bacteria (with catalytical ozone and ozone) as case and control repectively, according to the manufacturer's protocol (Cinnagen). The samples's cDNAs were synthetized and the alterations in the expression level of T4ss, ss and mip gene investigated.

Gene expression was followed by RT-PCR method (Corbet themocycler). Briefly; the reactions were conducted within 3 minutes at 95°C (1 cycle), 30 seconds at 95 °C (35 cycles), 30 seconds at 56 °C (35 cycles), 1 minute at 72 °C (35 cycles) and 10 minutes at 72°C for final extension. The housekeeping mip gene was used as an internal control[15].

RESULTS

Cataltyical ozonation were effective against *L.pneumophila* T4ss gene in the range of MIC=0.5¼g/ml. The efficacy of these processes on *L.pneumophila* eradication mechanism was approved with t4ss gene expression. The findings demonstrate that t4ss gene expression remarkably decreased after treatment via cataltyical ozonation disinfection processes while, housekeeping mip gene expression as a reference gene was not decreased.

CONCLUSION

Legionella is aerobic gram-negative bacil associated with respiratory infections. Of the 52 known species legionella, 20 species have been linked to pneumonia infections in humans. The species *L.pneumophila* (particularly serogroups 1-6) has been proved as the principal cause of human outbreaks of legionellosis, which includes legionnaires, disease and Pontiac fever[16]. Legionella are ubiquitous in natural aquatic environments, capable of existing in waters with varied tempreture,PH levels,and nutrient and oxygen contents. Contamination by legionella has occurred in the distribution systems of many hospitals. Their widespread survival in nature can be attributing to their relationships with other microorganisms in the environment. Symbiotic existence with algae and other bacteria, particularly in biofilms, increases the availability of nutrients. They also are able to infect protozoans and subsequently reproduce within these organisms. These relationships provide protection against adverse environmental conditions, including standard water disinfection techniques[17, 18].

Consequently, *legionella* are also preventing in anthropogenic waters such as potable water, cooling tower reservoirs, water distribution systems and whirlpools. Aerosol-generating systems such as faucet, showerheads, cooling towers, and nebolizers aid in the transmission of legionella from water to air. Human inhalation of contaminated aerosols leads to legionella infections and disease outbreaks[19].

L.pneumophila control is difficult to treat with existing disinfectants, but may in addition develop resistance after unsuccessful treatment. Thus, it is considered as an increasing threat to the community. The intrinsic antibiotic resistance of *L.pneumophila* is associated with the limited permeability of bacteria's outer membrane, over expression of efflux pumps. The use of innovation disinfectants to treat water contamination is common in many countries. Ozonation has been used as disinfection since the ancient times. Catalytical ozonation is one of the major compounds, which is easily enabling destruct this organism. Moreover, Cu catalytical ozonation could be attributed to the presence of this agent. Numerous studies have been published on ozonation and CU-Ag-ionization However, the influence of catalytical ozonation on the expression of T4ss has not yet been investigated and this is the first study reporting the inhibitory effect of this compound against this genes. Based on the results obtained in this study, this compound had an impeding role on T4ss by reducing the expression[19,20,21] . In the current study, RT-technique was applied because it is a rapid and

highly applicable technique for evaluating the expression profile of the target gene(s) and provides qualitative or semi quantitative information of mRNA levels. However, further studies are required to quantify the expression of the studied genes and identifying similar medicinal herbs that can block efflux and thus extend the life of existing antibacterial drugs could be beneficial[22,15].

In summary, our data suggest that catalytical ozonation may provide suitable compounds for clinical utility as inhibitors of secretory systems for *L. pneumophila*. According to results and due to the high resistance to disinfectants in *L. pneumophila* and high prevalence of nosocomial infections and enormous economic costs and the restrictions on the use of broad-spectrum disinfectants in hospital applications of native compounds against these pathogens resulted in these which can be effective enough to reduce the rate of virulence genes and consequence infection transmission.

The results indicated that catalytical ozonation have inhibitory effects on virulence genes *Legionella pneumophila*. So we can use it as an active disinfectan in hospital distribution systems.

REFERENCES

1. Lee HK, Shim JI, Kim HE, Yu JY, Kang YH. Distribution of *Legionella* species from environmental water sources of public facilities and genetic diversity of L. pneumophila serogroup 1 in South Korea. *Applied and environmental microbiology.* 2010; **76**(19):6547-6554.
2. Hsu SC, Martin R, Wentworth BB. Isolation of *Legionella* species from drinking water. *Applied and environmental microbiology.* 1984; **48** (4): 830-832.
3. Gruas C, Llambi S, Arruga MV. Detection of Legionella spp. and Legionella pneumophila in water samples of Spain by specific real-time PCR. *Archives of microbiology.* 2014; **196**(1):63-71. PubMed PMID: 24264468. Epub 2013/11/23. eng.
4. Edelstein, P. H., R. E. Whittaker, R. L. Kreiling, and C. L.Howell. Efficacy of ozone in eradication of Legionella pneumophila from hospital plumbing fixtures. *Appl. Environ.Microbiol.* 1982; **44**:1330-1334.
5. Stout, Janet E., PhD; Yu, Victor L., MD . "Experiences of the First 16 Hospitals Using Copper-Silver Ionization for *Legionella* Control: Implications for the Evaluation of Other Disinfection Modalities". *Infection Control and Hospital Epidemiology.*2003*;* **24**(8): 563–568.
6. Lin YE, Stout JE, Yu VL. Disinfection of water distribution systems for *Legionella*. *Semin Respir Infect* 1998; **13**:147-159.
7. Landeen LK, Yahya MT, Gerba CP. Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of *Legionella pneumophila*. *Appl Environ Microbiol* 1989; **55**: 3045-3050.
8. Huang SW, Hsu BM, Wu SF, Fan CW, Shih FC, Lin YC, *et al*. Water quality parameters associated with prevalence of *Legionella* in hot spring facility water bodies. *Water research.* 2010; **44**(16):4805-4811.
9. O'Neill E, Humphreys H. Surveillance of hospital water and primary prevention of nosocomial legionellosis: what is the evidence? *The Journal of hospital infection*. 2005; **59**(4): 273–279.
10. Tai J, Benchekroun MN, Mekkour M, Ennaji MM, Nader H, Cohen N. Investigation of *Legionella Pneumophila* in Hot Water Systems in Morocco. *International Journal of Science and Technology*. 2012; **1**(10):524-530.
11. Mojtaba mobarez A, Hosseini doust SR, Esmaili D. Identification of legionella in hospital water networks and ventilation systems. *Iranian Journal of Infectious Diseases and Tropical Medicine*. 2007; **12**(36): 33-37 .
12. Allegra S, Grattard F, Girardot F, Riffard S, Pozzetto B, Berthelot P. Longitudinal evaluation of the efficacy of heat treatment procedures against *Legionella* spp. in hospital water systems by using a flow cytometric assay. *Applied and environmental microbiology.* 2011; **77**(4):1268-1275.
13. Standards NCfCL. Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standards: National Committee for Clinical Laboratory Standards; 2006.
14. CLSI. Performance standards for antimicrobial susceptibility test-ing; twenty-first informational supplement. M100-S21. Wayne, PA: Clinical and Laboratory Standard Institute; 2014.
15. Ghotaslou R, Sefidan FY, Akhi MT, Soroush MH, Hejazi MS. Detection of Legionella Contamination in Tabriz Hospitals by PCR Assay. *Advanced pharmaceutical bulletin.* 2013; **3**(1):131.
16. Jalila T, Benchekroun MN, Ennaji MM,

Mekkour M, Cohen N. Nosocomial *Legionnaires*' disease: Risque and prevention. *International Journal of Environmental Sciences and Research.* 2012; **1**(3):72-85.

17. Bargellini A, Marchesi I, Righi E, Ferrari A, Cencetti S, Borella P, *et al*. Parameters predictive of *Legionella* contamination in hot water systems: Association with trace elements and heterotrophic plate counts. *Water research*. 2011; **45**(6):2315-2321.
18. Association APH. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, DC. 1998;1268.
19. Allegra S, Grattard F, Girardot F, Riffard S, Pozzetto B, Berthelot P. Longitudinal evaluation of the efficacy of heat treatment procedures against *Legionella* spp. in hospital water systems by using a flow cytometric assay. *Applied and environmental microbiology*. 2011; **77**(4):1268-1275.
20. Fields BS, Benson RF, Besser RE. *Legionella* and *Legionnaires'* Disease: 25 Years of Investigation. *Clinical microbiology reviews.* 2002; **15**(3): 506–526.
21. Serrano-Suarez A, Dellunde J, Salvado H, Cervero-Arago S, Mendez J, Canals O, et al. Microbial and physicochemical parameters associated with Legionella contamination in hot water recirculation systems. *Environmental science and pollution research international.* 2013; **20**(8):5534-44. PubMed PMID: 23436060. Epub 2013/02/26. eng.
22. Descours G, Cassier P, Forey F, Ginevra C, Etienne J, Lina G, et al. Evaluation of BMPA, MWY, GVPC and BCYE media for the isolation of Legionella species from respiratory samples. *Journal of microbiological methods.* 2014; **98**:119-21. PubMed PMID: 24462808. Epub 2014/01/28. eng.