International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319 – 6505, Impact Factor: SJIF: 5.995
Available Online at www.journalijcar.org
Volume 6; Issue 8; August 2017; Page No. 5637-5641
DOI: http://dx.doi.org/10.24327/ijcar.2017.5641.0767

Research article

DETECTION OF LEGIONELLA PNEUMOPHILA IN WATER OF SOME STATIONS ON HIGHWAY ROADS TO MAKKAH

Al Harbi N.A.S*., Al Zahrani S.H.M and Elbeeh N. Z

Faculty of Biological Science, Department of Microbiology, King Abdul-Aziz University, Jeddah, KSA

A R T I C L E I N F O

Article History:

Received 25th May, 2017
Received in revised form 13th June, 2017 Accepted 20th July, 2017
Published online 28th August, 2017

Key words:

Highway water, Makkah, *Legionella pneumophila*, Legionaries disease, pH, Antibiotic susceptibility

A B S T R A C T

The highway roads leading to Makkah are used by thousands of pilgrims every year because of the location of holy sites as a result of which the water running in restaurants located on these highways is largely consumed. This water is susceptible to be contaminated by Legionella pneumophila, a pathogenic bacterium that causes pneumonic illness called Legionaries disease, which affects the respiratory system. This study investigated the presence of pathogenic species of Legionella especially L .pneumophila in roadside water on way to Makkah. A total of 19 water samples were collected from different sites. Parameters like colony count, biochemical tests for isolation, ability to grow in different pH ranges, effect of iron concentration and antibiotic susceptibility of the bacterium were tested. Bacterial *isolates* were found in water samples except for three samples that were heated at 55°C for 15 min. In all water samples pH value ranged from 8.9 to 8.2 except for one sample which was 3.3. Colonies were most susceptible to Rifampicin. Water was found to be contaminated by L. pneumophila with specific characteristics. Hence, by using sanitation and clean water systems, pilgrims especially elderly people who have chronic diseases and weak immune exhibition of injury could be protected from getting infected by L. pneumophila

INTRODUCTION

Legionella pneumophila, a flagellated monopolar, gram-negative bacteria is the pathogen associated with pneumonia, both community and hospital acquired, especially in immune-compromised patients (Fields BS, 2002; Sabria M, 2006). It replicates within the protozoa and algae in the water system (Bartram J, 2007). Being highly prevalent in the water, *Legionella pneumophila* can be immensly threatful for public health. Pneumonia because of *Legionella* could occurs by two route; either by inhalation of infected aerosol particles or by exposure to contaminated water emitted by showers, taps and cooling towers. For the presence of this bacterium, temperature is the main factor, which is able to survive at temperatures upto 60°C. It has been reported that the most frequent source of infection involved hot water systems (Meenhorst PL *et al*, 1985; Berthelot P *et al*, 1998; Perola O *et al*, 2002).

When Muslims visit Makkah for Hajj, there occur huge gatherings, which make one more susceptible to catching respiratory infections. Studies have pointed towards an increase in the outbreak of nosocomial Legionnaires' disease in recent years (Chien, M *et al*, 2004). A few studies have been conducted in Saudi Arabia that have investigated the presence of Legionella sp. in cooling water tower and drinking water (Hatem Q, 2012; Esam IA *et al*, 2010). To the best of our knowledge, till date, there has been no epidemological study in Saudi Arabia that would determine the origin, characteristics and prevalence of this organism in water available for consumption along highway. Hence, this study aims to evaluate the prevalence and characteristic of *L. pneumophila* in water on highways leading to Makkah.

Corresponding author:* **Al Harbi N.A.S
Faculty of Biological Science, Department of Microbiology, King Abdul-Aziz University, Jeddah, KSA

MATERIALS AND METHODS

Water Samples

Water samples were collected from 19 stations on the highway roads to Makkah in five months. 5 Samples of water per station were collected from taps in sterile plastic bottles. Prior to collection, the tap was cleaned by a sponge to remove dirt and left open for two minutes followed by closing and reopening it for another two minutes. The sterilized flask was filled with water, space was left in bottles for air to ease shaking during inoculation and bottle was tightly closed. Water samples were kept in the refrigerator at 4°C until bacteriological analysis. Water samples were separated into two groups (A) and (B), group A was treated with heat while group B was left at the normal temperature.

Heat treatment of water samples

Group A water samples were treated at 55°C for 15 min in water bath to decrease the varieties of microorganisms in the sample (Qin T *et al*, 2013).

Test the presence of host organisms in water samples

All procedures used were approved by the King Abdulaziz City for Science and Technology (KACST) (Ethical Issue No:124-36-أط). 10ml of water sample was centrifuged at 4000 rpm for 30 min. A drop of water was taken on slide, covered with a glass cover and scanned by direct light microscopy at10× and 40×microscopic last view. Observations were taken in wet to test the presence of any pathogenic organism (magnification, × 40) (Bopp CA *et al*, 1981).

pH estimation

The probe and meter of instrument were calibrated following the manufacturer specifications and the pH value of sample water was taken.

Iron analyses

The water samples were send to Al-Mamlakka laboratory for estimating the iron content in them according to their standard protocol. The normal required value of iron content in water was taken as 0.3 mg/l as per standardization by the laboratory (Al-Mamlakka laboratory).

Impregnated antibiotic assay discs

The water samples were subjected to antimicrobial susceptibility testing using Erythromycin (E) 15 µg, Gentamicin (CM) 10 µg and Rifamycin (RA) 5 µg.

Estimation of bacteria in water samples

The samples were serially diluted in normal saline solution from (1×10^{-1}) to (1×10^{-4}) and 0.1 ml from each dilution was inoculated into Nutrient Agar Medium (CM0309) plates then incubated at 30°C in CO_2 incubator. The plates were examined after 24-48 hours of incubation. To detect *Legionella*, 75 ml of the water samples were centrifuged at 4000 ×g for 30 min and 0.1 ml from each was plated on GVPC agar (Oxoid, United Kingdom) following which the plates were subjected to incubation at 37°C for 72h. Colonies were identified as *Legionella* by using L-cysteine (gram staining).

Culture and morphological characteristics

All water samples were isolated on Buffered Charcoal Yeast Extract Agar BCYE-α, Charcoal Yeast Extract Agar CYE agar without L-cysteine, Nutrient Agar, and Blood Agar. All plates were examined every two or three days under dissecting microscope for the suspected *L. pneumophila* colonies with a cut-glass surface.

Antibiotic susceptibility

These isolates were tested for susceptibility to some common antibiotics and followed the way Bauer-Kirby Amended (Wikins, T.D *et al*, 1972) adopted by the World Health Organization (Vandepitte J, 2003) to conduct the test by using Mueller-Hinton agar. Susceptibility test was performed using antibiotics Rifampicin, Gentamicin, and Erythromycin. Colonies were suspended to a concentration of 107 CFU/ml in sterile water and adjusted to 0.5 McFarland. A swab was then dipped in the suspension and inoculated on Mueller-Hinton agar plates. The plates were then incubated at 37°C for 72 hours with increased humidity.

RESULTS

The maximum number of bacterial isolates found in the non-heated water sample was 63×10^{3}CFU/mL, while in the sample treated by heat at 55°C for 15 minutes, it was 45×10^{3}CFU/ml. However, no bacterial isolates were found in three samples that were heated at 55°C for 15 minutes. All the samples were tested by the standard culture method and colonies appeared as shiny gray color (Figure 1). An important characteristic of these colonies was that they were found to grow only on buffered charcoal yeast extract (BCYE) agar with cysteine but could not grow on Charcoal Yeast Extract Agar without cysteine, Nutrient Agar, and Blood Agar.

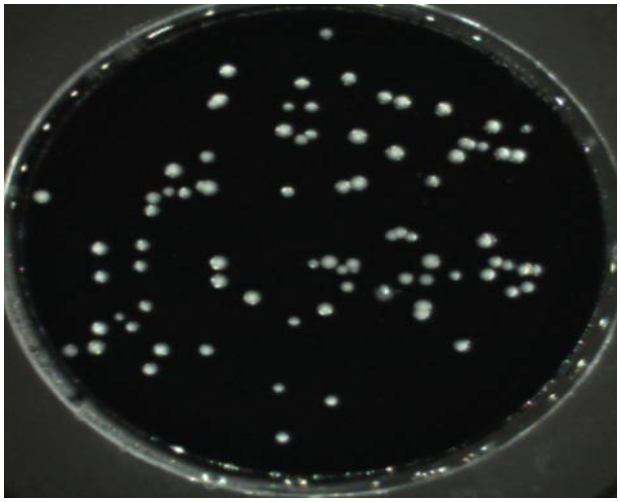

Figure 1 Appearance of colonies by standard culture

Samples in which the colonies were obtained were further tested for a primary identification by studying the key features and biochemical tests which appeared as a gram-negative poorly staining rods. The range represented as CFU/mL of *L. pneumophila*, *Legionella* spp. and prevalent colonies that were isolated from water samples are presented in Table 1.

Table 1 Count of *L. pneumophila* and *Legionella* spp. Group A includes water samples treated by heat and Group B indicates non-heated water samples.Values are presented as CFU/ml

Sample	*Legionella pneumophila*	*Legionella* spp.
Group (A)	31.5% (7/19)	68.4% (13/19)
Group (B)	84.2% (16/19)	100% (19/19)

Samples in which *L. pneumophila* was detected, 16 of them were direct samples and 7 were indirect. In both direct and indirect samples, *L. pneumophila* was detected after the heat treatment as well. By using the standard culture method, all positive samples from group (A) that had been treated with heat showed count less than the samples not treated with heat. Microscopic testing for other organisms showed the presence of 31.75% of algae and 5.62% of protozoa associated with this bacteria. The pH of the studied samples ranged between 8 to 8.9 except for one sample being 3.3 (Figure 2). When the pH was out of this range, isolation rates varied significantly. However, most of the tap water samples fell into the alkaline pH range.

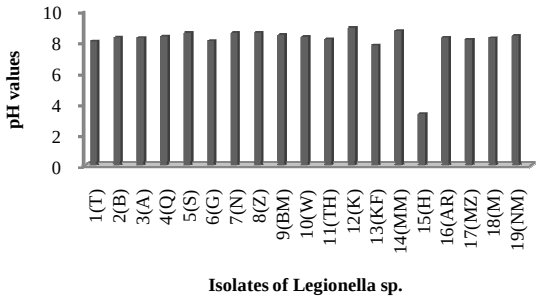

Figure 2 The relationship between pH value of water samples and presence of *Legionella* sp. Values are represented as numbers

The effect of iron on the presence of *L. pneumophila* was less clearly defi defined. The highest level of iron observed in water samples was 0.09 m/g whileas the lowest level was 0.01 m/g. Also no isolated *Legionella* from the water samples which contain iron level less than 0.01 m/g was detected.

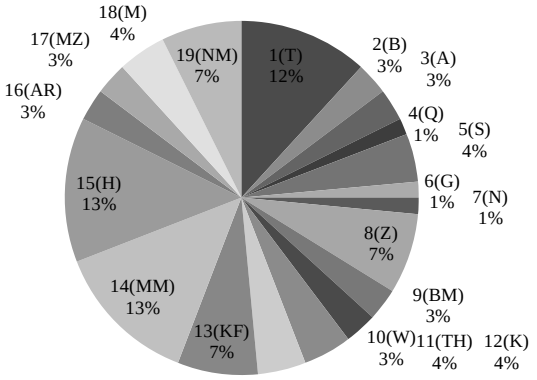

Figure 3 Effect of presence of iron on *Legionella* sp. The pie-chart represents prevelance of colonies of *Legionella* sp. with iron. All values are represented as percentages.

The isolates of *L. pneumophila* were highly sensitive to antibiotic Rifampicin, Gentamycin, and Erythromycin as summarized in Tables 2 and 3.

Table 2 Antibiotics susceptibility of *L. pneumophila* isolated from heated water samples. Values are represented in mm of area inhibited; * $p \leq 0.05$

Isolates	Symbol	Antibiotics	Diameter area antagonism (mm)
	(E)	Erythromycin	16.3*
1 (T)	(CN)	Gentamycin	10.4
	(R)	Rifampicin	15.6*
	(E)	Erythromycin	9.60*
2 (B)	(CN)	Gentamycin	11.5
	(R)	Rifampicin	10.4*
	(E)	Erythromycin	17.4*
3 (S)	(CN)	Gentamycin	22.2
	(R)	Rifampicin	24.8*
	(E)	Erythromycin	26.3*
4 (Z)	(CN)	Gentamycin	9.60
	(R)	Rifampicin	35.6*
	(E)	Erythromycin	28.9*
5 (AR)	(CN)	Gentamycin	12.6
	(R)	Rifampicin	12.2*
	(E)	Erythromycin	16.3*
6 (MZ)	(CN)	Gentamycin	10.4
	(R)	Rifampicin	15.6*
	(E)	Erythromycin	18.2*
7 (M)	(CN)	Gentamycin	16.7
	(R)	Rifampicin	18.9*

All isolates were inhibited by low concentrations of macrolides. In detail, isolates No.2 and 13 showed no antibiotic effect for Erythromycin. Whereas, isolates No.12 and 14 demonstrated a high response to Erythromycin with inhibition zone 29.6 mm and 29.3 mm respectively. However, the diameter reached 10.4 mm for isolates 7, 8, 15 and 16 respectively. Isolate No.3 had the highest response to Erythromycin with 37.8 mm inhibited zone. Isolates No. 5 and 16 showed a high susceptibility to antibiotic Gentamycin with inhibition zone 31.5 mm, and 29.6 mm respectively. Whereas, isolates 1, 3, 7, 10, 14 and 16 were showed a response to Erythromycin with inhibition zone ranged from 11.1 mm to 14.1 mm. Isolate No.2 and 9 had no response to antibiotic Gentamycin. However, isolates 1, 3, 7,10, 9, 12,14 and 16 were susceptible to Rifampicin with inhibited zones 21.5 mm,10 mm, 10 mm, 11.9 mm, 10 mm, 27.8 mm, 11.9 mm and 17 mm respectively. However, isolate 2 showed no susceptibility for Rifampicin. In general, gentamycin was the least active agent for all the isolates. Rifampicin and Erythromycin were found to have the most antimicrobial activity against all isolates.

DISCUSSION

An association of pneumonia due to *Legionella* and environmental samples has been documented in many studies but few such studies have been conducted in Makkah so far (Al-Ghamdi SM *et al*, 2003). A detailed study based on isolation, characterization, prevalence and antibiotic resistance of *Legionella* sp. from water bodies is the need of the hour in order to keep up with the good health of the pilgrims visiting the holy city of Makkah. In this study, we tried to carry a detailed investigation about the prevalence and characteristics of the pathogenic species of *Legionella* in the water being consumed on highway roads. *L. pneumophila* colonies were found to occur in both heated as well as non-heated water samples. The identification of *L. pneumophila* obtained from both groups of samples using chemical methods such as catalase and oxidase test gave weak results. This finding is in line with results obtained by Horwitz and Maxfield (Horwitz MA and Maxfield FR, 1984). They described that the members of the genus are relatively inert biochemically, catalase-positive (some may be only weakly catalase-positive), oxidase-variable and possess polar flagella.

Legionella comes into contact with cyanobacteria in open water sources, both natural, such as lakes and rivers, and engineered systems such as cooling towers. The lack of algae in many anthropogenic systems does not diminish the possibility that *Legionella* employs a similar survival technique of associated growth with other biofilm flora, be it fungal, bacterial, or protozoan (Tison DL *et al*, 1980). Legionella's ability to grow in association with cyanobacteria present in heated water systems that are exposed to light was demonstrated in this study as well. Definite range of physicochemical attributes are responsible for putting constraints on the growth and survival of all organisms. One of the important properties is pH of water, which is considered to have a major impact on contamination by *L. pneumophila*. This study indicated that pH values of water samples were between 8 and 8.9 that is in agreement with OSHA, United States Occupational Safety and Health Administration pH range supporting growth of *Legionella* (OSHA, 2017).

Table 3 Antibiotics susceptibility of *L. pneumophila* isolated from non-heated water samples. Values are represented in mm of area inhibited; * $p \leq 0.05$

Symbol	Antibiotics	Isolates of L. pneumophila															
		1 (T)	2 (B)	3 (A)	4 (Q)	5 (S)	6 (G)	7 (N)	8 (Z)	9 (TH)	10 (W)	11 (K)	12 (MM)	13 (H)	14 (AR)	15 (MZ)	16 (M)
		Diameter of inhibition zone (mm)															
(E)	Erythromycin	14.4	0.00	37.8*	31.3	31.1	23.7	10.0	10.4	18.5	27.4*	13.3	29.6*	0.00	29.3*	11.5	10.0
(CN)	Gentamycin	12.2	0.00	11.1	31.5	32.1	22.6	11.9	26.3	0.00	11.5	12.2	28.9	18.5	12.2	29.6	14.1
(R)	Rifampicin	21.5*	0.00	10.0	35.2*	35.2*	31.1*	10.0	9.60	10.0	11.9	37.8*	27.8	10.0	11.9	9.60	17.0

Many other studies show *Legionella* to be present in waters having pH near about the same range (Fliermans CB *et al*, 1981; Ohno A *et al*, 2003). In this study, there was no clear relationship between the count of *L. pneumophila* and iron concentrations in water samples. Some studies have found a positive correlation of iron and *Legionella* while others have found that it is negatively correlated (Borella P *et al*, 2004). Testing the antiobiotoc susceptibility, we found that gentamycin consistently proved to be the least active agent for most isolates from heat treated water samples and rifampicin was found to have the most antimicrobial activity against all isolates. These observations are in agreement with results obtained by other studies as well (Nguyen MH *et al*, 1991; Stout JE *et al*, 2005). Also, it was confirmed that the colonies of Legionella could grow only on buffered charcoal yeast extract (BCYE) agar with cysteine, which could be used as an isolation technique for this bacterium. This has been recently proved in other study as well (Subbaram K *et al*, 2017).

CONCLUSION

In conclusion, this study confirms the existence of *L. pneumophila* on water used on highway roads to Makkah with its thriving ability being high in pH ranging from 8 to 8.9.Water samples falling in this range are thus highly prone to contamination. Providing clean water is one of the essential solutions to prevent the pulmonary infection especially for children under two years old, elderly and immune-compromised people. If infected by *L. pneumophila*, rifampicin could be used as an efficient antibiotic for treatment.

Author Contributions

NASH contributed towards performing all the experiments and interpretation of data, SHMZ contributed towards the design of study and NZE contributed towards drafting the manuscript.

References

1. Fields BS, Benson RF and Besser RE. Legionella and Legionnaires' disease: 25 years of investigation. 2002. *Clin. Microbiol. Rev*. 15: 506-26.
2. Sabria M *et al*. A community outbreak of Legionnaires' disease: evidence of a cooling tower as the source. 2006. *Clin. Microbiol. Infect* 12: 642-47.
3. Bartram J. Legionella and the prevention of legionellosis. Geneva: World Health Organization. 2007. xxiv, 252 p.
4. Meenhorst PL *et al*. Water-related nosocomial pneumonia caused by Legionella pneumophila serogroups 1 and 10.1985. *J. Infect. Dis*. 152: 356-64.
5. Berthelot P *et al*. Nosocomial legionellosis outbreak over a three-year period: investigation and control. 1998. *Clin. Microbiol. Infec.t* 4: 385-391.
6. Perola O *et al*. Nosocomial Legionella pneumophila serogroup 5 outbreak associated with persistent colonization of a hospital water system. 2002. *APMIS* 110: 863-8.
7. Chien M *et al*. The genomic sequence of the accidental pathogen Legionella pneumophila. 2004. *Science* 305: 1966-8.
8. Hatem Q. Detection of Legionella from teaching hospital cooling tower water of air conditioning systems in Eastern Province of Saudi Arabia. 2012. *Asian J. Med. Sci*. 4: 99-102.
9. Esam IA *et al*, Legionella infection: an environmental and clinical study during hajj season. 2010. *Professional Med. J*. 17: 479-82.
10. Qin T *et al*. High prevalence, genetic diversity and intracellular growth ability of Legionella in hot spring environments. 2013. *PLoS One* 8: e59018.
11. Bopp CA *et al*. Isolation of Legionella spp. from environmental water samples by low-pH treatment and use of a selective medium. 1981. *J Clin Microbiol* 13: 714-9.
12. Wikins TD *et al*. Standardized single-disc method for antibiotic susceptibility testing of anaerobic bacteria. 1972. *Antimicrob. Agents Chemother*. 1: 451-9.
13. Vandepitte J. *Basic laboratory procedures in clinical bacteriology*. 2nd ed. (Geneva: World Health Organization. 167, 2003).
14. Al-Ghamdi SM *et al*. Pattern of admission to hospitals during muslim pilgrimage (Hajj). 2003. *Saudi Med. J*. 24: 1073-6.
15. Horwitz MA and Maxfield FR. Legionella pneumophila inhibits acidification of its phagosome in human monocytes. 1984. *J Cell Biol* 99: 1936-43.
16. Tison DL *et al*. Growth of Legionella pneumophila in association with blue-green algae (cyanobacteria). 1980. *Appl. Environ. Microbiol*. 39: 456-9.
17. OSHA United States Occupational Safety and Health Administration. 2017; Available from: http://www.osha.gov/dts/osta/otm/otm_iii/otm_iii_7.html.
18. Fliermans CB *et al*. Ecological distribution of Legionella pneumophila. 1981. *Appl. Environ. Microbiol*. 41: 9-16.
19. Ohno A *et al*. Factors influencing survival of Legionella pneumophila serotype 1 in hot spring water and tap water. 2003. *Appl. Environ. Microbiol*. 69: 2540-7.
20. Borella P *et al*. Legionella infection risk from domestic hot water. 2004. *Emerg. Infect. Dis*. 10: 457-64.
21. Nguyen MH, Stout JE and Yu VL. Legionellosis. 1991. *Infect. Dis. Clin. North. Am* 5: 561-84.

22. Stout JE *et al*. Comparative activity of quinolones, macrolides and ketolides against Legionella species using in vitro broth dilution and intracellular susceptibility testing. 2005. *Int. J. Antimicrob. Agents.* 25: 302-7.

23. Subbaram K, Kannan H and Massadeh MMA. Isolation, identification, characterization and antibiotic sensitivity profile of pathogenic *Legionella pneumophila* isolates from different water sources. 2017, *Asian Pacific Journal of Tropical Biomedicine*. 7: 411-15.

How to cite this article:

Al Harbi N.A.S *et al* (2017) 'Detection of Legionella Pneumophila in Water of Some Stations on Highway Roads to Makkah', *International Journal of Current Advanced Research*, 06(08), pp. 5637-5641.
DOI: http://dx.doi.org/10.24327/ijcar.2017.5641.0767

****