

## Isolation of *Legionella Pneumophila* Serogroups 1, 8 & 10 (Causative Agents of Legionnaires' Disease) from Water Sources in Tehran, Iran

Mojtaba Moosavian<sup>1\*</sup>, Bahram Fathollahzadeh<sup>2</sup>, Kazem Amoli<sup>3</sup> and Nasrin Moazzami<sup>4</sup>

<sup>1</sup>Dept. of Microbiology, School of Medicine, University of Medical Sciences, Ahvaz; <sup>2</sup>Dept. of Clinical Pathology, Imam Khomeini Medical Center (Vali-Asr Hospital), Tehran; <sup>3</sup>Bronchoscopy Division of Imam Khomeini Medical Center, Tehran University of Medical Sciences; <sup>4</sup>Dept. of Biotechnology, Iranian Research Organization for Science and Technology, Tehran, IR. Iran

### ABSTRACT

In this survey, 187 samples collected from 9 different sources of water in Tehran such as: water coolers, neonates incubators, water bathes (37° C), little pools in parks or squares, cooling towers, hot or cold water taps, were examined for Legionnaires' disease agents. Inoculating water samples on selective and nonselective media resulted in isolation of several strains of gram negative bacteria. Among them, seven strains of *Legionella pneumophila* which did not grow on any of the above media, were identified by indirect fluorescent antibody technique (IFA) and biochemical tests. Following confirmation by "Center National De Reference Des Legionelloses, Faculte of Medicine Alexis Carrel, Lyon", the Center identified *Legionella pneumophila* of serogroups of 1, 8 and 10. Out of 6 hospitals, 5 showed contaminated water having pneumonic Legionellosis agents. *Iran. Biomed. J.* 2: 83-87, 1998

Keywords: Legionnaires' disease, *Legionella*

### INTRODUCTION

*Legionella pneumophila*, the agent of Legionnaires' disease, was identified following an outbreak of pneumonia caused by contaminated aerosols originating from a cooling tower 22 years ago [1]. One strain of these bacteria had been isolated in 1947 [2] and the first outbreak of disease reported in 1957 [3]. The members of Legionellaceae are distributed in natural water such as lakes, rivers, thermal springs [31] and manmade sources including cooling towers, air conditioning systems, water distribution devices in hospitals or hotels, and equipment of respiratory treatments. It appears that human infections occur following contact with these environmental sources and by contaminated water aerosols which are present in the air [4, 5, 6, 7].

Among of more than 41 species, *Legionella pneumophila* is predominant causative agent of pneumonic Legionellosis [8,18]. This species in turn contains several serogroups and all of them are pathogenic for human. *L. pneumophila*,

serogroup-1 is responsible for more than 80% of infections caused by this species [9, 10]. In spite of many reports concerning Legionellosis and *Legionella* isolation from environmental sources of different countries, there are a few reports about them from Iran [32, 38]. Therefore we decided to study a number of water sources for these agents in Tehran, Iran.

### MATERIALS AND METHODS

**Locations of sampling.** In Tehran's hospitals, 187 samples of nine different water sources were collected and tested as follow: 75 water coolers (40.2%), 46 neonates incubators (24.6 %), 17 water bathes 37°C (9.1 %), 16 little pools in parks (8.5 %), hot and cold water tapes (11.8%), cooling towers (2.1 %), hot and cold water tanks(3.7 %), were also sampled.

**Processing of Samples.** Water sample (30 ml) from each source was collected into sterilized tubes and transferred to the Microbiology

\*Corresponding Author.

Laboratory of Vali-Asr Hospital in Tehran. They were concentrated by centrifuging at 2500 xg for 20 min. The supernatant was removed and the sediment was resuspended in 1 ml of the same water. Suspension (0.1 ml) was inoculated into each one of the following media, separately [11]: Buffered Charcoal Yeast Extract Agar (BCYE) containing 0.1%  $\alpha$ -ketoglutarate (Oxoid), and selective media such as BCYE containing polymyxin B, glycine, vancomycin and anisomycin (MWY-Modified Wadowsky & Yee (Oxoid, England) or BMPA (BCYE containing polymyxin, anisomycin and cephamandole (Oxoid). Plates were incubated at 35°C. in a humid atmosphere containing 5% CO<sub>2</sub> and examined daily for 2-weeks [12, 13]. The colonies were stained by Gram's method [12]. Gram negative bacilli which their colonies morphologically resembled *Legionella*, were subcultured on BCYE Agar and on the ordinary media such as Blood, MacConkey, EMB and Chocolate Agar [11, 14]. After incubation, *Legionella*-like colonies which failed to grow on any ordinary media were examined by indirect fluorescent antibody technique (IFA) for definitive identification.

**IFA Method.** IFA test performed using Merifluor *Legionella* Kit, (Meridian Diagnostic Inc.) [15] which contained: reagents of primary antibody (pool of lyophilized rabbit anti-*Legionella* antibodies), labeling (FITC-labeled goat anti-rabbit antibody), positive control antigen (a polyvalent pool of *Legionella* antigens) and negative control anti-body (lyophilized normal rabbit immunoglobulin). Briefly, suspensions of suspected colonies were prepared in 1% neutral formalin. After centrifugation, the supernatant was discarded and the sediment was washed 2x with buffer. Then, the sediment was resuspended in buffer and 10  $\mu$ l of bacterial suspension was placed in each of 2 wells on the slide. The slide was fixed gently by heat and the primary antibody and the negative control antibody were added to the corresponding wells. After 30 min at room temperature, the slide was washed with PBS buffer and after drying it was labeled. After 30 min of incubation, the slide was washed, dried and then examined under fluorescent microscope.

**Biochemical Tests.** The suspected isolates were identified as *Legionella* spp. by IFA test. Further-

more, number of biochemical tests such as catalase, oxidase, hippurate hydrolysis and P-lactamase were also used for definitive identification [12, 16, 17].

## RESULTS

187 water samples collected from diverse locations (Table 1), were inoculated to the selective and nonselective media. After incubation period, 244 strains of different bacteria including 129 (53%) strains of gram negative bacilli were isolated. Seven strains with the code of: 104, 105, 106, 110, 127, 155 and 174 were suspected to be *Legionella* spp. These gram-negative strains were identified based on morphology of the colonies and also based on their growth on the BCYE, MWY, BMPA media and lack of growth on ordinary media. All of these strains were identified as *Legionella pneumophila* by IFA [9] and by biochemical tests. These strains were positive by oxidase, P-lactamase, hippurate hydrolysis and by IFA tests. They had weaker reaction with catalase.

The isolated strains were lyophilized and sent to "Centre National De References Des Legionelloses Faculte De Medicine Alexis Carrel, Lyon, France" for confirmation of serogroups. This center confirmed three serogroups (1, 8 & 10) of *Legionella pneumophila*. The results also showed that 6 out of 17 water bathes and one out of 46 neonatal incubators were also contaminated with *Legionella*.

Table 2 shows that 3 out of 6 strains of *L. pneumophila* which were recovered from 37°C water bathes in Vali-Asr, Imam Khomeini, Dr. Shariati and Amirkabir Hospitals belonged to serogroup-1, 2 strains belonged to serogroup-8 and one strain of unknown serogroup. The 7th strain which was isolated from the neonatal incubator belonged to the serogroup-10.

## DISCUSSION

This study report, we have isolated seven serogroups of *L. pneumophila* from water sources. So far, more than 41 species and 63 serogroups of *Legionella* have been recognized worldwide through the research on patients and environmental sources which represent the distribution of the Legionnaires' disease agents in different countries [18,19, 20].

There are many natural water sources in h-an, including lakes, rivers, hot and mineral spring waters which are used by thousands of people every year. Contamination of these sources can serve as a focus for the spread of *Legionella* organisms. *Legionella* may also be distributed by contaminated equipment [18, 33, 37] such as water supplies of hospitals, hot water tanks, cooling towers, respiratory therapy devices, dental units, decoration fountains and any other sources in which water is left for a period of time.

In this study, 7 strains of *Legionella*-like organisms which were isolated from water sources, were later identified as *L. pneumophila*. Previous studies have demonstrated that *L. pneumophila* is the major causative agent of Legionnaires' disease [21, 22, 23], and about 85% to 90% of all cases were due to this species [8, 18]. The isolation of *L. pneumophila* from the environmental sources proves the existence of Legionnaires' disease in Iran. Although, all of the 15 known serogroups of *L. pneumophila* are pathogen for human, specially serogroup-1 is responsible for the 80% of the infections [10]. Isolation and role of *L. Pneumophila* serogroups 8 & 10 in infection have been reported previously [23].

Our data indicate that almost half of the isolated strains were serogroups-1 which show the higher prevalence of this serogroup in water sources. The majority of contaminated sources were 37°C water bathes. A survey on the mist-machine in a grocery store conducted by CDC (Center for Disease Control) showed 33 cases of Legionnaires' disease in persons who had been exposed for more than 30 min [24]. They had bought the items which located close to the mist-machine. The contamination of 1/3 of water bathes in our study and that of CDC study may indicate that the lab personnel who are in the close contact with the equipment such as water bathes are at high risk of acquiring the infection.

The outbreaks of Legionnaires' disease and Pontiac fever, Non pneumonic form, among young children and neonates have been previously reported [23, 25, 26]. Also, isolation of *L. pneumophila* from the humidification trays of five out of seven incubators in neonatology unit of a hospital [36] and inhalation of aerosols produced by contaminated humidifiers [33, 34] show that the neonates who are taking care of in these systems may be exposed to the Legionnaires' disease agents. In this study we have confirmed the presence of *L. pneumophila* by isolation of serogroup-10 from humidification tray of one neonatal incubator.

Distribution of *L. pneumophila* strains isolated from different therapeutic centers, (Table 2), shows that at least one strain of *L. pneumophila* was isolated from each studied center. This is a matter which should be noticed in view of the health of the lab personnel and hospitalized patients. Some factors which stimulate the growth and isolation of *Legionella*, such as water stagnation, favorable temperature, presence of commensal bacteria and combination of water sediment [27, 28, 36], may have been present in 37°C water bathes which were studied.

With attention to the conference of Legionellosis which presented in 4th Iranian congress of infectious and tropical disease (abstracts, Tehran University of Medical Sciences, 1993) and the other reports related to Legionnaires' disease from Iran [32] and recovery of a few strains of *L. pneumophila* in this study, it is necessary to do more research on different water sources and patients that are suspected to have Legionellosis in Iran. These studies will really determine the possible relation between presence of organisms in environment and disease outbreaks in the patients. According to our data, this is the first report of isolation of three serogroups of *L. pneumophila* in Iran.

## ACKNOWLEDGMENTS

We thank Dr. N. Bornstein, Centre National De Reference Des Legionelloses, Faculte De Medicine Alexis Carrel, Lyon, France for examining and typing our strains.

## REFERENCES

1. McDade, J.E., Shepard, C.C., Fraser, D.W., Tsai, T.R., Redus, M.A., Dowdle, W.R. (1977) Legionnaires' disease, isolation of a bacterium and demonstration of its role in other respiratory disease. *N. Engl. J. Med.* 297: 1197-1203.
2. McDade, J.E., Brenner, D.J. and Bozeman, FM. (1979) Legionnaires' disease bacterium isolated in 1947. *Ann. Intern. Med.* 90: 659-661.
3. Osterholm, M.T., Chin, D.Y., Osbourne, D.O., Dull, HB., Dean, AG., Fraser, D.W., Hayes, P.S. and Hall, W.N. (1983) A 1957 outbreak of Legionnaires' disease associated with a meat packing plant. *Am. J. Epidem.* 117: 60-67.
4. Swartz, M.N. (1990) *Legionella*. In: Microbiology (Davis, B.D., Dulbecco, R., Eisen,

- H.N. and Ginsberg, HS. eds), 4th ed. Philadelphia, Pennsylvania, J.B. Lippincott Company, PP. 717-19.
5. Joklik, W.K., Willett, H.P., Amos, D.B., Wilfert, CM. (1988) Zinsser Microbiology. 19th ed. USA, Appleton and Lange, Prentice-Hall, International Inc., PP. 588-591.
  6. Mastro, T.D., Fields, B.S., Breiman, R.F., Campbell, J., Plikaytis, B.D. and Spika, J.S. (1991) Nosocomial Legionnaires' disease and use of medication nebulizers. *J. Infect. Dis.* 163(3): 667-71.
  7. Ng, D.L., Koh, B.B., Tay, L., and Heng, B.H. (1997) Comparison of polymerase chain reaction and conventional culture for the detection of *Legionella* in cooling tower waters in Singapore. *Lett. Appl. Microbiol.* 24(3): 214-216.
  8. Wilkinson, H.W., Reingold, A.L., Brake, B.J., McGiboney, D.L., Gorman, G.W. and Roome, C.V. (1983) Reactivity of serum from patients with suspected Legionellosis against 29 antigens of Legionellaceae and *Legionella* like organisms by IFA. *J. Infect. Dis.* 147:23.
  9. Wilkinson, H.W. (1988) Hospital laboratory diagnosis of *Legionella* infections. Zed. Center for Disease Control. Atlanta, 1-35.
  10. Kuritsky, J.N., Reingold, A.L., Hightower, A.W., Broome, C.V. (1984) Sporadic Legionellosis in the United States, 1970-1982. In: *Legionella* proceedings of the 2nd International symposium., (Thornsberry, C., Belows, A., Feeley, J.C. and kubowski, W., eds), American Society for Microbiology, Washington, DC. P. 243.
  11. *Legionella* Selective Medium (1991) In: Selective Microbiology for the Clinical Laboratory, Unipath Limited, Basing Stoke, Hampshire, (Oxoid), UK., PP. 28-29.
  12. Baron, E.J. and Fingold, S.M. (1990) Bailey and Scott's, Diagnostic Microbiology. 8th ed. C.V. Mosby Company, St. Louis, USA.
  13. Sonnenwirth, A.C., Jarett, L. (1982) Gradwohl's Clinical Laboratory Methods and Diagnosis. C.V. Mosby Company, St. Louis, USA, Vol.2. PP.1842- 1843.
  14. Bezanson, G., Burbridge, S., Haldane, D., Yoell, C.H. and Marrie, T.H. (1992) Diverse populations of *L. pneumophila* present in the water of geographically clustered institutions served by the same water reservoir. *J. Clin. Microbiol.* 30(3): 570-576.
  15. Merifluor *Legionella* an indirect immunofluorescent detection procedure for 33 strains of *Legionella Spp.*, Meridian Diagnostics, Inc. Cincinnati, Ohio, USA. Catalog 221050.
  16. Hebert, GA. (1981) Hippurate hydrolysis by *Legionella pneumophila*. *J. Clin. Microbiol.* 13(1): 240-242.
  17. Fallon, R.J. (1989) Legionellaceae. In: Mackie and McCartney, Practical Medical Microbiology, (Collee, J.G. Duguid, J.P., Fraser, A.G. and Marmion, B.P. eds). 13th ed. Churchill Livingstone, UK, PP. 540-552.
  18. Mulazimoglu, L., Yu, V.L. (1998) *Legionella* In-fectons. In: Harrison's Principles of Internal Medicine, (Fauci, AS., Braunwald, E., Isselbacher, K.J., Wilson, J.D., Martin, JB., Kasper, DL., Hauser, S.L, and Longo, D.L. eds) 14th ed. Vol. 1, McGraw Hill, International Edition, PP. 928-933.
  19. Koneman, E.W., Allen, S.D., Dowell, Jr. V.R., Janda, W.M., Sommers, H.M., Winn, Jr. W.C. (1988) Color Atlas and Text book of Diagnostic Microbiology, 3rd ed. J.B. Lippincott Company, USA, PP.243-268.
  20. Bornstein, N., Marmet, D., Dumaine, M.H., Surgot, M., Fleurette, J. (1991) Detection of flagella in 278 *Legionella* strains by latex reagent sensitized with anti-flagellum immunoglobulins. *J. Clin. Microbiol.* 29(5): 953-957.
  21. Fingold, SM. (1988) Legionnaires' disease still with us. *New Engl. J. Med.* 318(9): 571-573.
  22. Falco, v., Sevilla, T.F., Aleg re, J., Ferrer, A., and Vazquez, J.M.M. (1991) *Legionella pneumophila*, A cause of sever community acquired pneumonia . *Chest* 100(4): 1007-1011.
  23. Aubert, G., Bornstein, N., Ryet, I., Pozzeto, B. , Lenormand, P.H. (1990) Nosocomial infection with *Legionella pneumophila*, serogroup 1 & 8 in a neonate. *Scand. J. Infect. Dis.* 22:367-70.
  24. Mahoney, F.J., Hoge, C.W., Farley, T.A., Barbaree, J.M., Breiman, R.F., Benson, R.F. and Mc-Farland, L.M. (1992) Community wide outbreak of Legion-naire's disease associated with a grocery store mist machine. *J. Infect. Dis.* 165(4): 736-739.
  25. Goldberg, D.J., Emslie, J.A., Fallon, R.J., Green, S.T. et al. (1992) Pontiac fever in children. *Pediatr. Infect. Dis.* 11(3): 240-241.
  26. Brady, M.T. (1989) Nosocomial Legionnaires' disease in a children hospital. *J. Pediatr.* 115: 46-30.
  27. Yu, V.I. (1988) *Legionella*: An environmental pathogen. In: Mechanisms of Microbial Disease,

- (Schaechter, M., Medoff, G., and Schlessinger, D. eds), Baltimore, MD, Williams & Wilkins, USA, PP. 346-352.
28. Yu, V.I. (1990) Legionnaires' disease. In: Principles and practice of infectious disease. (Mandell, G.L., Gordon Douglas, R. Jr., and Bennett, J.E. eds). 3rd ed. New York, Churchill Livingstone, PP.1764- 1782.
  29. Henke, M., Seidel, K.m. (1986) Association between *Legionella pneumophila* and Amoebae in water. *Isr. J. Med. Sci.* 22(9): 690-95.
  30. Bercovier, H., Fattal, B., Shuval, H. (1986) Seasonal distribution of *Legionella* isolated from various types of water in Israel. *Isr. J. Med. Sci.* 22(9): 644-46.
  31. Sommese, L., Scarfogliero, P., Vitiello, M., Catalanotti, P. and Galdiero, E. (1996) Presence of *Legionella* spp. in thermal springs of the Campania region of South Italy. *New Microbiol.* 19(4): 315-20.
  32. Moosavian, M., Agin, K.h., Fathollahzadeh, B., Amoli, K., Moazzami, N. (1994) First report on co-infection of Legionellosis and Tuberculosis in Iran. *Med. J. I.R.I.* 8(3): 191-193.
  33. Arnow, P.M., Chou, T., Weil, D., Shapiro, E.N. and Kretzchman, C. (1982) Nosocomial Legionnaires' disease caused by aerosolized tap water from respiratory devices. *J. Inf. Dis.* 146: 460-467.
  34. Zuravleff, J.J., Yu, V.L., Shonnard, J.W., Rihs, J.D. and Best, M. (1983) *Legionella pneumophila* contamination of a hospital humidifier demonstration of aerosol transmission and subsequent subclinical infection in exposed guinea pigs. *Am. Rev. Res. Dis.* 128: 657-661.
  35. Epidemiology, Prevention and Control of Legionellosis (1990) Memorandum from a WHO meeting. *Bull. World Health Organ.* 68(2): 155-164.
  36. Verissimo, A., Vesey, G., Rocha, G.M., Marrao, G., Colbourne, J., Dennis, P.J. and DaCosta, M.S. (1990) A hot water supply as the source of *Legionella pneumophila* in incubators of a neonatology unit. *J. Host. Infect.* 15(3): 255-263.
  37. Williams, H.N., Paszko-Kolva, C., Shahamat, M., Palmer, C., Pettis, C. and Kelley, J. (1996) Molecular techniques reveal high prevalence of *Legionella* in dental units. *J. Am. Dent. Assoc.* 127(8): 1188-93.
  38. Aslani, M.M., Pourmansour, M., Motavalian, M. (1997) Isolation of *Legionella pneumophila* from Tehran Hospital water samples. *Iran. Biomed. J* 1(1): 65-67.