Isolation and Identification of *Legionella pneumophila* from Environmental Water Sources in Khartoum State

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**ABSTRACT**

Legionnaire’s disease affects humans causing mild to severe pneumonia, particularly in immune-compromised individuals. The aim of this study was to identify the causative agent and to determine the frequency of *Legionella pneumophila* in potential water samples collected from various environmental sources in Khartoum State, Sudan.

A total of 120 samples were collected from different hospital settings during April to August 2012. Bacteriological examination of collected environmental water samples was performed to isolate *legionella pneumophila* by inoculation on buffered charcoal yeast extract (BCYE) agar. Several biochemical tests including; sodium hippurate, catalase, oxidase tests and gelatin liquefaction were further carried to confirm the identification. Antibiotic and beta-lactamase testing were done to detect resistance or susceptibility.

Out of 120 water samples; 8 (6.7 %) *Legionella pneumophilais* isolates were recovered from water environment in air conditions, shower heads and water coolers for the first time in Sudan. The organism was demonstrated as a beta-lactamase producer resistant to ampicillin and penicillin but susceptible to gentamicin, chloramphenicol and erythromycin.

This study concluded that *Legionella pneumophila* exists in the environment in Sudan. As an important environment water pathogen, it is vital to initiate further
research work with many samples to isolate and identify the organism from different water and food sources to assess prevalence and implications.

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**1. INTRODUCTION**

Legionnaire’s is a bacterial pulmonary disease caused by *Legionella pneumophila* (Shuman, 1998). The bacterium was identified as a causative agent of pneumonia in 1976 following an outbreak of pneumonia among people attended an American legion convention in Philadelphia, USA (Brenner et al., 1979). It contributes to 90% of legionellosis cases, while *L. micdadei* bacterium is the second most frequent cause of legionellosis (Shuman, 1998).

*Legionella pneumophila* belongs to the family legionellaceae. Currently, 50 valid published species and subspecies and total of 71 serologic types of *Legionella* have been isolated from either human specimens, environmental sources or both (King et al., 1988). The people who are most likely at risk to catch infection are over age 50. The risk is greater for people who suffer from health conditions such as malignancy, diabetes, lung disease or kidney disease. Other risk factors include immunosuppressive therapy and cigarette smoking. Legionnaire’s disease has occurred in children and particularly confined to newborns receiving respiratory therapy, children who have had recent operations and children who are immunosuppressed (King et al., 1988). People with HIV infection and AIDS do not seem to contract Legionnaires' disease with any greater frequency than the rest of the population, however, if it is contracted, the disease is likely to be more severe compared to other cases (King et al., 1988).
Legionnaire’s disease is caused by inhaling *Legionella* from the environment. Typically, the bacteria are dispersed in aerosols of contaminated water. These aerosols are produced by devices in which warm water can stagnate, such as air-conditioning cooling towers, humidifiers, shower heads and faucets. There have also been cases linked to whirlpool spa baths and water misters in grocery store produce departments. Aspiration of contaminated water is also a potential source of infection, particularly in hospital-acquired cases of Legionnaire’s disease. There is no evidence of person-to-person transmission of Legionnaire’s disease (Shuman, 1998).

Some legionellae cannot be grown on routine *Legionella* culture media, and have been called as *Legionella*-like amoebal pathogens (LLAPs). These organisms have been isolated and maintained by co-cultivating the bacteria with their protozoan hosts. One LLAP strain was isolated from the sputum of a pneumonia patient by enrichment in amoebae and is considered to be a human pathogen (Fry et al., 1999; Marrie, 2001). Additional LLAP strains may be human pathogens, but it is difficult to prove this, because they cannot be detected by traditional techniques used for legionellae. Recently, three discovered LLAP strains were named *Legionella* species (Adeleke, 2001; La Scola, 2004).

Although the pneumonia affects the lungs, Legionnaire’s disease is accompanied by symptoms that affect other areas of the body (Shuman, 1998). About half the victims experience diarrhea and a quarter have nausea and vomiting and abdominal pain. In about 10% of cases, acute renal failure and scanty urine production accompany the disease. Changes in mental status, such as disorientation, confusion and hallucinations also occur in about a quarter of cases (Shuman, 1998). In addition to Legionnaire’s disease, *L. pneumophila* legionellosis also includes a milder disease, Pontiac fever. Unlike Legionnaires' disease, Pontiac fever does not involve the lower respiratory tract. The symptoms usually appear within 36 hours
of exposure and include fever, headache, muscle aches and lethargy. Symptoms last only a few days and medical intervention is not necessary. Since the bacteria thrive in warm stagnant water, regularly disinfecting ductwork, pipes and other areas that may serve as breeding areas is the best method for preventing outbreaks of Legionnaire’s disease (Shuman, 1998). *Legionella* species are ubiquitous in soil and water and cannot realistically be eliminated. They are controlled by adequate chlorination of water, biocides, UV light, or by periodic superheating (> 60°C) of water. Bacteria living in amoebae are more resistant than free-living bacteria to biocides used for water treatment and may be better able to survive other adverse environmental conditions. Bacteria living outside of amoebae may be found in biofilms, which are also difficult to get rid of. Treatment must include antibiotics that penetrate intracellular. Erythromycin is most commonly used; others include rifampin, tetracycline, TMP-SMZ, or quinolones (Heath *et al*., 1996).

### 3. MATERIALS AND METHODS

#### 3.1. Study design

The present study is a cross sectional laboratory based study.

#### 3.2. Study area

This study was carried out in different hospitals in Khartoum State including Khartoum Hospital, Bashier Hospital, National Health Laboratory and Al kabashy Hospital.

#### 3.3. Study duration

The study was conducted during the period from April to September 2012.

#### 3.4. Study population

Water samples in environment, environmental samples
3.5. Sample size

A total of 120 water and swab samples were collected from water coolers, showerheads, meshes of air conditions.

3.6. Sample collection and processing

An environmental sampling protocol addressing selection of the appropriate sites to sample has been previously published (CDC 2005b). Whenever possible, one liter of water was collected, swab samples were collected from air conditions and showerheads, preferably with the aerator or showerhead removed. Swabs were submerged in 3-5 ml of collected water from the site to prevent drying during transportation (CDC 2005b). Whenever the water source has been recently treated with chlorine, 0.5 ml of 0.1N sodium thiosulfate was added to each 1 liter sample to neutralize the disinfectant. And added 0.5 ml of 0.001 N sodium thiosulfate to each 5ml sample to neutralize the disinfectant.

The HCl-KCl buffer used for pretreating water samples was prepared by mixing 3.9 ml of 0.2 M HCl with 25 ml of 0.2 M KCl to yield a buffer solution with a pH of approximately 2.2. Two procedures designated "A" and "B" for acid treatment were used.

In procedure A; 10 ml of the water sample was centrifuged at 4,000 rpm for 10 min in aerosol-free centrifuge containers. The supernatant was poured off, and the sediment was suspended in 0.5 ml of watersample. This suspension was diluted 1:10 with the HCl-KCl buffer. At intervals ranging from 5 to 60 min, 0.1-ml quantities were removed from the acid suspension and were plated on agar plates of BCYE supplemented with (dyes, glycine, vancomycin, polymixin B).

Procedure B; 0.5 ml of the swab sample was added to 4.5 ml of HCl-KCl buffer centrifuged at 4,000 rpm for 10 min in aerosol-free centrifuge containers. At intervals of 5 to 60 min, 0.1-ml of acid-treated water sample were inoculated onto
plated of BCYE with supplement (dyes, glycine, vancomycin, polymyxin B). (Pei-Yi et al., 2008).

3.7. Culturing of the samples

All the samples were inoculated in buffered charcoal yeast extract (BCYE) with supplement (dyes, glycine, vancomycin, polymyxin B) and blood agar, incubated at 37°C in aerobic incubator for 2 to 14 days.

3.8. Identification of bacteria

3.8.1. Colonial morphology

After incubation, the plates were observed for size, color, edges, side views and surface of the colonies.

3.8.2. Gram stain

The Gram stain was used for the identification of pathogens in cultures by determining their Gram reactions, cell shape and arrangement according to the Gram procedure. The method was performed as follows:

Thin smear was prepared on a clean slide and fixed by rapidly passing the slide over the Bunzen flame. The smear was covered with crystal violet, left for 1 min. and then rinsed carefully with water. It was then covered with lugol's Iodine left for 1 min., rinsed carefully with water, decolorized with alcohol and rinsed again carefully. Finally, the smear was covered with safranin, left for 2 min., rinsed as before and dried by blotting on a filter paper (Washington et al., 2006).

3.9. Biochemical identification

The environment isolates were subjected to different biochemical tests for their identification. The following tests were used.
3.9.1. Oxidase test

This test was done by moistening a filter paper with the substrate (1% tetramethyl-
P-phenylenediaminedihydrochloride) and small portion of a colony was rubbed on the filter paper by platinum wire loop, in the presence of cytochrome oxidase, dark blue color will developed indicating positive reaction (Betty et al., 2007).

3.9.2. Catalase test

Small amount of colony was transferred with sterile wooden stick and inoculated in sterile test tube contain 30 % hydrogen peroxide (in case of positive reaction bubbling of gas appeared indicating breakdown hydrogen peroxide catalase enzyme into oxygen and water (Betty et al., 2007).

3.9.3. Urease test

This test demonstrates the ability of a bacterium to produce the enzyme urease, capable of hydrolyzing urea and changing pH of urea to form ammonia. Urea agar inoculated with tested organism and incubated for 24 hrs. at 37°C (Betty et al., 2007).

3.9.4. Motility test

Motility test was made by single stab into the center of the medium concentrations of 0.4% or less to allow for the free spread, after incubation movement away from the stab line or hazy appearance throughout the medium indicates a motile organism (Washington et al., 2006).
3.9.5. Demonstration of β–lactamase production

β-lactamase (nitrocefin discs) provided by (Dickinson), the test was carried out according to the manufacturer instruction, were inoculated by isolates after moistened by sterile distill water and read immediately. The production of β-lactamase enzyme; contains nitrocefin which was a chromogenic cephalosporin ring that change from yellow to red color when the β-lactam ring is hydrolyzed by β-lactamase (Betty et al., 2007).

3.9.6. Hydrolysis of Sodium Hippurate

Broth containing sodium hippurate was inoculated with the organism and incubated overnight at 37°C, the tubes were centrifuged and the supernatant was removed. Ferric chloride reagent (0.2 ml; FeCl₃·6H₂O, 12g in 100 mL 2% aqueous HCL) was then added to the supernatant (0.8 ml), with the formation of a heavy precipitate. If the precipitate remains after 10 minutes, benzoic acid is present and the test is positive for sodium hippurate. Alternatively, ferric chloride reagent was added to the supernatant to detect free glycine (Washington et al., 2006).

3.10. Hydrolysis Gelatin liquefaction

Gelatin, a protein derived from the animal protein collagen. In tube contain gelatin was inoculated with the organism and incubated overnight at 37°C, One problem is that many bacteria have the ability to hydrolyze (liquefy) gelatin. This gelatin liquefaction ability forms the basis for this test. Some microorganisms possess an enzyme called gelatinase, which breaks down gelatin into amino acids. Which bacteria was protein produced by the hydrolysis of collagen (Washington et al., 2006).
4. RESULTS

4.1. Bacteriological investigation result

4.1.1. Colonial morphology

One hundred twenty suspected samples were collected from water of drinking coolers, swab samples taken from meshes of air conditions, and head showers. The samples were inoculated on BCYE selective medium and blood agar. After incubation period of 2 to 3 days, the colonies firstly appeared tiny and then increased in size as incubation had progressed (Figure 1). The colonies were grey to white with ground glassy and glistening in appearance and convex were observed on BCYE medium implying characteristic features suggestive of *Legionella pneumophila* (Figure 1).

Subsequent sub-culturing to maintain growth of the organism on selective medium was quite difficult since the organism quickly died and selective medium had run out. Following specimens streaking on blood agar, *L. pneumophila* did not grow (Figure 2).

![Figure 1 Culture of Legionella pneumophila on buffered charcoal yeast extract supplemented with dyes, glycine, vancomycin and polymyxin B.](image)
Figure 2 Inability of Legionella pneumophila to grow on blood agar.

4.1.2. Distribution of study sample and isolated organisms

Out of 120 collected samples, eight (6.7%) of the grown culture colonies on BCYE medium were found suggestive of Legionella pneumophila; while the remaining 112 cultures did not show any growth even after 7 days incubation (Table 1). Microscopic examination of smears revealed that the organisms were Gram negative rods.

Five (4.1%) out of the 120 samples were isolated from meshes of air conditioners, 2/120 (1.7%) were isolated from water coolers and 1 (0.8%) was isolated from a head shower (Figure 3).
4.1.3. Result of Biochemical Identification

*Legionella pneumophila* isolates were tested by biochemical tests including: oxidase, catalase, motility, sodium hippurate and gelatin liquefaction test. Results were as shown (Table 2; Figure 4).

**Table 2 Biochemical identification of *Legionella pneumophila***

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<th>Test</th>
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<td>Urease</td>
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<td>Motility</td>
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<td>Sodium Hippurate</td>
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<td>Grow on blood agar</td>
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Symbols: +, Positive; -, Negative; ±; most strains gave weak positive. Eight isolates # 1,2,3,4,5, 6, 7,8 identified as *Legionella pneumophila*. 
Figure 4 Biochemical identification of *L. pneumophila*.

(A) Positive hydrolysis of sodium hippurate test (violet colour) after addition of ninhydrin reagent to overnight, negative sodium hippurate in center. (B) Positive Catalase test of *L. pneumophila* isolate with characteristic elaboration of oxygen bubble. (C) Positive oxidase test (blue colour) of *L. pneumophila* isolates and the right is negative. (D) Positive (pink colour), hydrolyzing urea and changing pH of urea to from ammonia and the negative did not change the colour. (E) Positive was motility test away from the stab line or hazy appearance throughout the medium indicates a motile organism. (F) Gelatin liquefaction positive test, the medium was liquefied and negative medium remains solid.
4.2. β-lactamase testing

All isolates of *Legionella pneumophila* produced β-lactamase (pink colour) and the negative were non-β-lactamase producers (green or yellow colour).

![Nitrocefin Discs](image)

**Figure 5** Beta-lactamase testing

Positive β-lactamase test of *Legionella pneumophila* (nitrocefin discs showed red spots); while negative β-lactamase test of negative control (nitrocefin disc without red spots yellow or green colour) immediately after colonies of *Legionella pneumophila* were rubbed on the discs.
5. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion
Legionellae cause several syndromes affecting public health. The aim of the present study was to isolate and identify *Legionella pneumophila* from samples in environment probably had contamination such as meshes of air conditions, shower heads and drinking water from coolers.

From one hundred and twenty specimens, 8 (6.7%) were identified as *Legionella pneumophila* using colonial morphology, Gram staining reactions and biochemical tests. Five (4.1%) out of 120 samples were isolated from meshes of air conditions, 2(1.7%) were isolated from water coolers and 1(0.8%) was isolated from a head shower (Figure 3).

In the study conducted by Johnson *et al.*, (1985) they found that hospital potable water systems are the primary reservoirs for *Legionella pneumophila* with a percentage mounted to 36% which disagreed with the findings of present study. Furthermore, Erdogan and Arslan (2007) found *Legionella* contaminating water sources taken from apartments as 22.6%. Such high percentage of water contamination could be due to higher tower of water source which probably increased humidity and facilitated growth of *Legionella*.

In the present study project, *L. pneumophila* occurred in air conditions in comparable amounts as displayed by (Barrow *et al.*, 2005). Moreover by Tobin *et al.*, (1980) reported the isolation of 2 *Legionella* organisms from shower heads, that was in concordance with the finding this study.
5.2. Conclusion
In conclusion, *Legionella pneumophila* was found to constitute about 6.7% of the environment water samples among the study population. The eight isolates were found to be β-lactamase producers which explained their resistance to ampicillin. Therefore, it is necessary to look for beta lactamase production and sodium hippurate in all isolates as it will affect the type of antibiotic chosen for therapy.

5.3. Recommendations
- Further research is needed to study the epidemiology and the extent of *Legionella pneumophila* infections in Sudan.
- Immune fluorescent kits, molecular techniques and buffer charcoal yeast extract (BCYE) with and without supplement should be used for detection of *Legionella*.
- The present results suggest that a multi-professional approach must be taken for the control and management of *Legionella* spp. in water systems, with risk assessments and integrated risk management programs and training all those who have to be aware of the legionellosis problem.
Reference:


- Johnson, J. T; Yu, V. L; Best, M. G; Vickers, R. M; Goetz, A., and Wagner, R. (1985). Nosocomial legionellosis in surgical patients with head-


- **Pei-Yi, Y.U; Yusen Eason Lin; Wei-Ru, Lin; Hsiu-Yun, Shih; Yin-Ching, Chuang; Ren-Jy, Ben; Wen-Kuei, Huang; Yao-Shen, Chen; Yung-Ching, Liu; Feng-Yee, Chang; Muh-Yong, Yen; Ching-Chuan, Liu; Wen-Chien, Ko; Hsi-Hsun, Lin; Zhi-Yuan, Shi (2008)** The high prevalence of Legionella pneumophila contamination in hospital potable water systems in Taiwan *Intern j Infect Dis* 12:416—420.
