

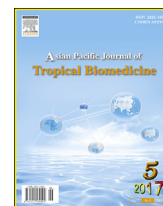
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journal homepage: [www.elsevier.com/locate/apjtb](http://www.elsevier.com/locate/apjtb)Original article <http://dx.doi.org/10.1016/j.apjtb.2016.12.023>Isolation, identification, characterization and antibiotic sensitivity profile of pathogenic *Legionella pneumophila* isolates from different water sourcesKannan Subbaram<sup>1\*</sup>, Hemalatha Kannan<sup>2</sup>, Majed Mohammad Ahmad Masadeh<sup>3</sup><sup>1</sup>Department of Medical Preparatory Year – Biology, Al-Ghad International Colleges for Applied Medical Sciences (Male), Riyadh, Saudi Arabia<sup>2</sup>Department of Medical Laboratory Sciences & Pathology, Jimma University, Jimma, Ethiopia<sup>3</sup>Department of Pharmaceutical Technology, Jordan University of Science and Technology, Irbid 22110, Jordan

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## ABSTRACT

**Objective:** To investigate the prevalence, isolation, identification, characterization, antibiotic profile and pathogenicity of *Legionellae* isolated from various set of waters.**Methods:** A total of 400 water samples were collected from different water sources. Water samples were pretreated using acid treatment followed by concentration and culture on buffered charcoal yeast extract agar. Parameters like ability of *Legionella* isolates to grow in various pH range, effect of different concentrations of chlorine and effect of different temperature optima were set up. Biochemical tests were performed to separate *Legionellae* into species. Antibiotic sensitivity tests and test for pathogenicity were also conducted on isolated strains.**Results:** The rates of isolation of *Legionella pneumophila* (*L. pneumophila*) in different water sources were found to be 20% (lakes), 10% (ponds), 8% (water-tanks) and 1% (rivers). Most of the isolates could grow in variable pH 6–8 and it could also survive the normal level of chlorination and even at temperature of 42 °C. Isolated species of *Legionellae* resulted in identification of 5 different species, *L. pneumophila* being the dominant one. Strains of *L. pneumophila* were resistant to many antibiotics. Inoculation of *Legionellae* into intracerebral route of suckling mice revealed that *L. pneumophila* was the most virulent.**Conclusions:** Serious and fatal *L. pneumophila* infections may be transmitted through water. *Legionella* can survive under various conditions in various water sources. *L. pneumophila* is the important pathogen causing human disease. Great challenge prevails to health care professionals because these *Legionellae* acquired antibiotic resistance to many routinely prescribed antibiotics.

## 1. Introduction

*Legionella pneumophila* (*L. pneumophila*) has frequently been isolated from patients with Legionnaires' disease and in

several instances also from epidemic-related environmental samples [1]. These include air conditioning cooling towers or evaporative condensers and hospital showers [2]. There is poor data available regarding the prevalence of *L. pneumophila* from surface waters like lakes, ponds, water-tanks and rivers [3].

In addition to *L. pneumophila*, the agent of Legionnaires' disease, there are now four other species of *Legionella*: *Legionella micdadei*, *Legionella dumoffii*, *Legionella bozemanii* and *Legionella gormanii* [4]. These studies till today state that out of these species of *Legionella*, only *L. pneumophila* is associated with disease. Other species are non-pathogenic saprophytes and also present in water bodies [5]. Serious and fatal *L. pneumophila* infections may be transmitted through water. Other infections caused by *L. pneumophila* include extrapulmonary abscesses

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and Pontiac fever [6]. Legionnaires' disease is a systemic illness, manifested primarily by pneumonia [7]. Pontiac fever is a nonpneumonic, systemic febrile illness, closely associated with aerosolization of *L. pneumophila*; it is most likely intoxication rather than an infection [7].

*L. pneumophila* are ubiquitous, water-borne, Gram negative bacilli belonging to one genus (*Legionella*) comprising at least 23 species [8]. Till today there are no elaborative studies conducted on isolation, characterization, antibiotic profiles and virulence potentials of *Legionella* species from different water bodies. Current study is aimed to isolate *L. pneumophila* and other species from different water bodies and to characterize them.

## 2. Materials and methods

### 2.1. Sample collection and transport

A total of four hundred water samples (hundred from each) were collected from different lakes, ponds, water-tanks and rivers in various seasons of the year. Each sample was collected in a sterile glass-stoppered bottle. Immediately after collection, the samples were kept in ice-pack and brought to the laboratory without delay.

### 2.2. Acid treatment of samples

To avoid the growth of other undesired bacteria and to facilitate the better isolation of *L. pneumophila*, all the samples were diluted (1:10) in a KCl–HCl solution (pH 2.2); this was mixed and incubated at room temperature for 4 min [9].

### 2.3. Concentration of samples

Concentrations of the diluted water samples were carried out by centrifugation at 400 r/min. Supernatant solution was discarded and the sediment was aseptically transferred and cultured on buffered charcoal yeast extract (BCYE) agar culture medium. This medium was supplemented with  $\alpha$ -ketoglutarate, vancomycin, polymyxin B, and anisomycin [10]. The culture media were incubated at 37 °C in 2.5% CO<sub>2</sub>. The culture plates were read after 5, 6, 7, and 10 days of incubation for colonies showing white, glistening, convex, circular, entire, and from 1 to 2 mm in diameter with a “ground glass” appearance under magnification [11].

### 2.4. Preliminary screening of suspected *L. pneumophila* colonies

Gram staining was performed on the suspected *Legionella* colonies. Basic fuchsin (0.1%) was used as a counter stain. Each colony was inoculated into nutrient broth tubes containing 1% glucose with Andrade indicator. Urease test and nitrate test were also carried out [12]. Oxidase testing was performed using 1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride. Catalase test was carried out with 3% H<sub>2</sub>O<sub>2</sub> in Tween 80. Test for gelatin liquefaction was performed in gelatin agar stab [13].

### 2.5. Confirmation of *L. pneumophila*

Each colony thought to be *Legionella* was streaked for isolation to BCYE agar plate without antibiotics and incubated for 2–5 days. From each plate, the isolate was streaked onto yeast extract-tyrosine agar and 5% sheep blood agar. If growth did not occur on blood agar after 5 days of incubation (*Legionella* characteristically fails to grow on blood agar) or if the isolate produced browning of the yeast extract-tyrosine agar, the isolate was examined by the fluorescent antibody staining method with conjugated antisera [14].

### 2.6. Ability of *L. pneumophila* to grow in various pH ranges

The isolates of *L. pneumophila* were separately tested to find out their ability to withstand different pH conditions. Each isolate was inoculated in nutrient broth water with L-cysteine in different pH ranges starting from 6 to 8. The tubes were incubated and observed for the growth by means of formation of turbidity in the tubes. This method used was a modified method suggested by Garrison *et al.* [15].

### 2.7. Effect of different concentrations of chlorine on *L. pneumophila*

Earlier study was conducted on various variety of pathogenic bacteria for their tolerance against various chemicals [16]. Confirmed *L. pneumophila* isolates were individually tested for their resistance to the action of chlorine in different concentrations. Dilutions of free chlorine starting from 0.1 mg/L to 1.5 mg/L in normal saline were prepared. The diluted tubes with chlorine were kept in room temperature for 30 min. Then from each tube one loopful was cultured on BCYE agar medium and observed for the growth.

### 2.8. Ability of *L. pneumophila* to grow at various temperature optima

The *L. pneumophila* isolates were further tested for their ability to grow in different temperatures. Each isolate was sub-cultured and incubated at 25 °C, 30 °C, 35 °C, 37 °C and 45 °C, respectively.

### 2.9. Speciation of isolated strains of *Legionella*

Different phenotypic and biochemical characterization tests were performed to identify the isolated *Legionella* to species level. Tests like Gram reaction, glucose fermentation, urease test, nitrate test, oxidase test, catalase test, gelatinase test and fluorescent pigment production test were carried out as per Maisa *et al.* [17].

#### 2.9.1. Antibiotic profile studies on *Legionella*

Antibiotic sensitivity tests were carried out by disc diffusion method developed by Jarraud *et al.* [18] using Mueller Hinton agar incorporated with antibiotic discs like azithromycin (25 µg), clarithromycin (30 µg), erythromycin (35 µg), doxycycline

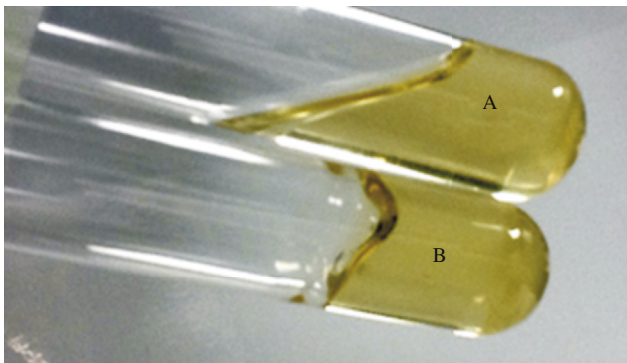
(32 µg), moxifloxacin (15 µg), cefuroxime (12 µg), cephalixin (14 µg), amoxicillin with clavulanate, ampicillin (23 µg) and vancomycin (20 µg) and incubated at 37 °C for 24 h.

### 2.9.2. Virulence and pathogenic potential of *Legionella*

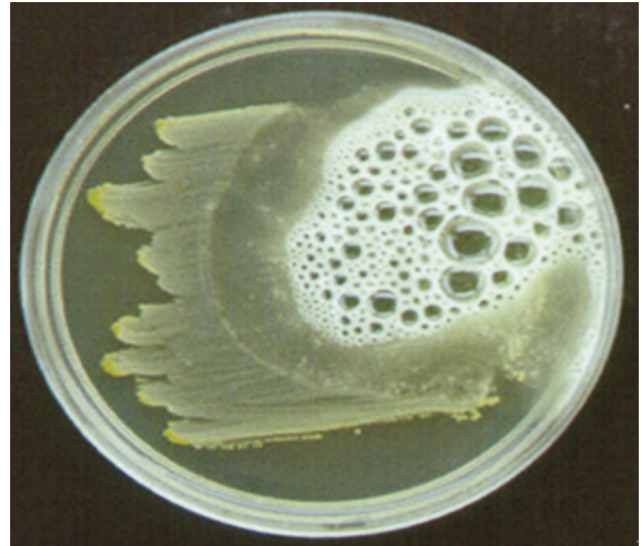
The virulence and pathogenic potential of isolated species of *Legionella* were carried out by injecting 0.1 µL of 24 h culture of *Legionella* in nutrient broth into intracerebral inoculation into suckling mice. Mortality of suckling mice was recorded.

## 3. Results

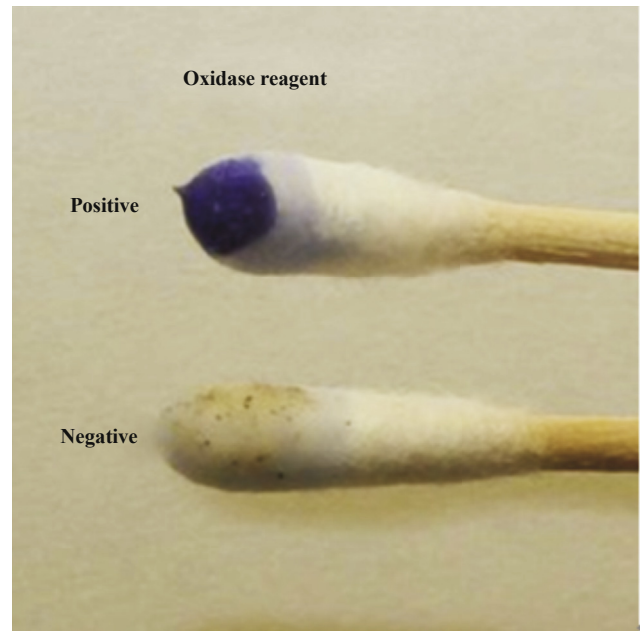
The rate of isolation of *L. pneumophila* in different water sources was found to be 20% (lakes), 10% (ponds), 8% (water-tanks) and 1% (rivers). It is also found that the isolation rate in summer seasons was higher when compared to winter and rainy seasons. Isolates of *L. pneumophila* showed the properties of pleomorphic Gram negative bacilli, asaccharolytic, urease negative, nitrate negative, gelatinase positive (Figure 1), catalase positive (Figure 2) and oxidase positive (Figure 3). The fluorescent pigments were best demonstrated by illuminating areas of heavy growth on tyrosine yeast extract medium with a long wave UV lamp. Phenotypic and biochemical characterization studies revealed that 80% isolates belonged to *L. pneumophila*, 7% belonged to *Legionella micdadei*, 6% belonged to *Legionella bozemanii*, 5% belonged to *Legionella feeleii* (*L. feeleii*) and 2% were *Legionella dumoffii*. The procedure employed in the current study of acid treatment of water samples prevented the growth of undesired bacteria thereby promoting the target bacteria (Figure 4). Since the water source is large, a new method for concentration of water sample was used in this study. The concentration of water sample after acid pre-treatment resulted in good growth of *Legionella* colonies on BCYE agar. All the isolates exhibited better growth in the pH range of 6–8. *L. pneumophila* showed growth even at normal level of chlorination of water. All isolates of *L. pneumophila* expressed good growth in the range of temperature between 25 °C and 45 °C. But it failed to grow at temperature below 25 °C and above 45 °C. Based on antibiotic sensitivity test, it was observed that *L. pneumophila* strains were resistant to all of the most commonly prescribed antibiotics. But *L. pneumophila* showed sensitivity to amoxicillin and clavulanate and vancomycin



**Figure 1.** Tube gelatinase test for *Legionellae*. A: Positive tube showing liquefied gelatin; B: Negative tube showing non liquefaction of gelatin.



**Figure 2.** Catalase test for biochemical characterization of *Legionellae*. Catalase positive test is indicated by brisk effervescence with air bubbles; catalase negative test is shown by no activity.



**Figure 3.** Oxidase test for *Legionellae*. Purple color indicates positive oxidase test. Light yellow color indicates negative oxidase test.

(Figure 5). Another species of *Legionella*, *L. feeleii*, also showed remarkable resistant patterns. This species *L. feeleii* expressed resistance to antibiotics like amoxicillin. We found that animal pathogenicity studies showed that *L. pneumophila* emerged as a crucial and most potent pathogen. All the suckling mice inoculated with *L. pneumophila* died suggesting *L. pneumophila* being the most virulent species. This clearly marked *L. pneumophila* as a dominant pathogen in *Legionellae* group of species. Interestingly, our studies also revealed another species of *Legionella*, *L. feeleii* which also caused 60% mortality in suckling mice. So this indicated that *L. feeleii* also may be a probable pathogen.



**Figure 4.** Culture of *L. pneumophila* on BCYE agar. Colonies of *L. pneumophila* are smooth with metallic sheen.



**Figure 5.** Antibiotic sensitivity profile of *Legionellae* on Mueller Hinton agar.

#### 4. Discussion

Initially when *Legionella* was discovered, it was revealed that many clinical cases were reported through the inhalation of aerosols from air handling coolers [19]. There were some cases also reported by human to human transmission [20]. Isolation of *Legionella* was conducted successfully from aerosols from air handling cooling towers [21]. Successful cultures were also made from sputum, bronchoalveolar lavage and tracheal aspirates of severely ill pneumonia patients [22]. Epidemiological studies also revealed that pneumonia outbreaks were due to inhalation of aerosols from air showers [23]. But today there were no attempts made to isolate pathogenic *Legionella* directly from water sources. There are also no studies available on speciation of

*Legionella* from water samples. Currently there are no successful attempts and established methods available to isolate *Legionella* followed by characterization, directly from water samples. Till today no scientific reports available on antibiotic profile of *Legionella* isolates from water bodies. Moreover, there is no study currently on identification of pathogenic potential of *Legionella* and its associated virulence.

This study expressed a good procedure and methodology using BCYE agar for the isolation of *Legionella* from different water samples. Isolation rates were also high by using this method. Good yield of *Legionella* was also facilitated by acid treatment of water samples. This acid treatment has rapidly decreased growth of unwanted bacteria that are present in the water samples thereby increasing the culture of *Legionella* isolates. Since the water samples were collected from water bodies like lakes, ponds, rivers and wells and water source is huge, it is extremely necessary to concentrate the water samples to obtain encouraging yield. The procedure employed for concentration of water sample also gives satisfactory results. The rate of isolation of *L. pneumophila* was found to be higher in lakes when compared to other water sources like ponds, water-tanks and rivers. This may be attributed to the large number of flora and fauna that support the growth of these bacteria. It is also found that the isolation rate in summer seasons was higher when compared to winter and rainy seasons. This may reveal the reason for more number of clinical cases of Legionnaire's disease and Pontiac fever during summer months when compared to winter and rainy months.

In this study, various parameters were assessed with regard to the growth of *Legionella*. It includes ability of *Legionella* to grow at various pH, different concentrations of chlorine and many different temperature optima. The studies on these criteria expressed the fact that *Legionella* can grow at wide range of pH, in the presence of high chlorine and many different temperature optima. This shows that *Legionella* can be a potential pathogen possessed with potent attributes of pathogenicity. Earlier studies indicated that *L. pneumophila* alone was the sole pathogen among the *Legionella* group. But the current study sparked a view that another species in *Legionella*, *L. feeleii*, can also be a potential pathogen. Concurrently our studies still emphasize that *L. pneumophila* is a major pathogen especially in public health point of view. All the isolates of *L. pneumophila* exhibited good resistance to various ranges of pH, chlorine and temperature. This may be the reason for this bacterium to survive in various water bodies under various parameters and cause severe disease in humans. Since this bacterium is transmitted mainly through water and causes water borne epidemics, stringent measures should be taken in controlling the infection.

Another major issue is antibiotic resistance among *L. pneumophila*. Isolated *L. pneumophila* strains directly from water samples were resistant to many commonly prescribed antibiotics. Another species of *Legionella*, *L. feeleii* also showed this property. Existence of antibiotic resistance among *Legionella* may be due to conjugation between *Legionella* or other unknown mechanisms.

All the isolates of *L. pneumophila* strains were highly pathogenic to suckling mice. Another species of *Legionella*, *L. feeleii* also showed significant pathogenicity. Among the group of *Legionella*, *L. pneumophila* and *L. feeleii* may be potential pathogens from water sources.

Serious and fatal *L. pneumophila* infections may be transmitted through water. Present study established that *Legionella* can

survive under various conditions in various water sources. Among the *Legionella* group, *L. pneumophila* is the important pathogen causing human disease. Our study also concluded that another species of *Legionella*, *L. feeleii* can also be a potential pathogen but to some lesser extent. Considering antibiotic resistance among *Legionella*, *L. pneumophila* and *L. feeleii* pose great challenge to public health care professionals because these *Legionellae* acquired antibiotic resistance to many routinely prescribed antibiotics. Since these *Legionellae* are commonly found in various water sources, and can be transmitted from these points to humans, extreme stringent precautions should be taken to prevent water borne outbreaks. Further research follow-up studies are needed on establishing molecular mechanisms behind virulence and antibiotic resistance of pathogenic *Legionellae*. Finally, presence of pathogenic *Legionellae* in water bodies especially in developing countries poses great public health risk and efficient and effective measures should be employed to curb the spread of this bacterial infection.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

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