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Microbiological features of virulent *Legionellae* detected from various water bodies

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ABSTRACT:

— ***Objective:*** *Legionellae* are free-living bacteria present in many types of water bodies. *Legionella pneumophila* can cause severe fatal pneumonia called Legionnaire's disease. These bacteria are commonly transmitted through air coolers. To examine the distribution and characterization of pathogenic *Legionellae* isolates from different water sources.

— ***Materials and methods:*** A sum of 500 water specimens (100 samples from each) was obtained from various water bodies in and around Jimma town, Ethiopia. Water specimens were treated with mild acidic solution, subsequently concentrated and cultured on *Legionella* selective BCYE agar medium. Criteria such as capacity of *Legionellae* to tolerate and produce growth in different pH optima, bactericidal effect of various levels of chlorine on *Legionella* isolates and lethal effects of divergent temperature conditions were framed. *Legionella* species distinctions were carried out using phenotypic (biotyping) tests. *Legionella* pathogenicity experiments on animal model and antibiotic susceptibility tests were performed on these bacterial isolates.

— ***Results:*** The prevalence and distribution of *Legionellae* in various types of water bodies were observed to be, wells (35%), lakes (30%), ponds (15%), water tanks (10%), and canals (10%). It was observed that many of the pathogenic *Legionellae* isolates can tolerate and multiply in a wide range of pH 5-9. *Legionella* can also withstand the usual concentration of chlorine in water bodies. Our experiments have also revealed that these bacteria can multiply at a variable range of temperature from 25 to 45˚C. The current study expressed that a total of 6 species of *Legionella* have been identified. Furthermore, it also suggested that *L. pneumophila* was identified more frequently than other species. Many isolated strains of *Legionellae* were also showing resistance to many commonly employed antibiotics. Instilling isolates of *Legionella* through intranasal route in guinea pigs proved that most of *L. pneumophila* strains were possessing high virulence followed by other species. Lethal and severe infections due to *Legionellae* can arise from the infection of these bacteria present in water bodies. These bacteria can tolerate and multiply in wide ranges of physicochemical conditions of water.

— ***Conclusions:*** Among the isolated species, *L. pneumophila* was the dominant and highly pathogenic species. Moreover, many isolates of *L. pneumophila* expressed multiple resistances to antibiotics. In spite of rapid development of medical field, health officials face a huge task ahead to curb the morbidity and mortality due to *Legionellae* especially in developing countries.

— ***Keywords:*** *Legionellae*, *Legionella pneumophila*, Distribution, Water bodies, Pathogenicity, Antibiotic sensitivity.

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INTRODUCTION

Many fatal infections due to *Legionella pneumophila* throughout the world were linked to a severe form of lung disease called Legionnaire's disease. *Legionellae* are Gram negative bacilli that usually have the habitat of living in freshwater bodies and most commonly humans acquire the infection through inhalation of water droplets contaminated with these bacteria[1].

In USA and developed countries, the main mode of infection is through air cooling showers and related sources[2]. But in developing countries, many clinical cases due to serious infection of *Legionellae* remain unreported due to improper detection strategies/misdiagnosis. In developing countries a few cases reported on *Legionella* are associated with its sources. In Ethiopia and other developing nations, people use fresh water sources like canals, lakes, wells, water tanks and ponds for their daily needs viz., drinking, cooking, bathing, swimming and other uses. These activities on water bodies containing contaminated *Legionella* can cause serious life-threatening consequences.

Due to lack of identification and isolation facilities *Legionellae* go unnoticed or misreported as other pneumonia causing microorganisms like *Streptococcus pneumoniae*, *Haemophilus influenzae* type-b, *Chlamydia trachomatis*, *E. coli*, *Mycoplasma pneumoniae*, *Klebsiella pneumoniae* or some viral etiology[3]. In most of the instances of Legionnaire's disease, *L. pneumophila* was the predominant pathogen encountered[4-6]. *Legionella* originally present in water bodies were transmitted through air handling equipment, air showers, hospital cooling towers etc[7]. Direct transmission of these bacteria from the water sources to humans without the mediation of cooling towers is not overruled.

Fewer studies have been conducted on the distribution and pathogenicity of *Legionellae* present in fresh water bodies. The genus *Legionella* consists of a total of 23 species, out of which predominant species include *L. pneumophila*, *Legionella gormanii*, *Legionella micdadei, Legionella bozemanii* and *Legionella dumoffii*[8]. Generally, apart from *L. pneumophila* other species are non-pathogenic saprophytes in fresh waters[9]. Severe diseases with high morbidity and mortality in developing countries can be caused by *Legionellae* present in water and may occur as water borne outbreaks. In addition to serious Legionnaire's disease, *L. pneumophila* may also cause Pontiac fever[10]. Other infections caused by *Legionella pneumophila* include lung sepsis, lung edema and lung necrosis[11]. The characteristic feature of Legionnaire's disease is typically a serious lung infection with pneumonia. Pontiac fever is a mild infection with fever without symptoms of pneumonia. *Legionellae* are omnipresent as aquatic, Gram negative, motile bacilli with different biochemical properties. Until now no extensive investigations have been formulated on identification, separation, classification, pathogenicity and antibiotic sensitivities of *Legionellae* isolated from various water sources.

The current study is directed to isolate *Legionella pneumophila* and other species from different water bodies and to characterize them.

MATERIALS AND METHODS

Collection of Water Samples

A sum of five hundred water specimens (100 samples from each) was collected aseptically from ponds, lakes, wells, water tanks and canals in and around Jimma town, Ethiopia. Every water specimen was collected in a sterile glass bottle and transported to microbiology laboratory in ice pack. Collections of these samples were carried out all throughout the year comprising of all seasons.

Pre-Treatment of Water Samples with Acid

To prevent the multiplication of unwanted bacteria and to increase the rate of isolation of *Legionellae*, every water specimen was pre-treated with 1:20 HCl-KCl (pH 2.0) treatment solution. This mixture was thoroughly mixed in a vortex mixture and incubated at room temperature for 30 min.

Strengthening and Culture of Water Samples

Strengthening of pre-treated water samples was executed by further centrifuging at 5000 rpm/15 min. Centrifuged deposits were aseptically inoculated onto buffered charcoal yeast extract (BCYE) agar plates. The BCYE agar plates were supplemented with polymyxin B, anisomycin and vancomycin for selection. These selective agars were placed in an incubator with 3% CO_2 at 37˚C. Slow growing bacterial colonies showing 'ground-glass' shape along with circular, convex, white, opaque, glistening and entire colony morphologies were isolated in pure culture, until 10 days in the incubator.

Presumptive Testing of Probable *Legionellae* Growth

Tentative *Legionellae* colonies were observed after Gram staining. For clear visibility, the counter stain employed was basic para fuchsin (0.2%). Every suspected *Legionellae* pure colonies were cultured in nutrient broth with 1.5% glucose sugar, containing Andrade's acid fuchsine indicator (0.1 gm/L). Biochemical tests for bacterial release of enzymes like nitrate reductase test and urease test were conducted. Test for synthesis of enzyme catalase was carried out with 2% H_2O_2 in 80.1% polysorbate, *N,N,N,N*-tetramethyl-*p*-phenylenediamine dihydrochloride was used as a substrate for testing the production of enzyme oxidase. Production of bacterial enzyme gelatinase was conducted in gelatin agar tubes, observed for liquefaction of gelatin after 24 hrs.

Authentication of *Legionellae* Colonies

Every suspected *Legionellae* colonies were grown on BCYE culture media without antibiotics with incubation up to 5 days at 37˚C. Pure colonies from these plates

were inoculated into 10% sheep blood agar and tyrosine yeast extract agar. Due to the fact that *Legionellae* cannot produce colonies on blood agar, and changes tyrosine yeast extract agar to brown color the isolates were confirmed by immune-fluorescent antibody [IFA] test using specific conjugated antibodies.

Separation of Isolated *Legionellae* into Separate Species

Various biochemical (Biotyping) tests were carried out to group the isolates of *Legionellae* into separate species using API 20NE and API ZYM rapid test kits (Biomerieux, Salt Lake City, UT, USA). In addition to these above-mentioned battery of tests, fluorescence emissions of *Legionellae* growth were also recorded.

Tolerance of *Legionellae* to Multiply in Various pH Conditions

Legionellae isolates were examined for its tolerance to multiply under various pH experimental setups. Isolates were allowed to grow in nutrient broth with amino acid L-cysteine in various pH setups ranging from 5-9, at 37˚C for 24 hrs. Culture tubes were examined for the production of 'turbid tubes'.

Effect of Various Chlorine Levels in *Legionellae* Growth

All *Legionellae* isolates were examined for their ability to withstand and grow in culture media containing various levels of chlorine. Different concentrations of chlorine ranging from 0.2 mg/L to 2.0 mg/L in physiological saline were made. The tubes were allowed to remain at 25˚C for 20 min. Every tube was sub cultured onto BCYE plates.

Capacity of *Legionellae* to Withstand Different Temperatures

Isolates of *Legionellae* were subjected to test their tolerance against various temperature conditions. All isolates were again inoculated onto BCYE agar plates and incubated at different critical temperatures like 20, 25, 30, 35, 37, 40, 45 and 50˚C.

Ability of *Legionellae* to Grow in Various Salt Concentrations

Isolated *Legionellae* strains were allowed to grow in nutrient broth containing different concentrations of sodium chloride. *Legionellae* isolates were inoculated and incubated in nutrient broth tubes added with different levels of sodium chloride (0.5, 1, 2, 2.5, 3 and 3.5%). *Legionellae* growths in the broth were examined by observing turbidity in the tubes.

Effect of Different Light Intensities on *Legionellae* Growth

Legionellae isolates were investigated for their ability to grow under various light intensities and their growths were recorded. All separate *Legionellae* strains were incorporated into nutrient broth tubes and exposed to different intensities of light (dark, 100 lux, 250 lux, 500 lux, 1000 lux, 1500 lux, 2000 lux, 2500 lux, 5000 lux and 10,000 lux). These broth tubes under these conditions were incubated at 37˚C for 24 hrs. Growth was noticed by observing the extent of turbidities in the tubes.

Antibiotic Sensitivity of Isolated Strains of *Legionellae*

Antibiotic susceptibility experiments were performed by standard antibiotic disc diffusion procedure on Mueller-Hinton agar culture plates incubated at 25˚C for 3 days. Antibiotic discs incorporated with erythromycin (35 µg), clarithromycin (30 µg), doxycycline (32 µg), vancomycin (20µg), cefuroxime (12 µg), cephalexin (14 µg), moxifloxacin (15 µg), amoxicillin with clavalanate, azithromycin (25 µg), ampicillin (23 µg) were included under this study.

Assessment of Pathogenicity of *Legionellae* Strains on Animal Model

Pathogenicity of the isolated *Legionellae* was assessed by instillation of 50 µl of *Legionellae* strains (grown in L-cysteine nutrient liquid broths) through intranasal inoculation into guineapigs. Animal deaths due to broncho-pneumonia were documented after 2-3 days.

RESULTS

The distribution and prevalence of *Legionella* spp. in various water bodies were observed to be, wells (35%), lakes (30%), ponds (15%), water tanks (10%), and canals (10%). During our study, we have observed that the frequency of isolation of *Legionella* spp was more in summer (75%) than rainy (15%) and winter (10%) seasons.

The phenotypic and biotypic properties of isolated stains of *Legionella* spp exhibited, Gram negative bacilli, pleomorphic, oxidase positive, nitrate reductase negative, gelatinase positive, catalase positive, urease negative and asaccharolytic. The ability of *Legionellae* strains to emit fluorescence under exposure of ultra violet (UV) lamp was exploited to recover the isolates of *Legionella* (grown on BCYE agar) from other contaminating bacteria in the water bodies.

This research project on *Legionellae* gave us information that out of all water samples analyzed (n=500), 100 water samples (20%) showed the presence of *Legionellae*. After detailed studies on biochemical characteristics and enzymatic experiments, we observed that

75% of *Legionellae* isolates were *Legionella pneumophila*, 12% L. *bozemanii*, 6% *L. feeleii*, 5% *L. dumoffii*, and 2% *L. micdadei*.

The present study on the isolation of *Legionellae* samples from large water bodies employed, pre-treatment of water source using mild acid prior to inoculation on to selective culture media. This acid treatment protocol suppressed the multiplication of unwanted bacteria and enhanced the growth of desired *Legionellae*. In this isolation strategy, the size of water sample used was too large enough for direct inoculation. To overcome this difficulty, we have introduced a novel, sample strengthening procedure followed by acid treatment of water samples. This sample strengthening method ensured significant growth of *Legionellae* colonies with emission of fluorescence upon exposure to UV light on BCYE culture plates (Figure 1).

In our study we demonstrated that every *Legionellae* isolate expressed good multiplication in the regulated pH set ups (5-9). In addition, we have also noticed that 90% isolates of *L. pneumophila*, 75% of *L. bozemanii*, 65% of *L. micdadei* and 50% of *L. feeleii* were able to multiply in the culture media containing 1 mg/L of chlorine. Every *Legionellae* isolate from all water sources indicated that they have the ability to withstand and multiply in the wide temperature range starting from 20 to 50˚C. *Legionellae* expressed no growth, below 20˚C and more than 50˚C.

When *Legionellae* strains were allowed to grow in nutrient broths with different levels of sodium chloride ranging from 0.5 to 3.5%, we have observed that very good turbid growths were seen in 0.5, 1, 2 and 2.5%. But less turbidity/growths were noticed in the concentration of sodium chloride, 3 and 3.5%. Experiments were also conducted to exhibit the effect of different light intensities on *Legionellae* growth. These experiments resulted in the nutrient broth tubes incubated in the dark, 100 lux and 250 lux showed less growth. Whereas the tubes incubated in the light intensities from 500, 1000 and 1500 lux exhibited a very high growth. But the tubes exposed to 2000, 2500, 5000 and 10,000 lux light intensities showed a very less growth.

To our surprise we have noticed that, based on the antibiotic susceptibility tests conducted on the isolates of *Legionellae*, most of the strains of *L. pneumophila* were resistant to many commonly used antibiotics against pneumonia and other respiratory infections. Fortunately, these strains were sensitive to vancomycin and augmentin. Another *Legionellae* member *L. bozemanii*, too exhibited significant antibiotic resistance. Experiments conducted on animal model to prove the animal pathogenicity of isolates of *Legionellae* on guinea pigs showed that *L. pneumphila* being the virulent member among the other *Legionellae*. These animal pathogenicity experiments also highlighted that the *Legionella pneumophila* was virulent compared to other species.

DISCUSSION

Legionellae and its association with human diseases were discovered in the year 1976 after an outbreak of deadly pneumonia in Philadelphia, USA[12]. Till then, numerous fatal pneumonia cases have been associated with *L. pneumophila* throughout the world[13]. All of these studies were conducted primarily in western and developed countries. Unfortunately, no data are available on *Legionellae* and its disease attribution in devel-

Figure 1. *Legionella* colonies on BYCE agar medium (Image source: Thermo Fisher Scientific).

oping nations. Previous investigation revealed that the transmission of *Legionellae*, occurred through air cooling showers, air coolers and air conditioning ducts that uses water contaminated with *Legionellae*[7].

There are no data currently present to correlate the presence and the transmission of these bacteria directly from their habitat, water bodies to humans. In large part of the world, especially in developing countries fatal pneumonia cases may be occurring due to the direct transmission of *Legionellae*[14]. But due to lack of sufficient lab diagnosis and scientific knowledge, this bacterial pneumonia might have been gone unreported or misreported.

Numerous data are available on the attribution of fatal pneumonia (Legionnaire's disease) with *L. pneumophila*[15]. *L. pneumophila* was associated with human cases of mild Pontiac fever. But no data is currently available to relate the pathogenicity of other *Legionellae* members with severe human disease.

During this study we have made efforts to bring out the 'missing gap' to bridge the distribution and pathogenicity of *Legionellae* present in various water bodies. *L. pneumophila* can be transmitted via aerosol through cooling showers. Our study demonstrated that *L. pneumophila* and other *Legionellae* are abundantly present in various water bodies in significant proportions.

In developing countries like Ethiopia, common people use the water sources such as lake well, ponds and river for their day-to-day livelihood[16]. So, these water bodies may act like a primary infective source for *Legionellae*. Earlier studies elaborated the isolation of *L. pneumophila* from contaminated aerosols[17]. The present study proved that both *L. pneumophila* and other *Legionellae* members can be isolated directly from these waters. An earlier investigation gave us information about the isolation protocols for *L. pneumophila* from human clinical specimens[18].

Our study used a combination of novel isolation strategies for *L. pneumophila* and other *Legionellae*, like pretreatment of water samples, concentration followed by inoculation onto selective media. It is a well-known fact that bacterial culture isolation from a large volume of water sample is very difficult. There are techniques like membrane filtration method of bacterial isolation from water sources[19]. But these isolation procedures are very expensive to afford by all diagnostic laboratories. So, we have introduced a novel, inexpensive culture of *Legionellae* directly from water samples after concentration and strengthening of the water samples.

Researchers conducted earlier clearly stated that *Legionellae* were present in various types of waters. Our study concluded that the distribution of *Legionellae* was high in wells (35%). The prevalence of these bacteria in other types of water sources were lakes (30%), ponds (15%), water tanks (10%), and canals (10%). The high isolation rate of *Legionellae* in well and lake waters may be due to its stagnancy, presence of flora & fauna and suitable environmental conditions that may enhance its biofilm formation[20]. But, as of today no reports are available to enumerate this pathogen's ability to withstand various parameters like pH, temperature, chlorine, salinity and exposure to light. Because these criteria are very crucial to determine the increased bacterial tolerance and survivability in the harsh environment. So, our study focused on the isolation of *Legionellae* and the ability of these bacteria to tolerate these conditions (Table 1). Bacteria present in environmental water sources showed that the most virulent bacteria possessed enhanced endurance to survive in harsh environmental waters[21]. These parameters include high pH, wide temperature range, various salt concentrations etc. In our study we have found that *Legionellae* can survive in water with pH range 5 to 9. This clearly showed that *Legionellae* can withstand low as well as high pH changes.

This study also suggested that isolates of *Legionellae* from different water bodies can tolerate low and high temperatures. This property of *Legionellae* concluded that these bacteria can withstand and multiply various water temperatures, suggesting the disease transmission in different seasons. The current study revealed that most of isolates of *Legionellae* can multiply in high concen-

Table 1. Distribution of *Legionellae* with analysis of physicochemical parameters.

	Physicochemical parameters analyzed							
Species of *Legionellae*	**Prevalence (%)**	**pH[a]**	**Temperature[a] (°C)**	**Chlorine[a] (mg L^{-1})**	**Salinity[a] (%)**	**Light intensity[a] (Lux)**	**Antibiotics[b]**	**Animal Pathogenicity[c] (%)**
Legionella pneumophila	75	5-9	20-50	1	0.5-2.5	500-1500	van, amc	97
L. bozemanii	12	5-8	30-40	0.8	0.5-2.0	500-700	van, amc, ery, cef	67
L. feeleii	6	6-7	35-45	0.7	0.5-1.5	500-1000	van, amc, ery, cef, clar	50
L. dumoffii	5	7 - 8	40-45	0.5	0.5-1.0	500-1500	van, amc, ery	20
L. micdadei	2	7 - 9	20-45	1	0.5-2.5	500-1000	van, amc, amp, amx	5

Keys to abbreviations:
[a]: These parameters in which best growth with maximum turbidities observed.
[b]: Isolated strains expressed sensitive to antibiotics.
[c]: Percentage of animal deaths.
van: vancomycin; amc: amoxicillin & clavulanate; ery: erythromycin; cef: cefotoxime; clar: clarythromycin; amp: ampicillin; amx: amoxicillin.

tration of chlorine (1 mg/L of chlorine). This suggested that there is a need for hyper chlorination of water bodies like wells, swimming pools, lakes etc., in order to eradicate *Legionellae* present, if any. To our surprise we have observed that *Legionellae* have the ability to grow in water containing low and high salt concentrations (0.5, 1, 2 and 2.5%). This property of *Legionellae* proved that these bacteria can be present in brackish or estuarine waters, and may be transmitted from them. During our study we have set an additional criterion to check *Legionellae* ability to grow in different light intensities.

The results of this test highlighted that *Legionellae* can grow and multiply in different intensities of sun lights in different seasons behaving like a highly virulent pathogen. Antibiotic susceptibility test conducted on isolated strains of *Legionellae* disclosed the fact that this group of bacteria possessed antibiotic resistance to many antibiotics normally employed against respiratory infections. Our animal model experiments conducted on isolates of *Legionellae* suggested that these bacteria are highly virulent. These animal studies gave us information that, along with *L. pneumophila,* other species of *Legionella* might have the ability to cause pneumonia in humans. Other *Legionellae* members like *L. bozemanii* and *L. feeleii* have also carried significant virulence on animal model suggesting that these species can also cause Legionnaire's disease or Pontiac fever in humans that need further evaluation.

Conflict of Interest:

The authors declare that they have no conflict of interests.

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