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Identification of *Legionella* from clinically diagnosed pneumonia patients and environmental samples

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Abstract

Legionnaires' disease is a multisystem disease with life-threatening acute and severe form of pneumonia which is responsible for 2-9% pneumonia with high mortality. Eighty six respiratory tract samples and urine were collected from clinically diagnosed pneumonia patients and 12 water samples were collected from different environment. Identification of *Legionella* was done by culture and Polymerase Chain Reaction (PCR) of respiratory tract samples and environmental samples and *Legionella* Antigen (Ag) in urine was detected by Immunochromatographic test (ICT). *Legionella was* identified from 4 (4.65%) clinically diagnosed pneumonia patients of which 1 (1.16%) case was culture positive, 1 (1.16%) case was urine ICT positive and PCR was positive in all four cases. Of the 12 water samples tested, 4 (33.33%) samples were *Legionella* positive by PCR but culture results of these samples were negative. Identification of *Legionella* should be done by PCR in parallel with culture and urine ICT. Detection of *Legionella* in environmental samples is also needed to explore possible links between the water sources and disease transmission in population.

Introduction

Legionnaires' disease (LD) is a multisystem disease with life-threatening acute and severe form of pneumonia caused by several species of *Legionella.*[1] It is responsible for 2-9% pneumonia with a mortality rate between 5% to 10%[1] and this rate may exceed 50% for elderly or immunocompromised patients.[2] Legionnaires' disease can account for up to 30% of pneumonia requiring admission to intensive care unit (ICU).[1] Currently, there are 50 species comprising 70 distinct serogroups in the genus *Legionella* of which 20 species have been reported to cause human pneumonia.[1] Approximately 90% *Legionella* induced pneumonia is caused by *Legionella pneumophila* which comprises 16 different serogroups (sg).[1] There is strong evidence that the risk of Legionnaires' disease (LD) might be higher under certain environmental condition like warm, humid and rainy weather, thus this weather shown associated with higher incidence of Legionnaires' disease in tropical countries.[3,4] Bangladesh is a tropical country with monsoon-type climate including hot, humid and rainy summer which might be associated with higher incidence of Legionnaires' disease.

In Bangladesh, annual rate of new pneumonia cases is six million which is the world's fourth highest annual pneumonia rate.[5] A study has been carried out in Bangladesh which reported 3% children with pneumonia admitted in a hospital had high antibody titre against *Legionella* species.[6] However, no study has been done in Bangladesh to identify the *Legionella* in adult pneumonia patients. In many studies, *Legionella* has been found to be a common cause of pneumonia and the clinical manifestations were more severe and the mortality was higher when compared with pneumonias of other aetiology.[7] However, this is due to delay in diagnosis and suboptimal antibiotic therapy, rather than enhanced virulence of *Legionella.*[7] The rate of *Legionella* pneumonia amongst adults in our country may be underestimated owing to practical difficulties such as lack of a productive cough and availability of diagnostic methods.[8] Pneumonia in adult and elderly patients is a common and serious problem that has different clinical presentation and higher mortality.[9] Elderly patients may present with atypical symptoms other than the typical respiratory symptoms of pneumonia that lead to delay in diagnosis and initiation of treatment and may be responsible for higher observed mortality.[10]

Considering long summer climate, wet humid weather patterns and high incidence of pneumonia in Bangladesh, it is imperative to study the existence of *Legionella* by early detection for timely intervention and the correct choice of antimicrobial therapy to reduce the mortality. Moreover, *Legionella* pneumonia is acquired by

inhalation of aerosolized water or microaspiration of water droplets contaminated by *Legionella* from natural or man made water sources such as ponds, lakes, potable water systems (e.g., showerheads, taps) and air conditioning systems.[11] Besides, as the transmission of *Legionella* from person to person has never been observed[12], prevention needs to concentrate on the elimination of this pathogen from water and aerosol producing systems. Thus, rapid and precise detection of *Legionella* in water systems is very important for risk prediction and the elimination of *Legionella* from possible infection sources.[1] So, this study has been designed to identify *Legionella* from clinically diagnosed pneumonia patients of adult age group by culture, urine ICT and PCR and from environmental samples by culture and PCR.

Materials and Methods

Specimens: Respiratory tract samples were collected from 86 clinically diagnosed pneumonia patients admitted in General Medicine ward and ICU of Bangabandhu Sheikh Mujib Medical University (BSMMU) during the period of August 2012 to July 2013. Of which 50 were sputum samples and 36 were tracheal aspirates (TA). Urine was collected from all 86 patients.

A total of 12 water samples were collected from different environment. Three water samples were obtained from three ponds, one from a hotel, five from General Medicine ward and ICU of BSMMU and two from biosafety laboratory of Department of Microbiology and Immunology, BSMMU. One shower head swab sample was collected from General Medicine ward, BSMMU.

Sample processing[13]: Thick sputum samples were diluted by PBS. Sputum or TA was mechanically homogenized by vortexing for five minutes. After vortexing, sputum and TA were centrifuged at 3000g for 10 minutes. Supernatant was discarded using a sterile pipette and concentrated sediment was resuspended to be used for culture and PCR.

Water samples were concentrated by filtration by pouring the samples into sterile 47 mm filter funnels assembly containing a 0.22 µm polycarbonate filters. After filtration, the filters were removed aseptically from the holder with sterile forceps and placed into a sterile 50 ml centrifuge tube containing 5ml of sterile water. The centrifuge tube was then vortexed for one minute to free bacteria and organic material from the filters and the water was kept for culture and PCR. Swab containing tube was shaken vigorously for two minutes to release the bacteria. Swab stick was discarded and the water was kept for culture and PCR[14].

Culture[14-16]: The sputum, TA and water were divided into two categories: untreated and acid-treated by 0.2 M KCl-HCl buffer (pH-2.2). For acid treatment, approximately 0.5 ml of specimen was added to 2.5 ml of a 0.2 M KCI-HCI solution to achieve a 1:5 dilution and kept for five minutes. Approximately 0.01 ml of both processed sample without decontamination and processed sample decontaminated by acid buffer were inoculated on the selective buffered charcoal yeast extract (BCYE) medium (HiMedia, India) supplemented with L-cysteine, iron and antibiotics and BCYE medium without antibiotics. The media were incubated at 37°C and were examined daily for seven days for the presence of growth. The plates were held for a maximum of two weeks before being discarded as negative. If growth was present then identification of the *Legionella* was done by colony morphology, gram staining, biochemical test and PCR. Round, glistening, convex, 3-4 mm diameter, frosted glass colonies were used for identification. Gram staining was done to see the thin, faintly stained gram-negative morphology of *Legionella*. *Legionella* were catalase-positive, oxidase positive, liquefy Gelatine and *Legionella pneumophila* hydrolyses sodium hippurate.

Urine Ag Immunochromatographic test (ICT): Legionella pneumophila serogroup one urinary antigen was detected by *Legionella* ICT kit (Binax NOW) following the manufacturer's instructions.

PCR: The test was carried out by amplification of *Legionella* gene fragment 16SrRNA with the use of primers pair JFP and JRP. The sequences of the used primers were as follows[17]:
Forward primer JFP:
5' –AGGGTTGATAGGTTAAGAGC-3';
Reverse primer JRP:
5' –CCAACAGCTAGTTGACATCG-3'.

PCR was performed in a final reaction volume of 25 µl in a PCR tube, containing 12 µl of master mix (mixture of dNTP, taq polymerase, $Mgcl_2$ and PCR buffer), 1 µl of forward primer and 1 µl of reverse primer (promega corporation,USA) of *Legionella*, 3 µl of extracted DNA and 8 µl of nuclease free water. After a brief vortex, the PCR tube was centrifuged in micro centrifuge for few seconds. Amplification was carried out in DNA Thermal Cycler and comprised initial denaturation at 95°C for 15 minutes followed by 36 cycles consisting of 94°C for 30 s, 59°C for 40 s, and 72°C for 40 s and a final extension at 72°C for 20 min. Then the product was held at 4°C. After amplification product was processed for gel documentation or kept at - 20°C till tested. The size of the amplified DNA fragments was 386 bp. PCR products were

identified by electrophoresis in 2% agarose gel with ethidium bromide.

Results

Out of 86 pneumonia patients, 4(4.65%) cases were *Legionella* positive of which 1(1.16%) case was culture positive, 1(1.16%) case was urine ICT positive and PCR was found to be positive in 4(4.65%) cases. Out of 86 pneumonia patients, 2(2.33%) cases were only PCR positive, 1(1.16%) case was both culture and PCR positive and 1(1.16%) case was both urine ICT and PCR positive. No case was only culture positive or urine ICT positive. Both culture and urine ICT positive cases were also positive by PCR (Table I).

Table I: Frequency of *Legionella* detection by culture, urine ICT and PCR in pneumonia patients (n=86)

Methods	Positive	
	No.	%
Only culture positive	0	0
Only Urine ICT positive	0	0
Only PCR positive	2	2.33
Culture and PCR positive, ICT negative	1	1.16
Urine ICT and PCR positive, Culture negative	1	1.16
Total	4	4.65

Identification of *Legionella* from tracheal aspirate and sputum by culture and PCR and from urine by ICT is shown in Table II. Among 86 respiratory tract samples, 36 were tracheal aspirates and 50 were sputum. Out of 36 tracheal aspirates, *Legionella* was positive by culture in 1(2.77%) case and 3(8.33%) cases were identified by PCR. Out of 50 sputum specimen, no case was *Legionella* culture positive but 1(2%) case was identified by PCR. Out of 86 urine samples, 1(1.16%) case was positive by urine ICT.

Table II: Identification of *Legionella* from tracheal aspirate and sputum by culture and PCR and from urine by ICT

Name of specimen	No. of specimen	Culture positive No. (%)[a]	PCR positive No. (%)[a]	ICT positive No. (%)[a]
Tracheal aspirate	36	1(2.77)	3(8.33)	NA*
Sputum	50	0 (0)	1(2.00)	NA
Urine	86	NA	ND**	1(1.16)

[a] Percentage done on individual total sample
* NA- Not applicable, ** ND-Not done

The distribution of *Legionella* detected from environmental samples by culture and PCR is shown in Table III. Out of 12 environmental samples, 4(33.33%) samples were found to be *Legionella* positive of which *Legionella* was detected in 3(25%) ponds and 1(8.33%) supply water in General Medicine ward by PCR, but the culture results of these samples were negative.

Table III: Distribution of *Legionella* detected from environmental samples by culture and PCR (n=12)

Source		Total	Culture positive	PCR positive
			No. (%)	No. (%)
Pond water		3	0 (0)	3 (25)
Hotel water		1	0 (0)	0 (0)
ICU	Supply water (n=1)	3	0 (0)	0 (0)
	Air condition (n=1)		0 (0)	0 (0)
	Dialysis Machine (n=1)		0 (0)	0 (0)
General Medicine ward	Supply water (n=1)	3	0 (0)	1 (8.33)
	Air condition (n=1)		0 (0)	0 (0)
	Shower swab (n=1)		0 (0)	0 (0)
Biosafety lab	Supply water (n=1)	2	0 (0)	0 (0)
	Air condition (n=1)		0 (0)	0 (0)
Total		12	0 (0)	4(33.33)

Discussion

The mortality associated with pneumonia due to *Legionella* makes it the "atypical" pathogen.[8] Many first-line antibiotics commonly used to treat typical bacterial pneumonias (i.e., beta-lactams) are ineffective against *Legionella* species.[18] This is at least partially due to the fact that *Legionella* are intracellular pathogens. So, there is a need for a more rapid and specific methods for timely intervention and the correct choice of antimicrobial therapy to reduce the mortality.

In this study *Legionella* were identified in 4.65% pneumonia patients which is a close finding of a large study in Germany among 2503 pneumonia patients showing 3.8% *Legionella* infection by culture, urine Ag detection and PCR.[19] In the present study isolation of *Legionella* was 1(1.16%) in 86 pneumonia patients. This finding is consistent to a large study conducted in Germany that reported 0.6% *Legionella* isolated from 479 respiratory materials of pneumonia patients.[19] Another study in Iraq also found 0.6% *Legionella* positive by sputum culture in 295 lower respiratory tract infection patients.[15] Though a study in India in 1991 reported a higher rate (9%) of *Legionella* in respiratory specimen of pneumonia patients by culture[20] but recent study in South India isolated 2.55% *Legionella* in 470 respiratory samples by culture and biochemical analysis.[16] Another study at New Delhi reported all 51 respiratory tract samples of pneumonia patients to be negative by *Legionella* culture.[21]

Low yield of *Legionella* was mentioned in various studies attributed to some reasons. *Legionella* pneumonia is often accompanied by little sputum or other respiratory secretions.[1] As a result, low number of organisms present outside of lung tissue and inhibitory effect of other bacteria of the lower respiratory tract present in the sample may reduce the sensitivity of culture.[22] Moreover, other reasons for poor sensitivity of culture may be that some

Legionella become viable but cannot be grown on routine *Legionella* culture media.[1]

In this study, out of 86 pneumonia cases, *Legionella* urine ICT was positive in 1(1.16%) case. This result is consistent with the study by Song et al. that reported 1.1% positive *Legionella* urine ICT among 648 hospitalized adult pneumonia cases in eight Asian countries.[9] Miyashita et al. in Japan also reported 1.5% positive urine ICT in 400 hospitalized pneumonia patients.[23] Though higher rate of infection by *Legionella pneumophila* sg 1 was reported in some studies[21], sensitivity of the urinary antigen detection depends on several circumstances. If the proportion of pneumonia cases included in the respective studies is not definitively caused by *L.pneumophila* sg 1, the urine Ag test will be negative[24] as urine ICT detects only *L. pneumophila* sg 1 infections. Moreover, it has been reported that 60% of *Legionella* pneumonia patients excrete the antigen intermittently.[24] So, a negative urinary antigen result does not exclude *Legionella* infection.

In the present study, *Legionella* was detected in 4(4.65%) pneumonia patients by PCR which was almost similar to the results reported by Jonas et al. and Weir et al.[17,25] The *Legionella* specific PCR assay described in this study targets the 16S rRNA gene which is very sensitive.[13] In the present study among the four PCR positive cases, culture was positive in one case. PCR finding of this study is corroborated by other studies using conventional PCR methods and many of these authors showed PCR to have a higher rate of detection than culture-based methods.[13,17, 26-28]

Respiratory samples may be contaminated with oropharyngeal organisms that overgrow and mask *Legionella* to grow in culture.[24] Another contribution to a lowered sensitivity of culture is the use of samples collected from patients currently being treated with antibiotics. A PCR test is likely to overcome these issues and indeed, PCR is considered the test of choice for patients who produce sputum by some authors.[26] Though culture of respiratory samples can detect multiple species, but it may take up to five days for results. *Legionella* specific PCR of respiratory samples that targets a 386-bp portion of the 16S rRNA gene can be performed rapidly in 6-8 hours.

In the present study, out of 12 environmental samples, 4(33.33%) were positive by PCR but not in culture. This finding is similar to the study by Pasculle et al., Anbumani et al., and Al-Matawah et al.[16,29,30] Discrepancies between PCR and culture results in this study can be explained by several factors. *Legionella* growth can be inhibited or masked by overgrowth of contaminating microorganisms that leads to the poor sensitivity of water culture (10 to 30%).[30] Furthermore, *Legionella* can enter a viable but noncultivable (VBNC) state, from which it can recover after passage in amoebae.[31] These VBNC *Legionella* may be detected by PCR, along with dead bacteria, possibly explaining, why PCR values are usually higher than those obtained by culture.

This study identified high rate of *Legionella* (33.33%) in environmental samples but the rate was 4.65% in pneumonia patients. A possible explanation for the relatively decreasing rate of *Legionella* in pneumonia patients may be a more widespread indiscriminate use of fluoroquinolones and macrolides as they are available over the counter in our country[32] and *Legionella* is susceptible to these drugs. Prospective studies on larger number of patients are required to substantiate these findings.

This study was not designed specifically to address the epidemiologic link between presence of *Legionella* in environmental and public water sources and acquisition of *Legionella* pneumonia. This study was conducted to determine if the natural and man made water sources are contaminated with *Legionella* and to give the decision maker a view about the situation. In this study, *Legionella* has been identified from tracheal aspirates and sputum of patients admitted in the hospital with pneumonia acquired in the community. So, different water samples from ICU and medicine ward were studied so as to alert hospital infection control committee about its consequences. In Bangladesh, this study is first time performed to detect *Legionella* in environmental, public and hospital water samples as well as in clinical samples of pneumonia patients to identify the bacteria in these sources. A possible link between the water sources and disease transmission in population may need to be explored as there is no human to human transmission and the disease is acquired by inhalation or microaspiration of water droplets contaminated by *Legionella* from environmental sources.

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