Urinary antigene and PCR can both be used to detect *Legionella pneumophila* in children's hospital-acquired pneumonia

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Abstract

Legionella pneumophila is the causative agent of more than 95% cases of severe *Legionella* pneumonia. Nosocomial pneumonias in different hospital wards is an important medical and pharmaceutical concern. This study aimed to detect *Legionella* with two methods: polymerase chain reaction (PCR) and detection of urine antigenic test (UAT) in patients suffering from nosocomial pneumonia admitted to pediatric intensive care unit (PICU) of children hospitals. This study was conducted in PICU wards of Rasool Akram and Bahrami children hospitals, Tehran, Iran during 2013 - 2014. In patients diagnosed with hospital-acquired pneumonia, intratracheal secretion samples for PCR and urine sample for UAT were taken. Simultaneously, PCR and urinary antigen test were conducted using commercial kits. The results of urinary antigen test and PCR were analyzed by SPSS v.19 for statistical comparison. In this study, 96 patients aging 2.77 years on average with two age peaks of less than 1 year and 7-8 year were enrolled. More than half of the patients were under 1 year old. The most common underlying diseases were seizure, Acute Lymphoblastic Lymphoma, Down syndrome and metabolic syndromes. The positivity rate of Legionella urinary antigen test was 16.7% and positivity rate of PCR test was 19.8%. There were no significant associations between the results obtained by both assays with age, gender or underlying diseases. In conclusion, PCR is a better detection method for *Legionella* infection than urinary antigen test, but the difference between the two methods was not significant.

Key Words: *Legionella pneumonia*, Polymerase chain reaction, Urinary antigen test, hospital-acquired pneumonia, pediatric intensive care unit.

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L*egionella* are Gram-negative, aerobic bacteria residing in aquatic environments as an intracellular parasite of free-living amoeba which can cause varying degrees of respiratory infections in communities, hospitals and health-care-retirment homes.[1] Legionellaceae family contains over 50 species and 64 serogroups. *Legionella Pneumophila* species cause approximately 90% of *Legionella* infections,[2] and Legionnaires' diseases and of nosocomial pneumonia.[1,2] Serogroup 1 of *Legionella Pneumophila* is considered to be responsible for the majority of the reported cases of *Legionella* infection.[3] Approximately 1 to 30 percent of hospital-acquired pneumonias arise from *Legionella*.[1, 2] Mortality rate varies in different patients and this rate is higher in elderly and immunocompromised people in whom 80 percent mortality rate is reported.[3] One of the peculiar features of this bacterium is hardly staining. Thus, base fusin is recommended instead of safranin for its staining. Isolation and culture of this bacterium is possible in charcoal yeast extract selective medium.[4] This bacteria can act in sporadic and epidemic forms, simultaneously. On the other hand, simultaneous infection of *Legionella* with other respiratory pathogenic agents such as

streptococcus pneumonia, *haemophilus influenza* and *mycobacterium tuberculosis* can lead to more complicated health problems. Patients suffering from *Legionella* do not have serious risks if they diagnosed and treated in the initial phase of the infection. Treatment course lasts 10-14 days.[5] People with normal immune system show good response to therapy while treatment in immunocompromised patients have delayed or even failured treatment and diagnosis.[6] Bronchial aspiration is the best candidate specimens for diagnostic which have approximately 90% of sensitivity, while sputum specimens show sensitivity around 70 percent.[7] Generally, bacterial culture, direct fluorescent antibody staining and tracking antigen in urine are used to indentify *Legionella* in clinical samples.[8] The gold standard method for diagnosing *Legionella* is bacterial culture, but delayed incubation time and the negative effect in culture when antibiotic therapy is present, are disadvantages of the method. Bronchoalveolar Lavage (BAL) samples can be stained with Giemsa solution and check with an optical microscope; but direct fluorescent antibody test is more accurate with a sensitivity of 70 %. Newer assays have been developed for identification of *Legionella pneumophila* soluble antigens in urine which show 75 to 90 percents of sensitivity.[9,10] A comprehensive and complete diagnostic method with higher sensitivity rate, capable of diagnosing the initial disease phases and asymptomatic patients, is essential. In this study we compared the PCR and urinary antigen methods to detect *Legionella pneumophila* in hospitalized patients.

Materials and Methods

This cross-sectional study was conducted in pediatric intensive care unit (PICU) wards of Rasool Akram hospital complex and Bahrami hospital in Tehran through 2013 to 2014. Intratracheal secretion samples for PCR and urine sample for urinary antigen test were taken from patients with diagnosed hospital-acquired pneumonia. Samples were collected in sterile tubes and stored in -20'C until analysis. Urinary antigen test was performed with Alere CORIS BioConcept® Legionella Urinary Antigen Card. Real time PCR investigations were executed base on the manufacturer's instructions in order to These PCR tests were run for detection of the 16s ribosomal gene in *L. pneumophila* species. The sequence of the forward primer was 5′-GTTAAGTCCCGCAACGA-3′ from GenBank entry N315 (NC-002745), positions 1100 to 1116. The sequence of the reverse primer was 5′-AGGAGGTGATCCAGCC-3′, from GenBank entry N315 (NC-002745), positions 1551 to 1536.The PCR was performed on a model 9600 GeneAmp PCR system (Applied Biosystems). 10 µL of RT-PCR Master mix, 1 µL of the primer/probe, 4 µL of distilled water free of RNAse/DNAse and 5 µL of the samples from patients were added to each reaction. After 10 minutes of denaturation at 95°C, Polymerase chain reaction was run for 35 cycles at 90°C for 20 seconds and 65°C for 50 seconds. The results of urinary antigen test and PCR were analyzed by SPSS v.19 for analytical and descriptive comparisons.

Results

In this study 96 patients were evaluated which 52.1% of them were females. The patients' had an age with an average 2.77±2.72, of which 56.2% had an age lower than 1 year age. Two age peaks were observed. The first peak was under 1 year age (32%) and the other one was between 7-8 years of age (19%). The most common underlying diseases were seizure (n= 24), acute lymphoblastic leukemia (ALL) (n=12), Down syndrome (n=10) and metabolic syndrome (n= 8) respectively. The remaining 45 patients were affected with other diseases presented in table 1. There was no association between occurrence of underlying disease with age (p=0.338) or gender (p=0.710). *Legionella* urine antigen test was positive in 16 cases (16.7%) whereas PCR was positive in 19 patients (19.8%). All positive cases in urinary antigen test were also positive for PCR. No significant association was found between urinary antigen test/PCR positivity with age, gender and underlying diseases, but there was a trend toward a strong association in the case of "drowning". There 6 cases of drowning, which 2 cases of them were positive for both urinary antigen test and PCR (33.3%) but this figure didn't reach statistical significance (p=0.09). 12 cases (12.5%) acquired pneumonia after initiating ventilation while 84 cases (87.5%) had pneumonia before ventilation. Out of 12 post-ventilation pneumonia cases, 3 cases occurred less than 5 days after ventilation (25%) while 9 other pneumonia cases appeared after 5 days of ventilation. There was a strong association between the time of onset of post-ventilation pneumonia and disease course. The mortality rate in after-5-days pneumonia patients (6 out of 9) was significantly higher than the other group of patients (0 out of 3) (P<0.0001). The final clinical outcome displayed death in 12 cases (12.5%), while the remaining patients were discharged either without complications (55.2%) or with some health complications (32.3%). There was no significant association between disease course with gender (p=0.223), age (p=0.157), urinary antigen test positivity (p=0.162) or PCR positivity (p=0.728). Overally, the most common symptoms of patients were respiratory distress in 51 cases followed by abnormal X-ray in 41 cases and cyanosis in 26 cases.

Discussion

Our patients could be categorized into two age peaks. The first peak in our study was similar to Huoug Ple et al. study,[11] in which they concluded that the highest rate of atypical pathogens pneumonia occurs in patients under 2 years of age. The second peak in our study was not reported in any other study. The disease complications in these patients were milder than those in the first age peak,

Table 1. Basic Characteristics of the study

Underlying condition	*No. of patienrs (Percent)*	*No. of positive PCR cases*	*No. of positive Urinary Antigen test cases*	*Age Interval (Mean)*	*Gender*	
					Male	*Female*
Cardiomyopathy	4 (4.16%)	1	1	2 mo – 11 mo (5.3 mo)	1	3
SMA	1 (1.04%)	0	0	8 years	0	1
Hypoglycemia	3 (3.12%)	0	0	2 mo – 11 years (5.8 yr)	2	1
LOC decrease	5 (5.2%)	1	0	6 mo – 9 years (4.5 yr)	4	1
Poor Feeding	3 (3.12%)	0	0	3 mo	2	1
Myopathy	3 (3.12%)	0	0	6 yr – 8 yr (6.9 yr)	3	0
ALL	12 (12.48%)	3	3	2 yr – 10 yr (7.3 yr)	4	8
Asphyxia	2 (2.08%)	0	0	45 d – 2 mo (1.7 mo)	0	2
CP	3 (3.12%)	1	1	2 yr – 7 yr (5 yr)	3	0
Seizure	26 (27.08%)	5	4	3 mo – 11 yr (6.9 yr)	11	15
Drowning	4 (4.16%)	2	2	4 yr – 10 yr (8.1 yr)	2	2
Down Syndrome	10 (10.4%)	1	0	5 mo – 10 yr (6.9 yr)	4	6
Metabolic Disease	8 (8.32%)	3	3	3 mo – 6 yr (4.5 yr)	5	3
Gastroentritis	3 (3.12%)	1	1	1 yr – 8 yr	2	1

Note: SMA (Spinal Muscular Atrophy); LOC (level of conciousness); ALL (Acute Lymphoblastic Lymphoma); CP (Cerebral Palsy); GERD (Gastro-esophageal reflux disease); HLH (Hemophagocytic Lymphohistiocytosis)

which may occur due to higher pathogens exposure in community by entrance of children to school. Our data shows that the most common underlying conditions of hospital-acquired pneumonias are seizure, ALL, Down syndrome and metabolic syndromes. Other studies do not support our findings,[12, 13] but this apparent discrepancy may be due to higher rate of pneumonia attributed to aspiration in epilepsy and seizure. On the other hand, ALL found to be one of the most common underlying conditions of hospital-acquired pneumonias, probably because of defect in immunity system and general predisposition to infections. *Legionella pneumophila* has

been reported as the cause of 7.22% of severe atypical pneumonias.[1] The prevalence of pneumonias due to *chlamidya pneumonia* and *Legionella* is rapidly increasing. Considering the importance of this organism in pneumonia pathogenesis, its early identification is important for rapid and accurate therapeutic interventions. In the present study we employed UAT and PCR methods to detect *Legionella* species in patients with hospital-acquired pneumonia. The traditional gold standard method for identification of this organism has been culturing the *Legionella*. However, culture of *Legionella* bacteria from clinical samples could be impaired bacause of the long incubation time, and possible inhibition of the bacteria growth in competition with other faster-growing organisms in the culture medium.[14] These limitations have prompted to use newer methods. One of such methods is *Legionella* urinary antigen test. Kanavaki et a1[15] declare that 80% of patients with *Legionella* secrete soluble antigens of this microorganism in urine 1 to 3 days after infection. These antigens are detected in urine for at least 42 to 50 days post infection. This assay has been found to be an appropriate method with several advantages, such easy urine sample collection, high volume collection of sample, and the possibility of identify the antigen in urine even after antibiotic therapy. Short time span to have results is a further advantage of the method.[16] PCR is a semi-quantitative method which uses to detect DNA sequences based on amplification of targeted section. This method has been widely used to detect *Legionella pneumonia*. Lim et al.[17] have conducted a study on 267 patients with pneumonia symptoms which yielded a 3.3% positivity rate for Legionella urinary antigen test. Similarly, Goudarzi et al.[18] have reported a 5.7% rate of positivity by urinary antigen test. However, 18.4% of the samples tested by Arnouts et al were positive by UAT, a finding similar to our results (16.7%). Jonas et al.[19] conducted PCR and culture on 250 BAL samples. Their results showed that 8 samples were culture and PCR-positive, whereas 6 other samples were PCR-positive, but culture-negative. Wellinghausen et al.[20] performed Quantitative PCR on 67 water sewage samples collected from different hospital sections which revealed 98.7% positivity in PCR while this figure in culture was 70.1%. The sensitivity of urinary antigen test was reported to be 86 to 90% while its specificity reached 100 percent.[15] Cloud et al.[21] in a study on 212 samples reported that 100% of culture-positive samples were PCR-positive and out of 181 culture-negative samples, 12 were PCR-positive leading to specificity of 93% for PCR. In another study, Wilson et al[22] reported 27 PCR positive and only 7 culture positive samples out of 150 patients. Their results together with our findings indicate higher sensitivity of the PCR assay as compared to better specificity of the urinary antigen test. Furthermore, the differences observed between our results and those reported by others might be due to differences in methods of sample collection, clinical conditions of patients, patients' ethnicity, commercial kits and diagnostic materials. Several other studies have stated that the sensitivity of PCR method for L.pneumophila detection is greater or equal to culture method.[23-26] We have not been able to culture our samples due to technical shortcomings. This limitation does not allow us to estimate sensitivity and specificity of UAT and PCR techniques. Another finding in our study was relatively higher positivity rate of *Legionella pneumophila* infection in patients with an history of drowning; this phenomenon can be attributed to *Legionella*'s growth in water environments. We also observed a higher rate of mortality in patients who developed pneumonia 5 days after ventilation compared to those who developed pneumonia within 2 days post-ventilation. Regarding this issue, there was nothing found in the literature, but it can be speculated that patients with more than 5 days intubation are usually more susceptible subjects of concomitant infections and they were discharged from the hospital while they have worse general condition.

In conclusion, the gold standard method to identify *Legionella* infections is microbial culture that might show false negative results by many causes, including culture condition and microorganism features and properties. These limitations have prompted employment of newer methods. Present study compared Legionella urinary antigen test and PCR to detect the presence of *Legionella pneumophila* in hospital-acquired pneumonia. PCR method was more sensitive in the detection of *Legionella* than urinary antigen test; however the difference between these two methods was not statistically significant.

List of acronyms

ALL - acute lymphoblastic leukemia
BAL - bronchoalveolar lavage
CP - Cerebral Palsy
PICU - pediatric intensive care unit
UAT - urinary antigen test

Authors contributions

Sayed-Yousef Mojtahedi supervised the implementation of the study and corrected the final version of the manuscript, Aliakbar Rahbarimanesh participated in selecting patients and carrying out the project, Samileh Noorbakhsh participated in selecting patients and carrying out the project , Hossein Shokri cooperated in statistical consultation and designation of the study, Saeedreza Jamali-Moghadam-Siyahkali wrote the draft of manuscript, Anahita Izadi presented the project's original design and she is the corresponding author of the article.

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Conflict of Interest

The authors report no conflict of interest.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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