Sero diagnosis of *Legionella* infection in community acquired pneumonia

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Received July 23, 2008

*Background & objectives:* *Legionella pneumophila* has been increasingly recognized as an emerging pathogen responsible for community acquired pneumonia (CAP) worldwide. In India, the actual burden is not known. The present study was thus undertaken to see the presence of *Legionella* infection in patients with community acquired pneumonia admitted in a tertiary care centre in north India.

*Methods:* Both children and adults (n=113) with symptoms of pneumonia were included in the study. Clinical samples (blood, urine, nasopharyngeal aspirates, bronchoalveolar lavage, sputum, etc.) were collected and subjected to culture and other tests. Enzyme linked immunosorbent assay (ELISA) was done by commercial kits for all the three classes of immunoglobulins (IgG, IgM & IgA). Urinary antigen was also detected using commercial kits. Culture was performed on 51 respiratory tract fluid samples. Serum samples of 44 healthy controls were also screened for the presence of anti-legionella antibodies (IgG, IgM & IgA).

*Results:* Thirty one of the 113 cases (27.43%) were serologically positive. Anti-legionella IgG, IgM and IgA antibodies were positive in 7.96, 15.92 and 11.50 per cent patients respectively. In controls, seropositivity was 9.09 (4/44). IgA was positive in 3 and IgM, IgG combined in one. Antigenuria detection by Microwell ELISA kit showed 17.69 per cent positivity. Four antigenuria positive patients were also serologically positive; of these two patients were positive for IgM, hence considered as confirmed cases of *Legionella* infection. None of the sample was culture positive.

*Interpretation & conclusions:* Combination of serology and antigenuria detection may be a valuable tool for the diagnosis of *Legionella* infection in absence of culture positivity. In order to evaluate the actual burden of *Legionella* in community acquired pneumonia, further studies with larger samples need to be done.

**Key words** Antigenuria - community acquired pneumonia - ELISA - immunoglobulins - *Legionella pneumophila*

Legionnaire’s disease is acute pneumonia caused by *Legionella* spp., which are responsible for 2-5 per cent of community acquired pneumonia (CAP)¹. Patients are infected by inhalation of aerosols containing *Legionella* species or by aspiration of water contaminated with *Legionella* species. Over 90 per cent of Legionnaire’s disease cases are caused by *Legionella pneumophila*, of which 70-80 per cent belongs to serogroup type 1².³

Diagnosis of Legionnaire’s disease is mainly based on culture, antigen detection in urine and
antibody detection in serum. Of the various antibody detection methods available to detect Legionella infection, indirect immunofluorescence (IIF) assay and enzyme-linked immunosorbent assay (ELISA) are the most commonly used \(^3\)\(^4\). Previous studies showed that serology yields good sensitivity and specificity data \(^3\)\(^6\), four-fold or greater increase in the titre of IgG antibody is considered diagnostic. Immunoglobulin M (IgM) detection is widely used in infectious serology as it appears earlier in the course of a disease. However, despite its reported validity for the diagnosis of legionellosis\(^2\)\(^7\)\(^8\), some investigators consider it of limited value \(^3\)\(^9\). ELISA, which generally shows higher sensitivity and better characteristics in terms of both automation and objective measurement than immunofluorescence does, has not been thoroughly studied for the detection of IgM antibodies in legionellosis.

We undertook this study to detect three classes of antibodies (IgG, IgM, IgA) and urinary antigen simultaneously along with culture to diagnose Legionella infection in clinical samples of patients who were diagnosed with pneumonia based on clinical symptoms and radiography at their first visit to a tertiary care hospital in north India.

**Material & Methods**

**Study group:** All 113 clinical samples of paediatric (27) and adult (86) patients were collected during May 2005 to January 2008 on their first visit to the All India Institute of Medical Sciences, New Delhi, with the symptoms of pneumonia (Table I), and were admitted in the wards. The samples were collected after obtaining informed consent. Of these, 75 were male and 38 were females, with age ranging from 8 months and 78 yr (mean age 37.96±24.08).

A total of 113 samples of blood, random urine and 51 respiratory tract fluids (31 sputum, 12 nasopharyngeal aspirates, 6 endotracheal aspirates, 2 broncho alveolar lavage) were collected. All the samples were stored at -20°C until use.

**Control group:** Serum samples were also collected from 44 healthy individuals. The adult controls were from the hospital and laboratory staff and pediatric controls were children attending OPD with no history of lower respiratory tract infection. Out of 44 controls 10 were children and 34 were adults with age ranges from 2 to 55 yr with mean age of 25.56±15.80. Out of 44 controls, 33 were males and 11 females.

**Case definition:** The diagnosis of community acquired pneumonia (CAP) requires the following to be present\(^10\): new pulmonary infiltrate seen on chest radiograph taken within 24 h of presentation, the confirmatory clinical finding is presence of at least one of the major criteria which include, cough, sputum production, temperature >37.8°C or at least two of the minor criteria: pleuritic chest pain, dyspnoea, altered mental status, pulmonary consolidation by physical examination and white blood cell count of >12000/µl.

Presumptive case of Legionnaire’s disease was defined as positive test in ELISA by any class of antibody and confirmed case was defined as culture/urinary antigen positive in addition to positive IgM antibody test\(^3\).

**Procurement of standard strain:** Standard strain of *L. pneumophila* (ATCC 331522) was obtained from American Type Culture Collection (ATCC), USA. Strain was revived and maintained in Buffered Charcoal Yeast Extract agar (BCYE) (BD, Becton, Dickenson & Co. USA).

**Culture:** All respiratory fluids were cultured on selective BCYE agar. Before inoculation, all sputum samples were treated by simple dilution method\(^11\). One hundred µl homogenized sputum and 900 µl trypticase soya broth were mixed and vortexed. One or two drops of this suspension were inoculated on selective BCYE agar, incubated at 37°C in microaerophillic environment. After 72 h, plates were examined for legionella like colonies which are round, with an entire edge, glistening, convex, gray white in colour, measure 1-4 mm in diameter and resembling ground and cut glass appearance.

**Antigen detection test:** Legionella urinary antigen was detected in urine samples using the Legionella urinary antigen detection assay Microwell ELISA (IVD Research, USA) and Binax NOW Legionella urinary antigen ICT kit (Binax Inc.) following the manufacturers instructions.

**Serological test:** Three commercial ELISA kits (EUROIMMUN Medizinisch Labordiagnostika AG, Netherland) were used for detection of serum IgG, IgM and IgA present in patients and controls against *L. pneumophila* serogroup 1-7 according to the manufacturer’s protocol. The kits included microtiter strips each with 8 breaks-off reagent wells coated with lipopolysaccharide (LPS) of the *L. pneumophila* strain 1-7. These assays use single 1:101 dilutions of serum in sample buffer and include cut-off calibrators to score samples as negative or positive. Samples with
borderline results were retested and if same result was obtained, it was scored as uncertain.

The study protocol was approved by the Institute Ethics Committee.

**Statistical analysis:** Data were analysed using Chi square test/Fisher exact test.

**Results**

Of the 113 serum samples, 27.43 per cent (31) were scored serologically positive. Of these 31, IgM was positive in 11.50 per cent (13), IgG in 4.42 per cent (5), IgA in 4.42 per cent (5) cases, IgM and IgA in 3.53 per cent (4), IgG and IgA in 2.65 per cent (3), and IgM, IgG and IgA all together were positive in 0.88 per cent (1). Of the 44 control samples, 9.09 per cent (4) samples were serologically positive; IgA was positive in 6.8 per cent (3) both IgM and IgG were detected in one case (2.2%), the difference between patients and controls was significant ($P<0.05$) (Table I).

Of the 113 serum samples, IgM was found positive in 18 patients, significantly higher than in controls ($P<0.05$), (15.92%), IgA in 11.50 per cent (13) and IgG was positive in 7.96 per cent (9) cases.

Of the 113 urine samples, 17.69 per cent (20) were positive for *Legionella* antigen by Microwell ELISA kit; none was found positive by Binax NOW antigen detection ICT kit.

Of the 20 urinary antigen positive cases, 3.53 per cent (4) patients had antibodies in their serum samples (2 positive for IgM antibody and one each for IgG and IgA). Among 51 respiratory tract fluid samples, none were found positive for *L. pneumophila*. Standard strain of *L. pneumophila* (ATCC 331522) is maintained on BCYE agar and used as a control for culture of clinical specimens.

**Discussion**

Serological testing for *L. pneumophila* is often the primary method of screening for possible *L. pneumophila* infections and high sensitivity is paramount in a screening assay. However, non availability of commercial assays such as immunofluorescence assay (IFA), microagglutination, indirect haemagglutination test and ELISA$^4,12$ in India has resulted in limited studies in community acquired pneumonia patients. Some studies have reported, IgM detection by ELISA presented a sensitivity in first sample and the value of IgM detection as an early serological marker of legionnaires disease has been reported previously$^2,7,8$.

In our study, patients showed significantly higher seropositivity for *Legionella* infection in acute phase samples as compared to controls. IgM antibody detection was also found to be statistically significant in patients compared to controls. Usually both IgM and IgG can be detected in samples of patients during the course of their illness, but in a significant proportion of patients, no IgG is detectable$^{16}$. Some studies reported comparatively high detection of IgG antibodies (in 43 to 50% of cases)$^{13,14}$ but these studies were carried out on outbreak samples.

Consequently a positive result in the ELISA for IgM should be considered as presumptive and confirmation should be done by highly specific antigenuria detection or seroconversion$^3$. In our study, only 4 seropositive patients were found positive for urinary antigen, and only two of these were IgM positive. Based on both antigen as well as IgM antibody positivity, only two (1.77%) were confirmed cases. *Legionella* spp. are responsible for 2.5-31 per cent of sporadic community acquired pneumonia cases using variable detection assay as reported from different geographical location$^{10,15-23}$ (Table II).

<table>
<thead>
<tr>
<th>Table I. Clinical symptoms and clinical signs for all the patients and patients positive by any serological test.</th>
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<tbody>
<tr>
<td>Clinical symptoms</td>
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<tr>
<td>Fever</td>
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<tr>
<td>Cough</td>
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<td>Vomiting</td>
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<tr>
<td>Headache</td>
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<tr>
<td>Abdominal Pain</td>
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<tr>
<td>Diarrhoea</td>
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<tr>
<td>Dyspnoea</td>
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<tr>
<td>Plural rub</td>
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<tr>
<td>Wheezing</td>
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<tr>
<td>Crepitation</td>
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<td>Bronchial breathing</td>
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<tr>
<td>Whispering pectoriloquy</td>
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<td>Dbro</td>
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<tr>
<td>Significant chest X-ray Findings</td>
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</tbody>
</table>
The first report of the isolation on *L. pneumophila* from India\(^4\) was reported in 1991, on 45 patients of pneumonia and 17 randomly selected environmental samples. This showed presence of *L. pneumophila* in 4 of 45 (9%) clinical specimens and 13 of 17 (76%) environmental samples. Bahl \(et\ al\)\(^{25}\) in 1997 reported low antibody titter of *Legionella* in 21 of 100 patients in their prospective study in lower respiratory tract infection (LRTI) while 50 environmental samples and 50 controls tested negative. Chaudhry \(et\ al\)\(^{20}\) in 2000 first time reported 15 per cent seropositivity by IgG ELISA in 60 community acquired pneumonia patients and showed that *L. pneumophila* may be an important cause of CAP in adults.

Over recent years, antigenuria detection has emerged as a reference method for the diagnosis of legionnaire’s disease since it enables early diagnosis: antigen has been detected in urine as soon as 1 day after the onset of symptoms. This method shows good sensitivity values in the detection of serogroup 1 infection\(^{26}\). In our work we initially performed Microwell ELISA test for antigen detection, (which was the only available kit in the market), and 20 (17.69%) samples were positive. Later on, urine samples were tested by Binax NOW ICT antigen detection kit (At -20°C for more than 2 to12 months) with but antigen was not detected in previously positive specimens. It is possible that failure to detect specimens that had previously given positive result could be related to, decay of the urinary antigen in the stored frozen specimens.

Several studies reported that concentration of urine increases the sensitivity of the tests but the reported increase shows great variations\(^{27,28}\). Chang \(et\ al\)\(^9\) reported a decrease in sensitivity for EIA and RIA after long term storage of urine. Guerrero \(et\ al\)\(^{13}\) in their study on 178 unconcentrated urine samples reported different positivity in antigen detection using different commercial ELISA kits. They also reported that after concentrating negative urine samples, sensitivity increased. In our study inability to detect antigen by Binax NOW kit could be attributed, firstly due to delay in testing, secondly use of unconcentrated and frozen urine samples.

Although culturing remains the gold standard for the diagnosis of legionellosis, its sensitivity may be limited in clinical routine laboratories\(^2,3\). In our study, legionellae were not isolated in any of the respiratory tract fluid samples. Diagnosis by culture of respiratory tract secretions requires 3 to 5 days of incubation. Laboratories in India routinely do not attempt culture for *Legionella* spp. Studies from other countries reported the sensitivity of culture from respiratory tract secretions varying from 17 to 75 per cent.\(^{15,14}\).

Helbig \(et\ al\)\(^6\) reported that antigenuria detection remains the best method for the early diagnosis of Legionnaires’s disease; but also supported the earlier recommendations of Hoge \(et\ al\)\(^{10}\) that use of serodiagnosis by antibody detection provides a valuable complementary test to antigenuria detection in diagnosis of legionella infection.

In conclusion, seropositivity based on positive IgM antibody was 15.92 per cent in community acquired pneumonia whereas confirmed legionella infection was detected only in 1.77 per cent cases. In order to evaluate the actual burden of *Legionella* in community acquired pneumonia, further studies are required.

**Acknowledgment**

Authors thank shri Pramod Kumar for technical support, and acknowledge Department of Biotechnology, Ministry of Science and Technology, New Delhi, India, for providing financial support.

**References**


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**Table II. Seropositivity of *Legionella pneumophila* in community acquired pneumonia patients in different geographic areas by different detection methods**

<table>
<thead>
<tr>
<th>Study</th>
<th>Place</th>
<th>Seropositivity (%)</th>
<th>Detection methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pachon (et\ al)(^{15})</td>
<td>Spain</td>
<td>21.8</td>
<td>IFA</td>
</tr>
<tr>
<td>Fang (et\ al)(^{16})</td>
<td>USA</td>
<td>6.7</td>
<td>IFA</td>
</tr>
<tr>
<td>Moine (et\ al)(^{17})</td>
<td>France</td>
<td>1</td>
<td>IFA</td>
</tr>
<tr>
<td>Riquelme (et\ al)(^{18})</td>
<td>Spain</td>
<td>6</td>
<td>IFA</td>
</tr>
<tr>
<td>Ruiz (et\ al)(^{19})</td>
<td>Spain</td>
<td>8</td>
<td>IgG ELISA and urinary antigen</td>
</tr>
<tr>
<td>Chaudhry (et\ al)(^{20})</td>
<td>India</td>
<td>15</td>
<td>IgG ELISA</td>
</tr>
<tr>
<td>Bochud (et\ al)(^{21})</td>
<td>Switzerland</td>
<td>0.6</td>
<td>IFA</td>
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<tr>
<td>Arancibia (et\ al)(^{22})</td>
<td>Spain</td>
<td>12</td>
<td>IgG ELISA and urinary antigen</td>
</tr>
<tr>
<td>Miyashita (et\ al)(^{23})</td>
<td>Japan</td>
<td>1.6</td>
<td>IFA</td>
</tr>
<tr>
<td>Wattanathum (et\ al)(^{10})</td>
<td>Thailand</td>
<td>8.2</td>
<td>Urinary antigen</td>
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</tbody>
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