

Development of an indirect sandwich ELISA for detection of urinary antigen, using *Legionella pneumophila* PAL protein

Abolfazl Gholipour · Mojtaba Moosavian ·
Manoochehr Makvandi · Hamid Galehdari ·
Amirhooshang Alvandi · Seyyed Ali Mard

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Abstract *Legionella pneumophila* peptidoglycan-associated lipoprotein (PAL) protein is an extremely conserved antigen among *Legionella* species. In this study, rabbit and rat anti-PAL immunoglobulin G antibodies were produced by immunization with purified, recombinant PAL (r-PAL) protein of *L. pneumophila* serogroup 1 and used as capture and detection antibodies in the PAL antigen-based enzyme-linked immunosorbent assay (ELISA) to detect urinary PAL antigen. Urine samples were obtained from rats experimentally infected with *L. pneumophila* serogroup 1. The PAL antigen was measured in urine samples of 40 infected and 40 uninfected rats. After choosing the cut-off

value of 0.192, the sensitivity and specificity of the PAL antigen-based ELISA were 87.5 and 97.5 %, respectively. The results obtained by PAL antigen base ELISA were compared with those obtained by Biotest. The PAL antigen was detected efficiently by both of the assays and all of the control human urine samples were negative by the ELISA test. The PAL antigen-based ELISA assay was relatively simple to perform, precise, highly sensitive and specific, and reproducible. Based on our data the PAL antigen-based ELISA described here is the first indirect sandwich ELISA for urinary antigen detection which could easily be applied for diagnosis of Legionnaires disease.

A. Gholipour
Department of Microbiology and Immunology, School of
Medicine, Molecular and cellular Research center, Shahrekord
University of Medical Sciences, Shahrekord, Iran

M. Moosavian (✉)
Infectious and Tropical Diseases Research Center, Department
of Microbiology, School of Medicine, Ahvaz Jundishapur
University of Medical Sciences, Ahvaz, Iran
e-mail: moosavian_m@yahoo.com

M. Makvandi
Department of Medical Virology, School of Medicine, Ahvaz
Jundishapur University of Medical Science, Ahvaz, Iran

H. Galehdari
Department of Genetics, Faculty of Science, Shahid Chamran
University, Ahvaz, Iran

A. Alvandi
Department of Microbiology, Faculty of Medicine, Kermanshah
University of Medical Sciences, Kermanshah, Iran

S. A. Mard
Department of Physiology, School of Medicine, Ahvaz
Jundishapur University of Medical Science, Ahvaz, Iran

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Introduction

Legionnaire's disease (LD), a form of severe pneumonia, caused by *Legionella* spp. was originally believed to be an exotic plague. LD still occurs, both in sporadic and epidemic form, sometimes involving many hundreds of victims and can cause high morbidity and mortality (range 30–50 %) if treated improperly (Edelstein and Cianciotto 2005; Winn et al. 2006). Although *Legionella pneumophila* ranked as the third most common etiologic agent for community-acquired pneumonia, diagnosis of *Legionella* pneumonia can be difficult. Failure to diagnose LD is largely due to an absence of clinical awareness. Clinical manifestations of the patients and radiographic features are nonspecific, and it is not possible to clinically distinguish patients with LD from patients with other types of pneumonia (Fang et al. 1990; Edelstein 1993).

The special laboratory tests available for the diagnosis of LD consist of detection of organisms in respiratory secretions by a direct fluorescent antibody (DFA) test; antibody determination in serum samples by indirect immunofluorescent assay (IFA) test; detection of *Legionella* DNA in respiratory secretions by polymerase chain reaction (PCR); culture of clinical specimens such as blood, sputum, biopsy of tissue etc.; and detection of *Legionella* antigen in urine specimens (Waterer et al. 2001; Forgie and Marrie 2009).

Some problems with many accessible diagnostic tests for legionellae are: insufficient sensitivity and specificity, and inability to provides a result in a clinically useful time period (Fields et al. 2002). While DFA supplies a rapid method of identifying *Legionella* spp., immunofluorescent microscopy is technically very demanding and is very liable to error if not carried out by experienced personnel (Waterer et al. 2001; Fields et al. 2002). In the clinical setting, serology is restricted in its usefulness as a diagnostic instrument for legionellosis due to the length of time required, the need for paired sera, and the difficulty of getting proper convalescent samples (Stout and Yu 1997). In genotypic methods, the results of PCR for the diagnosis of *Legionella* infections seem very hopeful and show that this method may be more sensitive than other diagnostic methods. PCR is costly and there are no FDA (the Food and Drug Administration) approved reagents. In addition, PCR is used in few laboratories to diagnose LD (Maiwald et al. 1998). A main limitation of culture-based methods is that 25–75 % of patients with *Legionella* pneumonia do not expectorate sputum, although culture-based diagnosis remains the gold standard for diagnosis of legionellosis. Besides, *Legionella* organisms grow slowly on culture media, so laboratories that throw away negative cultures after 5 days may also miss some *Legionella*-containing specimens (Waterer et al. 2001; Fields et al. 2002).

Nearly 80 % of patients with *L. pneumophila* serogroup 1 (SG1) infection excrete *Legionella* antigens in their urine at some stage of their illness (Helbig et al. 2003). The urinary antigen testing revolutionized the laboratory diagnosis of LD, making it the most common laboratory test ordered for diagnosis of such a disease, since this test can be easily done by those without specific skills. Urine antigen testing allows primitive diagnosis and beginning of suitable antibiotic therapy (Kashuba and Ballow 1996; Edelstein and Cianciotto 2005). Three commercial enzyme immunoassay (EIA) kits have been reported to be sensitive and specific in many clinical studies. However, several authors mentioned that the available commercial EIA kits revealed excellent sensitivity to *L. pneumophila* SG1 antigen, but they had changable sensitivity to *L. pneumophila* non-SG1 and other *Legionella* species (Dominguez et al. 2001; Fields et al. 2002; Kim et al. 2003). Although

the *L. pneumophila* SG1 was the cause of 70–90 % of all cases of LD in most geographic areas, other *L. pneumophila* serogroups and other *Legionella* spp. are being identified with increment of frequency, therefore questioning the broad-spectrum utility of these tests (Reingold et al. 1984; Yu et al. 2002). It is still necessary to develop antigen capture assays to diagnose infections with all species and serogroups of *Legionella*. Expansion of a genus-wide urinary antigen test looks practicable and would provide a distinct diagnostic advantage (Harrison 2005). In addition, if a *Legionella* species-common, surface antigen is targeted, the value of urinary antigen detection assays will be significantly increased to diagnose *Legionella* pneumonia which results from all *Legionella* species (Tang and Toma 1986; Kim et al. 2003). Among the *L. pneumophila* SG1 antigens, the peptidoglycan-associated lipoprotein (PAL) of *L. pneumophila*, as an extremely conserved portion between all *Legionella* species, was identified as the most eminent surface antigen. In addition, this antigen is a strong antibody inducer in rabbits and mice, also since it is excreted in infected urine specimens; it will be diagnostically useful to design an ELISA kit to diagnose LD (Yoon et al. 2002; Kim et al. 2003).

This paper delineates the development of an indirect sandwich ELISA for the detection of the PAL antigen from rats which were infected with *L. pneumophila*. It demonstrated that the *Legionella* PAL antigen was detected efficiently by the PAL antigen-based ELISA.

Materials and methods

Periplasmic expression and purification of r-PAL protein

Recombinant PAL (r-PAL) protein was previously expressed and purified by Gholipour et al. (2010, 2012). Briefly, Luria–Bertani (LB) broth medium (Himedia), supplemented with kanamycin, (LBB-K) was inoculated with *E. coli* BL21 (DE3) carrying the recombinant pET26b-pal plasmid and the r-PAL protein expression was induced by adding 1 mM Isopropyl- β -Dthiogalactopyranoside (IPTG). To prepare the periplasmic extract, the bacterial cells were harvested by centrifugation and the pellet was re-suspended in TES buffer (0.02 M Tris–Cl, 0.5 M EDTA, and 20 % sucrose, pH = 8.0). Lysozyme (Boehringer Germany) and complete protease inhibitor cocktail (Roche) were added to the cell suspension, followed by adding ice cold deionized water. The suspension was agitated on ice and the cells were centrifuged. In order to purify r-PAL protein, the supernatant was dialyzed overnight against chromatography buffer, and then the concentrated dialyzed sample containing r-PAL protein

added to the Ni-NTA resin, mixed gently and transferred to the column. The column was washed with chromatography buffer, the r-PAL protein was eluted and analyzed by SDS-PAGE and Western blotting.

Rat and rabbit anti-PAL antigen IgG preparation

For acclimatization, two rats and rabbits (New Zealand White) were held for one week before the beginning of the procedures. Rats and rabbits back (the sites of injection) were swabbed down with ethanol cotton balls and subdermal injection was done with 20 and 100 µg of r-PAL protein, respectively at 2-weeks intervals for 2 months (Drenckhahn et al. 1993). Hyperimmune sera were collected, and IgG was purified by Protein A antibody purification kit (Sigma-Aldrich, USA) based on the manufacturer's protocol. The purified IgG was identified by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), and the concentration was determined by Bradford protein assay and finally used for experiments.

Rat infection and urine sample collection

L. pneumophila SG1 (ATCC33152); obtained from the American Type Culture Collection (ATCC), Manassas, VA, was cultured on selective modified Wadowsky-Yee medium (MWY) (Oxoid) at 37 °C and 5 % CO₂ under humidified air for 3–4 days. After incubation, *L. pneumophila* colonies were harvested to prepare the inoculums for rat infection.

Intratracheal inoculation of rats were performed with 1×10^6 CFU/animal of *L. pneumophila* which led to *Legionella* pneumonia 48 h after infection (Brieland et al. 1994). Ten male rats (250–350 g) were held for one week before inoculation. The rats were lightly anesthetized with ketamine and xylazine, they were tied then a vertical cut was made through the skin of the ventral neck. Then 200 µL of the bacterial suspension was injected directly into the trachea with an insulin syringe followed by 200 µL of air. The skin incision was closed and then the rats were put to their cages. Ten control rats were also similarly inoculated with 200 µL of sterile phosphate-buffered saline (PBS). The rats were watched and controlled for signs of illness, therefore, urine samples from the infected and uninfected (control) rats were gained at 3, 6, 9, 15 days after inoculation and kept in aliquots at –70 °C before use (Kim et al. 2003; Saha et al. 2005). After urine collection, the rats were gently killed and the lungs were removed, finely minced in sterile PBS, homogenized and cultured on MWY agar and incubated at 37 °C (5 % CO₂ under humidified air) for 4 days (Brieland et al. 1998).

Chessboard titrations

Many ELISA systems require optimizing the used reagents. Chessboard titrations (CBTs) are key features to help the process. (Crowther 2001). We designed indirect sandwich ELISA based on four parameters for optimization; capture antibody, antigen, detecting antibody and anti-antibody conjugated with enzyme. We used *L. pneumophila*-PAL recombinant protein which had been optimized and purified before (Gholipour et al. 2012). Rat anti-PAL IgG was used as capture and rabbit anti-PAL IgG was used as detection antibodies. In order to titrate the capture antibody and antigen (Stage I), the diluent (borate coating buffer) (pH 9.6) containing boric acid (3.1 g), potassium chloride (3.5 g), sodium hydroxide (1.3 g) and Phenol red (2 mg) was added in 50-µL volume to all wells of the microplate (Nunc, Denmark). 50 µL of the capture antibody (40 µg/ml) was added to all wells in column 1 of the microplate and it was diluted to column 11. There was no antibody in column 12 and it only contained borate coating buffer which it was considered as blank. After incubation at 37 °C for 2 h, the microplate was washed three times with PBS (pH 7.4) and a dilution range of antigen (0.039 to 5 µg/ml) was made from column 1 to column 11 in blocking buffer (PBS containing 1 % bovine serum albumin -BSA, 0.05 % Tween 20). The microplate was incubated at 37 °C for 1 h. Then the microplate was washed and 50 µL of detection antibody (2 µg/ml) was added to each well. After incubation at 37 °C for 2 h, the microplate was washed and 50 µL of conjugate (1/1,000 in blocking buffer) was added to each well. Finally after incubation at 37 °C for 2 h, the microplate was washed, 50 µL of BM Blue POD substrate (Rocche, Germany) was added to each well and microplate was incubated in the dark at room temperature for 20 min. To stop the color reaction, 50 µL of stop solution (HCl 2 N) was added to each well and the optical density (OD) read using a microplate reader (Tecan, Australia) set to 450 nm. To titration of detection antibody and conjugate (Stage II), the concentration of capture antibody and antigen was fixed and the concentration of the detection antibody and conjugate optimized was the same as mentioned above.

PAL antigen-based ELISA design

An indirect sandwich ELISA was designed to detect PAL antigen in urine samples of infected rats, after finding optimum concentration of capture antibody, antigen, detection antibody and conjugate from Stage I and II. After coating the microplate (with optimum concentration of capture antibody), all urine samples were boiled for 5 min, centrifuged at 1,200×g for 10 min, and 50 µL of infected urine specimens, obtained from different days, added to

duplicate wells. 50 μL of purified r-PAL protein (at optimum concentration found from CBT in Stage I) and 50 μL of uninfected urine samples were added to copy wells as positive control and negative controls, respectively. The next steps of PAL antigen-based ELISA were performed as described above for CBTs. Human urine samples were included in this study for cross-reactivity testing.

Biotest Legionella urinary antigen EIA

The Biotest *Legionella* urinary antigen EIA is a direct sandwich assay that uses polyclonal rabbit antibodies as the capture and detection antibodies which react with antigens of *L. pneumophila* serogroups, as well as with antigens of other species of *Legionella*. The test was performed according to the manufacturer's instructions.

Statistical analysis

For antigen determination, all specimens were examined in duplicate and the results were expressed as the mean absorbance for each determination. The mean optical density of the negative samples plus 2* standard deviations (SDs) was used to calculate cut-off value. The Biotest *Legionella* urinary antigen EIA results used as the standard test. The results were compared by Pearson correlation analysis which were achieved by the Biotest *Legionella* urinary antigen EIA and the PAL antigen-based ELISA. For sensitivity and specificity value calculations of the PAL antigen-based ELISA a total of 40 negative urine samples were collected from uninfected rats and the sensitivity and specificity values were calculated by using each cut-off value. All statistical analyses were performed by using the SPSS version 15 for Windows and Microsoft Excel 2003 software.

Results

Protein A antibody purification kit was used to purify anti-PAL antigen IgG from serum sample of immunized animals. The concentration of purified IgG was 1,000 $\mu\text{g}/\text{ml}$. To confirm specificity of the purified IgG, Western blot analysis was done by using the purified IgG as an initial antibody and anti-Rabbit IgG (whole molecule) peroxidase conjugate (Sigma, A 9169) as a secondary antibody. The blotted r-PAL protein showed specific signal detection as well as using anti-His₆ peroxidase antibodies.

Recovery of *L. pneumophila* from infected rats

The lung tissue homogenates of infected rats were cultured on MWY agar and *L. pneumophila* colonies observed after

4 days of incubation at 37 °C in a humidified 5 % CO₂. This finding confirmed that all rats were infected with *L. pneumophila*.

Titration of capture antibody and antigen with constant detection antibody and conjugate

Two experimental combinations were examined. In the first one rabbit anti r-PAL IgG was used as capture and detecting antibody that provided very high optical densities (0.18–3.87) (Fig. 1) when r-PAL antigen was applied. In the second combination rat anti r-PAL IgG was used as the capture antibody and rabbit anti r-PAL IgG as the detecting antibody that yielded lower optical densities (0.05–1.56) (Fig. 2) which it was suitable for PAL antigen-based ELISA. The variation in the capture antibody and antigen concentration was assessed in multiple assays. The results confirmed that a 2.5 $\mu\text{g}/\text{ml}$ capture antibody (rat anti r-PAL IgG) concentration and a 0.625 $\mu\text{g}/\text{ml}$ r-PAL antigen concentration both had better results. Accordingly, these concentrations of capture antibody and r-PAL antigen were applied for later assays.

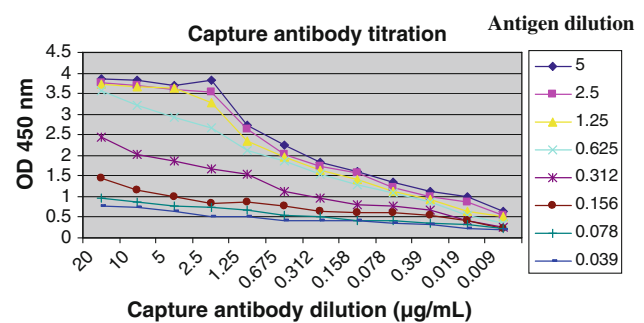


Fig. 1 Determination of optimal amounts of capture antibody (rabbit anti r-PAL IgG) and antigen for PAL antigen-based ELISA. Each line represented titration of the same dilution range of antigen using a different concentration of capture antibody. The detection antibody and conjugate dilution was constant

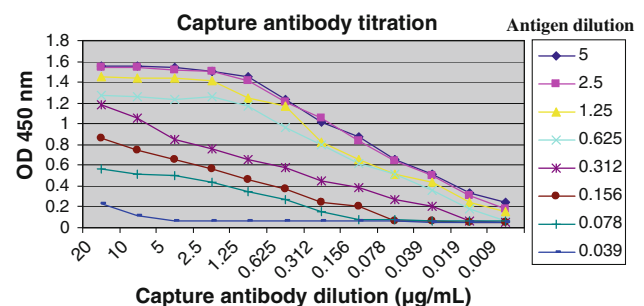


Fig. 2 Determination of optimal amounts of capture antibody (rat anti r-PAL IgG) and antigen for PAL antigen-based ELISA. Each line represented titration of the same dilution range of antigen using a different concentration of capture antibody. The detection antibody and conjugate dilution was constant

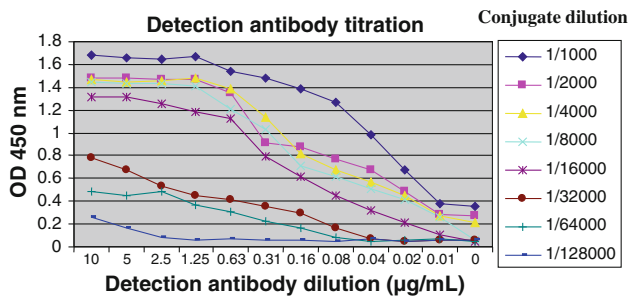


Fig. 3 Determination of optimal amounts of detection antibody and conjugate for PAL antigen-based ELISA. Each line represented titration of the same dilution range of conjugate using a different concentration of detection antibody. The capture antibody and antigen dilution was constant

Titration of detection antibody and conjugate with constant capture antibody and antigen

To determine the optimal concentration of the detection antibody and conjugate using wells coated with 2.5 µg/ml capture antibody and also using a 0.625 µg/ml antigen another chessboard assay was performed. By using the above concentrations, the optimal values for detection antibody and conjugate were determined at 1.25 µg/ml and 1/8,000, respectively (Fig. 3).

PAL antigen-based ELISA

The results of the CBT proposed that a 2.5 µg/ml concentration of capture antibody, a 0.625 µg/ml concentration of r-PAL antigen, a 1.25 µg/ml concentration of detection antibody and a 1/8,000 dilution of conjugate were suitable for use in the diagnostic assay. Rat anti-PAL IgG was recognized as a satisfactory capture antibody since it was capable to capture the respective antigen with a range of 0.078–5 µg/ml; therefore, the limit of detection was determined to be approximately 78 ng of r-PAL antigen/ml. To recognize whether the PAL antigen was excreted in infected urine specimens or not, the PAL antigen capture ELISA was performed in duplicates on 40 urine samples obtained from infected rats—after finding optimum concentration of capture antibody, detection antibody, and conjugate. The purified r-PAL protein and urine samples from rats which were injected with sterile PBS were employed as positive control and negative controls, respectively.

Biotest *Legionella* urinary antigen EIA

The experiment was done in duplicates on 40 urine samples from infected rats and 40 urine samples from rats which were injected with sterile PBS based on the producer's instructions. The cut-off value was computed as the mean optical density of the negative controls plus 0.200. Urine

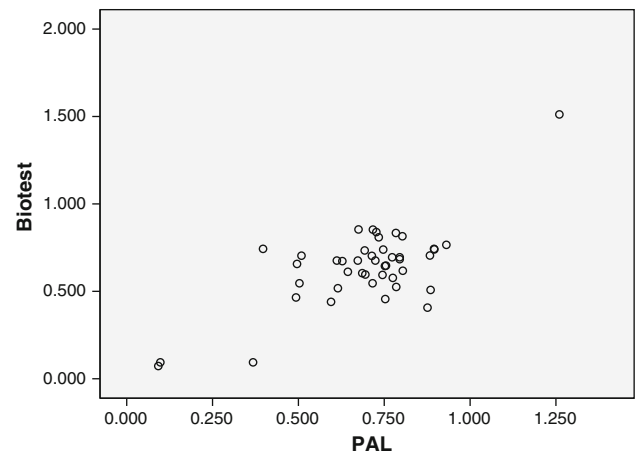


Fig. 4 Correlation between the absorbance values of the Biotest *Legionella* urinary antigen EIA and the PAL antigen-based ELISA. The Pearson correlation coefficient was 0.72 ($P < 0.001$). circle *L. pneumophila*

samples including PAL antigen with an extinction value equal to or greater than the cut-off value were regarded positive.

Calculation of cut-off values, sensitivity and specificity

The negative and positive urine samples were tested by both PAL antigen-based ELISA and Biotest *Legionella* urinary antigen EIA. The cut-off values calculated for mean + SD and mean + 2*SD and to establish the sensitivity, specificity and cut-off value, the urine samples got from the uninfected control rats were used as negative samples. PAL antigen-based ELISA OD readings from samples of these rats ranged from 0.065 to 0.356 (Mean = 0.098, SD = 0.047). The OD for positive urine samples from infected rats were between 0.148 and 0.937 (Mean = 0.677, SD = 0.21).

The results obtained were compared with those from the Biotest *Legionella* urinary antigen EIA to settle the evaluation of the PAL antigen-based ELISA as a diagnostic assay. Figure 4 shows the correlation of the absorbance values of both antigen assays. The correlation coefficient was 0.72 ($P < 0.001$). In line with the results from the Biotest *Legionella* urinary antigen EIA, as a standard, the PAL antigen-based ELISA PAL antigen was detected efficiently by both of the assays.

The accomplishment of the PAL antigen-based ELISA was assessed on both 40 infected urine samples and 40 controls obtained from the uninfected rats. When a cut-off value of 0.145 was chosen, all 40 infected urine samples were positive within the absorbance range between 0.148 and 0.937 and 3 out of 40 controls were also positive (sensitivity, 100 %; specificity, 92.5 %). When the cut-off value of 0.192 was chosen, 35 out of 40 infected urine samples were positive

Table 1 Ranges of sensitivity and specificity of the PAL antigen capture ELISA by each cut-off value

Cut-off	% sensitivity	% specificity
0.145	100	92.5
0.192	87.5	97.5

The cut-off value was calculated by using the mean absorbance of negative controls plus SDs

Table 2 Ranges of sensitivity and specificity of the Biotest *Legionella* urinary antigen EIA by cut-off value

Cut-off	% sensitivity	% specificity
0.289	100	100

The cut-off value was calculated by using the mean absorbance of negative controls plus 0.200

Table 3 Ranges of absorbance values in two control groups determined by the PAL antigen-based ELISA

Group	No. of urine samples	Absorbance value		
		Mean \pm SD	Min	Max
Patients with urinary tract infection	30	0.0665 \pm 0.0091	0.054	0.085
Healthy adults	10	0.070 \pm 0.013	0.051	0.088

Min minimum, *Max* maximum

and 1 out of 40 controls were also positive (sensitivity, 87.5 %; specificity, 97.5 %) (Table 1).

The fulfillment of the Biotest *Legionella* urinary antigen EIA was also evaluated on 40 infected urine samples and 40 controls got from the uninfected rats. The cut-off value was 0.289 and all 40 infected urine samples were positive within the absorbance range from 0.407 to 0.854 (Mean = 0.675, SD = 0.117) and all 40 controls were negative within the absorbance range between 0.073 and 0.095 (Mean = 0.089, SD = 0.01) (sensitivity, 100.0 %; specificity, 100 %). (Table 2).

The PAL antigen-based ELISA was also evaluated for cross-reactivity. A total of 40 human urine samples were included in the experiment: 30 urine samples were collected from patients who had urinary tract infection due to *E. coli* and *Klebsiella pneumoniae* and 10 samples from healthy adults. All of these control samples were negative by the PAL antigen-based ELISA (Table 3).

Discussion

Legionnaire's disease (LD) is a prevailing type of serious pneumonia, but these infections are rarely diagnosed. The

clinical symptoms of infection with *Legionella* are indistinguishable from the symptoms of other causes of pneumonia. Besides, *Legionella*, the bacteria that cause this disease, are fastidious and not easily cultured (Bartlett 1993; Fields et al. 2002). To recognize *Legionella* and to supply timely and suitable therapy, correct diagnostic methods are required. Specialized laboratory tests must be conducted to improve diagnosis, by the clinical microbiology laboratory, on patients in a high-risk category (Bartram et al. 2007).

Among the particular laboratory tests at hand for the diagnosis of LD, tests proposed by the Panel are culture on selective media, which identifies all *Legionella* strains but is technically hard and a urine antigen assay for *L. pneumophila* SG1, a test that is technically not difficult and trustingly and quickly diagnoses up to 70 % of cases of LD (Bartlett et al. 1998).

The necessity of alternative diagnostic test to culture for LD diagnosis is based on urinary antigen detection. Urine antigen testing is very useful, particularly for patients who do not produce sufficient sputum for culture (Winn et al. 2006). Urine antigen tests have been represented to be sensitive (70–100 %) and specific (approaching 100 %) for detecting *L. pneumophila* SG1 (Murdoch 2003); other feasible benefits of the tests are the technical ease of carrying out of them and the validity of results after several days of effective antibiotic treatment (Bartlett et al. 1998).

Notwithstanding the accessibility of immunological and molecular genetic methods, detection of LD is usually efficacious only for *L. pneumophila* SG1 and the sensitivity and specificity of methods for diagnosing other *L. pneumophila* serogroups and species of *Legionella* are far from perfect (Tartakovskii 2001). At present, accessible kits are shown to be greatly specific and reliable in usual use, but the major disadvantage of the kits is that they solely detect the soluble antigen of *L. pneumophila* SG1 but do not effectively detect *L. pneumophila* non-SG 1 and other *Legionella* species (Harrison et al. 1998; Benson et al. 2000). The sensitivity may have been affected by strain differences of LPS because the soluble antigen detected by these tests is LPS. Furthermore, the Biotest EIA kit produced to detect all the serogroups of *L. pneumophila* also antigens from other *Legionella* species do not find out non-*pneumophila* *Legionella* strains as efficiently as *L. pneumophila* SG1. Hence, the value of urinary antigen tests would be increased if *Legionella* infections other than *L. pneumophila* SG1 infections could also be detected (Domínguez et al. 2001).

The characterization of different antigens of *L. pneumophila* in bacterial cells has been studied by several research groups. Initial investigations propose that a *Legionella* genus common PAL target is detectable in the urine of experimentally infected guinea-pigs, whereas the

successful detection of common protein antigens like major outer membrane protein (MOMP) or macrophage infectivity potentiator (MIP) in urine has not been reported (Engleberg et al. 1984; Engleberg et al. 1991; Ludwig et al. 1991; Yoon et al. 2002; Kim et al. 2003). The periplasmic expression of PAL protein, as the most important superficial antigen of *L. pneumophila* (Engleberg et al. 1991), was reported (Gholipour et al. 2010), which can be used in succeeding evaluation of diagnostic studies.

The purpose of the study was to design a *Legionella* recombinant PAL antigen-based ELISA kit for detection of PAL antigen in the urine of infected rats, assessment of the sensitivity and specificity of the provided *Legionella* r-PAL antigen ELISA kit, and comparing the sensitivity and specificity of this kit with those of the standard commercial *Legionella* urinary antigen ELISA kit for detection of LD.

The first step to establish a sandwich ELISA is to access to enough amounts of capture antibody that is specific for the antigen you want to assess, in this case r-PAL protein. On the other hand, the amount of capture antibody coated on the microplate wells influences the specificity and sensitivity of the assay (Crowther 2001). Hence, the ideal amounts of anti-PAL IgG for coating were settled and according to the results got from the CBT, the PAL antigen-based ELISA was planned for detection of *L. pneumophila* PAL antigen from urine sample.

Lebrun et al. (1983) assessed the detection of *L. pneumophila* antigen in urine samples by a sandwich ELISA. They displayed that urinary antigen was present from the start of the acute phase of the illness. According to forthcoming and past studies, the most significant characteristic of urinary antigen assay emerged to be its high specificity and a moderate-to-high sensitivity for *L. pneumophila* infections, ranging from 56 to 99 %. The distinctions in sensitivities may be expounded by differences in test and patient features, the serogroup with which the patient was infected, and if the urine was concentrated before testing. A main disadvantage with these tests is their incapability to reliably detect organisms other than *L. pneumophila* SG1, and development of a genus-wide urinary antigen would prepare a clear diagnostic advantage (Birtles et al. 1990; Murdoch 2003).

For this purpose, the PAL antigen, a *Legionella* genus common antigen, was considered to plan the PAL antigen-based ELISA. Showing the ability of an indirect sandwich ELISA for the diagnosis of LD was the first published data, as far as we know. The results showed that PAL antigen was not detected in uninfected rat's urine and urine samples from rats infected with *L. pneumophila* SG1 got positive about 3 days after infection and stayed positive for up to 2 weeks. This is nearly in agreement with the results reported by Berdal et al. (1979) for detection of *L. pneumophila* antigen in urine by ELISA.

Sensitivity and specificity values were considered according to 40 known positive and 40 known negative samples. The PAL antigen-based ELISA test using the rat anti-PAL IgG had sensitivity of 100 % and specificity of 92.5 % when a cut-off value of 0.145 was selected and the test detected antigen in urine from 40/40 experimentally infected rats. The sensitivity of the test reduced to 87.5 % and the specificity enhanced to 97.5 % when a cut-off value of 0.192 was selected and the test detected antigen in urine from 35/40 experimentally infected rats. No cross-reactions with other bacteria in urine samples from patients with urinary tract infections were noticed and from healthy adults like other authors (Kohler et al. 1981; Tang and Toma 1986). Comparison between analytical sensitivity of the PAL antigen-based ELISA and broad-spectrum ELISA reported by Tang and Toma (1986) displayed that the PAL antigen-based ELISA was more sensitive than broad-spectrum ELISA (with a sensitivity of 70 % and a specificity of nearly 100 %).

Comparison between analytical sensitivity of the PAL antigen-based ELISA made in our study and sandwich ELISA reported by Kim et al. (2003) for diagnosis of PAL antigen from urine sample of infected guinea pigs displayed that the PAL antigen-based ELISA was more sensitive than the sandwich ELISA (with a sensitivity of 76.5–88.2 % and a specificity of 95.5–98.5). ELISA which was recently described for the detection of *Legionella* PAL antigen applied a cytoplasmic expressed protein, PAL, as antigen. A probable disadvantage of the sandwich ELISA is that cytoplasmic expressed PAL protein may be contaminated with bacterial cytoplasmic proteins and may not lead to appropriate refolding and described undiagnosed non-*pneumophila* species of *Legionella* (Kim et al. 2003).

The *Legionella* Urine Antigen EIA (Biotest) is planned to detect legionellae other than *L. pneumophila* SG1, but it does so less reliably than it detects *L. pneumophila* SG1 (Harrison et al. 1998). The disadvantage of the Biotest EIA exists in its incapability to recognize the special *Legionella* species and/or serogroup that causes the infection. Nevertheless, the Biotest EIA is useful instrument for helping physicians in the diagnosis and treatment of patients with LD (Dominguez et al. 1998).

The results of testing the 40 infected rat urine samples with the Biotest EIA represented that all 40 infected urine samples were positive and all 40 controls were negative (sensitivity, 100 %; specificity, 100 %). This was in accordance with the results reported by Kim et al. (2003) for detection of *L. pneumophila* antigen in urine of infected guinea pigs by Biotest EIA. Test specificity is of paramount importance for an infection with low prevalence, such as LD. Harrison et al. (1998) showed that the specificity of the Biotest EIA was excellent at least for patients with lower respiratory tract infections, with no false-positive results

being found among the 123 non-LD patients examined (Harrison et al. 1998). By comparing of the PAL antigen-based ELISA and the Biotest EIA results, we deduced that the efficiency of the PAL antigen based-ELISA for PAL antigen was very good, providing values almost in close proximity to those got by the Biotest *Legionella* urinary antigen EIA.

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