Characterization of a Lipoprotein Common to Legionella Species as a Urinary Broad-Spectrum Antigen for Diagnosis of Legionnaires’ Disease

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We have previously identified the Legionella 19-kDa peptidoglycan-associated lipoprotein (PAL) as a species-common immunodominant antigen. We describe here for the first time the excretion and detection of the PAL antigen in infected urine specimens, which is useful for the diagnosis of Legionnaires’ disease. Rabbit anti-PAL immunoglobulin G (IgG) antibody was produced by immunization with the purified, recombinant PAL of Legionella pneumophila serogroup 1 and used in the PAL antigen capture enzyme-linked immunosorbent assay (ELISA) to detect urinary PAL antigen. A soluble-antigen capture ELISA using rabbit IgG antibodies against Legionella soluble antigens was prepared independently and used as a broad-spectrum standard test to detect soluble antigens of several Legionella species. Urine samples were obtained from guinea pigs experimentally infected with each of L. pneumophila serogroups 1, 3, and 6, and other Legionella species. The absorbance values of the PAL antigen ELISA highly correlated with those of the soluble-antigen ELISA in infected urine samples, with a correlation coefficient of 0.84 ($P < 0.01$). When applied to 17 infected urine samples and 67 negative controls from guinea pigs, the sensitivity and specificity of the PAL antigen capture ELISA were 88.2 and 95.5%, respectively. Compared to the commercial Biotest enzyme immunoassay, the PAL antigen ELISA was more efficient for detecting pneumophila non-serogroup 1 and nonpneumophila species. None of the 161 control human urine specimens obtained from healthy adults and patients with either non-Legionella pneumonia or urinary tract infections tested positive in the PAL antigen ELISA. The present study shows that the Legionella PAL is a very useful broad-spectrum antigen for urinary diagnostic testing. Moreover, since recombinant PAL antigen can be produced more efficiently than the soluble antigens, the development of a broad-spectrum diagnostic immunoassay based on the detection of the PAL antigen appears to be warranted.

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Legionella is an important cause of both community-acquired and nosocomial pneumonia. Legionella pneumonia may be severe and is potentially fatal in elderly and immunocompromised patients, and rapid diagnosis and early antibiotic treatment are required (17, 23). However, the diagnosis of Legionella pneumonia can be difficult because clinical manifestations and radiographic findings are nonspecific, and conventional laboratory tests, including culturing of the organism, direct fluorescent antibody staining of the bacterium, and serum antibody detection have suboptimal sensitivities, with results that are not rapidly available (8, 9).

Detection of soluble antigens in the urine of patients with Legionella pneumonia was first described in 1979 (3, 26). Over the intervening years, Legionella urinary antigen detection methods using the techniques of enzyme immunoassay (EIA) and radioimmunoassay have been extensively studied and have proven to be the most powerful diagnostic methods (4, 5, 18, 22, 24). The specificity for these tests has been reported to be 100%, and the sensitivity has been shown to vary between 70 and 100% (15). The advantages of these methods include ease of urine collection, the ability to detect antigen after initiation of antibiotic therapy, and the ability to obtain results quickly. Two commercial EIA kits, Binax EIA (Binax, Portland, Maine) and Biotest EIA (Biotest AG, Dreieich, Germany), have been widely used since being marketed in 1996 and 1997, respectively, and a new Bartels EIA (Bartels, Inc., Trinity Bio-tech Company, Wicklow, Ireland) has been introduced recently. These EIAs have been reported to be sensitive and specific in many clinical studies (2, 6, 7, 11, 12, 16, 21). However, several authors pointed out that the available tests showed excellent sensitivity to L. pneumophila serogroup 1 antigen but variable sensitivity to L. pneumophila non-serogroup 1 and other Legionella species (2, 6, 7, 12, 16). Although the L. pneumophila serogroup 1 is the predominant cause of legionellosis in most geographic areas, other pneumophila serogroups and other Legionella species are being recognized with increasing frequency, therefore questioning the broad-spectrum utility of these tests (27). The commercial EIA tests are direct sandwich assays that use polyclonal rabbit antibodies specific to L. pneumophila serogroup 1 or reactive to soluble extracts of L. pneumophila serogroups and other species as the capture and detection antibodies. Therefore, the value of urinary antigen detection assays would be significantly enhanced to diagnose Legionella pneumonia if a Legionella species-common, surface antigen is targeted.

The 19-kDa peptidoglycan-associated lipoprotein (PAL) of L. pneumophila was sequenced and characterized as the most prominent surface antigen in 1991 (10, 19) and retrospectively
seems to be same as the 19-kDa common Legionella outer membrane antigen that was cloned before (13). However, whether or not the L. pneumophila PAL will be diagnostically useful is open to investigation. We have previously overexpressed and purified the recombinant Legionella PAL and demonstrated that the Legionella PAL is a strong antibody inducer in rabbits and mice. The PAL is also highly conserved among Legionella species (30). The aim of the present study is to characterize the Legionella species-common PAL as a potential broad-spectrum urinary antigen to diagnose Legionnaires’ disease. We developed an enzyme-linked immunosorbent assay (ELISA) using the capture antibody specific to the PAL antigen and demonstrated that the Legionella PAL antigen, excreted in urine samples from guinea pigs infected with pneumo- phila and nonpneumophila species and serogroups, was detected efficiently by the PAL antigen capture assay.

MATERIALS AND METHODS

Guinea pig infection and urine sample collection. Male Hartley strain guinea pigs (300 to 400 g) were inoculated intratracheally with ca. 10^7 CFU of legionellae suspended in 0.5 ml of phosphate-buffered saline (PBS), as previously described (29). A couple of guinea pigs in a group were inoculated with each strain of pneumo- phila and nonpneumophila species and serogroups. Animals were observed twice daily after inoculation for signs of illness: fever, ruffled fur, labored respirations, weight loss, and crusting of conjunctival fluid. Fever was defined as two consecutive rectal temperatures that exceeded 40°C. Urine samples were collected from the control and infected animals by using a metabolic cage and stored in aliquots at −70°C before use. Urine samples from the infected animals were obtained between days 1 and 5 after inoculation.

A couple of guinea pigs infected with L. pneumophila serogroup 1 were sacrificed 3 days after infection. Lungs were fixed in 10% formalin. Tissues were embedded in paraffin, sectioned at 6 mm, stained with hematoxylin and eosin, and examined for the pathological findings of Legionnaires’ disease. Urine samples obtained from these animals were used as positive controls in the ELISA.

Human urine samples. Human urine samples were collected from three control groups: 86 healthy adults who visited the health center of Korea University Hospital, 59 patients with nonlegionella pneumonia enrolled in the prospective, multi-center study to identify the etiology of community acquired pneumonia in Korea, and 16 patients with urinary tract infection. Urine samples were stored at −70°C before use.

Preparation of the recombinant Legionella PAL. The recombinant PAL antigen of L. pneumophila serogroup 1 was overproduced in Escherichia coli and purified by affinity chromatography, as described previously (30). The resultant truncated recombinant PAL was filtered and kept at −70°C in PBS before use as an antigen in experiments.

Preparation of Legionella soluble antigens. Soluble antigens from Legionella ATCC strains (L. pneumophila serogroups 1, 3, 4, 5, and 6; L. micdadei; L. dumoffi; L. jordani; L. oakridgensis; L. anisa; L. gormanii; and L. saintheleniensis) were prepared by the method described previously (3, 24). Each Legionella strain was grown on buffered charcoal yeast extract–alpha-ketoglutarate (BCYE) agar for 48 h and harvested in 3 ml of 0.05 M PBS (pH 7.4). The cell suspensions were centrifuged, and 0.5-ml samples of wet packed cells were autoclaved for 1 h at 121°C, and stored at −20°C before use.

Preparation of soluble antigens extracted from several Legionella species (<L. pneumophila serogroups 4, 5, and 6; L. micdadei; L. jordani; L. oakridgensis; L. anisa; L. gormanii; and L. saintheleniensis> were prepared by SDS-PAGE and immunoblotting with the anti-PAL antibodies, as described previously (30). The resultant truncated recombinant PAL was filtered and kept at −70°C in PBS before use as an antigen in experiments.

Anti-PAL antibody and anti-soluble-antigen IgG preparation. Production and purification of anti-PAL immunoglobulin G (IgG) of L. pneumophila serogroup 1 has been described previously (30). An adult New Zealand White rabbit was injected with 100 µg of truncated recombinant PAL into the subcutaneous space and several subcutaneous sites on the back at 2-week intervals for 2 months. Hyperimmune sera were collected, and IgG was purified by fast-protein liquid chromatography (Pharmacia Biotech, Inc.) in a protein A-Superose HR 10/2 column (Pharmacia LKB). The protein concentration was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). The purified IgG was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and used for experiments.

Production of anti-soluble-antigen IgG was done by the method described previously (24). A patient isolate of L. pneumophila serogroup 1 was prepared as whole-cell antigen at a density of 40 IU, and combined with soluble antigens from the 12 Legionella strains in an equal volume. One milliliter of antigen was injected into a rabbit according to the immunization protocol described earlier (24). The following purification steps were the same as described above.

RESULTS

PAL antigen is an immunodominant component of Legionella soluble antigens. To demonstrate the Legionella PAL antigen as a component of soluble antigens of Legionella species, soluble antigens extracted from several Legionella species (<L. pneumophila serogroups 4, 5, and 6; L. micdadei; L. jordani; L. oakridgensis; L. anisa; L. gormanii; and L. saintheleniensis> were analyzed by SDS-PAGE and immunoblotting with the anti-PAL antibodies, as described above. 
PAL IgG antibody of *L. pneumophila* serogroup 1 (Fig. 1). All species tested showed an ~19-kDa prominent protein that reacted strongly to anti-PAL IgG, even though each PAL was not distinctive in the SDS-PAGE. We also identified that the purified, recombinant *Legionella* PAL antigen showed strong reactivity to anti-soluble-antigen IgG antibody in an immunoblot analysis (data not shown). This result indicates that the *Legionella* PAL antigen is an immunodominant component of soluble antigens and is a highly conserved common antigen among pneumophila and nonpneumophila species. This observation supports the idea that the *Legionella* PAL can be used as an alternative target antigen in the urinary diagnostic immunoassay for Legionnaires’ disease.

**Preparation of soluble-antigen capture ELISA as a broad-spectrum standard test.** For the identification of positive urine samples, the soluble-antigen capture ELISA with anti-soluble-antigen IgG was prepared independently in the present study as a broad-spectrum standard test. The positive threshold for the assay was 0.22 (0.12 ± 0.10 SD) (unless otherwise stated, results are given as the mean absorbance plus 3 SDs), determined by using 67 samples from uninfected control animals. Urine samples were obtained from guinea pigs inoculated with *L. pneumophila* serogroups 1, 3, and 6, *L. jordanis*, *L. oakridgensis*, *L. anisa*, *L. gormanii*, and *L. sainthelenensis*. All animals inoculated, except for one inoculated with *L. gormanii*, showed signs of illness. A couple of animals infected with *L. pneumophila* serogroup 1 were sacrificed and confirmed for the pathological changes: lung tissue exhibited focal, acute bronchiolitis characterized by alveolar and septal infiltration with neutrophils and macrophages on the light microscopic findings (data not shown). The soluble-antigen ELISA was performed on 17 specimens from each of the infected animals collected between days 1 and 5 after inoculation. All 17 urine samples were determined to be positive, with an absorbance range of 0.47 to 3.0 (Fig. 2), indicating that the soluble-antigen ELISA could serve as a valid standard test.

**Evaluation of the *Legionella* PAL antigen capture ELISA as a diagnostic assay.** Preliminary experiments were performed to evaluate anti-PAL IgG for the ability to serve as an adequate *Legionella* PAL antigen-capture reagent, and we identified it as a satisfactory capture antibody because it was able to capture the respective antigen with a range of 0.01 to 10 μg/ml (data not shown). To identify whether the PAL antigen was excreted in infected urine specimens, we performed the PAL antigen capture ELISA on 17 infected urine samples from guinea pigs and compared the results obtained with the PAL antigen and to those obtained with the soluble-antigen assays. The mean absorbance values ± the SDs obtained by the soluble-antigen and the PAL antigen capture assays were 1.46 ± 0.89 and 0.25 ± 0.15, respectively. The correlation of the absorbance values from the two antigen assays is shown in Fig. 2. The correlation coefficient was 0.84 (*P* < 0.001). On the basis of the result from the soluble-antigen capture ELISA as a standard, the PAL antigen was excreted in urine as a component of *Legionella* soluble antigens and detected efficiently by the PAL antigen capture assay.

The performance of the PAL antigen ELISA was evaluated on 17 infected urine samples (8 from pneumophila serogroups and 7 from nonpneumophila species) and 67 controls obtained from the uninfected animals. The overall sensitivity and specificity were 88.2 and 95.5%, respectively, when a cutoff value of 0.10 was chosen. The positive predictive value was 83.3%, and the negative predictive value was 97.0% (Table 1). With this cutoff value, all 8 pneumophila urine samples were positive, with an absorbance range of 0.13 to 0.49, and 3 of 67 controls also were positive (sensitivity, 100.0%; specificity, 95.5%). In the case of nonpneumophila species, 7 of 9 infected urine samples were positive, with an absorbance range of 0.10 to 0.35 (sensitivity, 77.8%; specificity, 95.5%).

On the basis of these findings, we compared the results of
the PAL antigen ELISA with those obtained by the commercial Biotest Legionella urinary antigen EIA (Biotest AG) (Table 2). For 17 infected urine samples, the Biotest EIA could detect only \emph{L. pneumophila} serogroup 1-infected samples. Three samples from \emph{L. pneumophila} non-serogroup 1 and five samples from non-pneumophila species that were positive in the PAL antigen ELISA were negative in the Biotest EIA.

We also evaluated the PAL antigen capture ELISA for cross-reactivity. A total of 161 human urine samples were included in the experiment: 16 urine samples collected from patients with urinary tract infection due to \emph{E. coli}, \emph{Klebsiella pneumoniae}, methicillin-resistant \emph{Staphylococcus aureus}, \emph{Enterococcus faecium}, or \emph{Pseudomonas aeruginosa}, etc.; 59 samples from patients with non-Legionella pneumonia; and 86 samples from healthy adults. All of these control samples were determined to be negative by the PAL antigen ELISA (Table 3).

**DISCUSSION**

In this study we characterized the 19-kDa PAL antigen of \emph{Legionella} species as a potential urinary diagnostic antigen for Legionnaires’ disease. We developed the Legionella PAL antigen capture ELISA to detect the PAL antigen excreted in infected urine, and we evaluated the sensitivity and specificity of the assay with urine samples obtained from guinea pigs infected with pneumophila and nonpneumophila species and serogroups.

Legionnaires’ disease has been described as overtreated and underdiagnosed (1), a reflection of the limitations of available diagnostic tests. Urinary antigen testing to detect \emph{Legionella} antigen has proven the most powerful diagnostic method (4, 5, 15, 16, 18, 22, 24), and the commercially available tests have been widely used. However, the main drawback to the available urinary antigen tests is that they only detect the soluble antigen of \emph{L. pneumophila} serogroup 1 but do not efficiently detect \emph{L. pneumophila} non-serogroup 1 and other \emph{Legionella} species (2, 12). Moreover, concerns have been raised over the sensitivity of urinary antigen testing, as the prevalence of serogroup 1 decreases (27). Since the soluble antigen detected by these tests has been known as lipopolysaccharide (LPS) (28), the sensitivity may have been influenced by strain differences of LPS or other immunogens, as suggested elsewhere (4, 6, 14). Therefore, the value of urinary antigen tests would be enhanced if \emph{Legionella} infections other than \emph{L. pneumophila} serogroup 1 infections could also be detected. A broad-spectrum ELISA was developed by using IgG antibodies raised by soluble culture extracts from multiple \emph{Legionella} species and serogroups and detected antigenuria in patients with laboratory-diagnosed Legionnaires’ disease caused by \emph{L. pneumophila} serogroups 1, 3, 4, 6, and 8; by \emph{L. micdadei}; or by \emph{L. longbeachae} serogroup 1 (24). The test is not yet commercially available.

We have shown that \emph{Legionella} PAL is an immunodominant component of the soluble extracts from \emph{Legionella} species and also highly conserved among the \emph{Legionella} species. We have previously shown that this lipoprotein most strongly reacted to the rabbit anti-soluble-antigen polyclonal antibody absorbed with \emph{Legionella} LPS during characterization of \emph{Legionella} soluble antigens in an immunoblot analysis (data not published), allowing us to consider the PAL as a potential urinary diagnostic antigen. Although the \emph{pal} gene has not been amplified from \emph{L. micdadei} and \emph{L. jordanis} by using the primer set derived from the pal gene sequence of \emph{L. pneumophila} serogroup 1 (30), the 19-kDa protein antigen has been clearly shown with \emph{Legionella} species tested, including \emph{L. micdadei} and \emph{L. jordanis}. This suggests that the anti-PAL IgG used contains antibodies to the common epitopes of the PAL harbored by all \emph{Legionella} species.

We have prepared independently and used the soluble-antigen capture ELISA as a broad-spectrum standard test to validate the PAL antigen for a diagnostic urinary antigen. The

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**TABLE 1. Ranges of sensitivity and specificity of the PAL antigen capture ELISA by each cutoff value**

<table>
<thead>
<tr>
<th>Cutoff</th>
<th>% Sensitivity</th>
<th>% Specificity</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.11</td>
<td>76.5</td>
<td>98.5</td>
<td>92.9</td>
<td>94.3</td>
</tr>
<tr>
<td>0.10</td>
<td>88.2</td>
<td>95.5</td>
<td>83.3</td>
<td>97.0</td>
</tr>
<tr>
<td>0.09</td>
<td>88.2</td>
<td>89.6</td>
<td>68.2</td>
<td>96.8</td>
</tr>
<tr>
<td>0.08</td>
<td>100.0</td>
<td>77.6</td>
<td>50.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*The cutoff value was calculated by using the mean absorbance of negative controls plus 3 SD. PPV, positive predictive value; NPV, negative predictive value.*

**TABLE 2. Comparison between the results of the PAL antigen capture ELISA and the Biotest EIA in 17 urine samples obtained from guinea pigs infected with \emph{Legionella}**

<table>
<thead>
<tr>
<th>Legionella strain infected</th>
<th>PAL ELISA result</th>
<th>Biotest EIA result</th>
</tr>
</thead>
<tbody>
<tr>
<td>\emph{L. pneumophila} SG1 (AA100)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\emph{L. pneumophila} SG1 (AA100)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\emph{L. pneumophila} SG1 (isolate)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\emph{L. pneumophila} SG1 (isolate)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\emph{L. pneumophila} SG3 (ATCC 33155)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\emph{L. pneumophila} SG3 (ATCC 33155)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\emph{L. pneumophila} SG6 (ATCC 33215)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\emph{L. sainthelensi} (ATCC 35248)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>\emph{L. sainthelensi} (ATCC 35248)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>\emph{L. sainthelensi} (ATCC 35248)</td>
<td>–</td>
<td>NT*</td>
</tr>
<tr>
<td>\emph{L. anisa} (ATCC 35292)</td>
<td>+</td>
<td>NT*</td>
</tr>
<tr>
<td>\emph{L. anisa} (ATCC 35292)</td>
<td>+</td>
<td>NT*</td>
</tr>
<tr>
<td>\emph{L. oaktidensis} (ATCC 35761)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\emph{L. oaktidensis} (ATCC 35761)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\emph{L. jordanis} (ATCC 33623)</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>\emph{L. jordanis} (ATCC 33623)</td>
<td>+</td>
<td>NT</td>
</tr>
</tbody>
</table>

*NT, not tested.

**TABLE 3. Ranges of absorbance values in three control groups as determined by the PAL antigen capture ELISA**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of urine samples</th>
<th>Absorbance valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with urinary tract infection</td>
<td>16</td>
<td>0.058 ± 0.015</td>
</tr>
<tr>
<td>Patients with non-Legionella pneumonia</td>
<td>59</td>
<td>0.047 ± 0.013</td>
</tr>
<tr>
<td>Healthy adults</td>
<td>86</td>
<td>0.053 ± 0.007</td>
</tr>
</tbody>
</table>

*Min, minimum; Max, maximum.*

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broad-spectrum ELISA using the polyclonal antibodies against the soluble antigens from multiple Legionella species has been previously reported to show more sensitivity for detecting urine antigen from L. pneumophila of different serogroups and other Legionella species compared to the commercial assays (2, 18). The prepared soluble-antigen capture ELISA could detect soluble antigens successfully in all urine samples from guinea pigs infected with pneumophila and nonpneumophila species and serogroups. We have shown that the PAL antigen was detected in most of these urine samples by the PAL antigen capture ELISA. The absorbance values obtained by the PAL antigen assay also correlated with those of the soluble-antigen capture ELISA. This further supports the view that the PAL antigen is excreted in urine as a general component of Legionella soluble antigens and could be used as an alternative diagnostic urinary antigen to the soluble antigens.

The PAL antigen capture ELISA was ca. 76.5 to 88.2% sensitive and ca. 95.5 to 98.5 specific according to the cutoff values determined and was thus comparable to that of the broad-spectrum assay reported previously (with a sensitivity of 70% and a specificity of nearly 100%) (24). The reasons for this remain to be elucidated, but sensitivity may depend on the concentration of the antigen excreted in urine samples. There is a lack of information on the factors that influence the concentration of the soluble antigen released and achieved in urine specimens. We did not apply the PAL antigen capture ELISA to patients’ urine samples infected with Legionella in the present study due to a lack of specimen availability. The clinical performance of the PAL antigen assay should be evaluated in the future.

We have also shown that the PAL antigen capture ELISA showed better sensitivity than the commercial Biotest EIA, especially in detecting urinary antigen from L. pneumophila nonserogroup 1 strain and other Legionella species. These findings are similar to those of previous reports, although the commercial kit is manufactured to detect all of the serogroups of L. pneumophila, as well as antigens from other Legionella species. Further evaluation is needed for comparative test performance on culture-proven Legionnaires’ disease.

We have observed that cross-reactivity was not noted in analyses when 161 human urine samples from healthy subjects and from subjects with non-Legionella pneumonia and urinary tract infections were tested with the PAL antigen capture ELISA, but a few negative control urine samples from guinea pigs tested positive near the cutoff value of absorbance. These reactions may be due to diagnostically irrelevant components of guinea pig urine specimens or other technical factors in performing ELISA, which needs to be reevaluated in further studies with larger sample series and clinical Legionnaires’ disease.

In conclusion, this is the first study demonstrating the excretion and detection of Legionella PAL antigen in infected urine specimens and evaluating the PAL antigen capture ELISA for the diagnosis of Legionnaires’ pneumonia. The Legionella PAL antigen capture ELISA was able to diagnose 88.2% of urine samples tested and should be considered as a broad-spectrum assay to detect both pneumophila and nonpneumophila species and serogroups. Its diagnostic accuracy could be enhanced to 100% if concentrated urine samples are used. The results reported here suggest that the Legionella PAL is a circulating soluble surface antigen and is excreted in urine similar to the dynamics of other soluble antigens. Therefore, it can serve as an alternative broad-spectrum antigen to the soluble antigens to enhance the value of the urinary antigen assay. In addition, the PAL antigen is readily available as a recombinant protein that acts as a strong antibody inducer. Thus, developing the PAL antigen capture ELISA is warranted to improve the diagnosis of Legionnaires’ disease.

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