Detection of *Legionella* Antigenuria by Reverse Passive Agglutination

PATRICK W. TANG, DON DE SAVIGNY, AND SANDU TOMA*

Clinical Bacteriology Section, Laboratory Services Branch, Ontario Ministry of Health, Toronto, Ontario M5W 1R5 Canada

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A reverse passive agglutination method was developed to detect soluble antigens of *Legionella* spp. By this method *Legionella* antigens were detected in urine specimens from 14 of 15 antigenuric patients with clinically diagnosed Legionnaires disease and in none of 263 urine samples from healthy subjects or patients with urinary tract infections. Intra-genus cross-reactivity was observed only between *L. pneumophila* serogroups 2, 3, and 6. The *Legionella* reverse passive agglutination method was also evaluated with reference to reagent concentrations, test conditions, and subjectivity of reading test results. The method is rapid and does not require special equipment.

There are currently three established methods for laboratory diagnosis of Legionnaires disease: indirect fluorescent antibody, direct fluorescent antibody, and culture isolation. The indirect fluorescent antibody test may not seroconvert until 4 to 6 weeks after the onset of disease, and its diagnostic value is diminished when immunosuppressed patients are tested. Both direct fluorescent antibody and culture methods require clinical specimens often obtained by invasive procedures (2, 4). A need remains for a rapid and practical diagnostic method that is sensitive to recent legionellosis but negative for past infection or exposure. An attractive diagnostic approach is the detection of *Legionella* antigens in urine specimens. This has been achieved by enzyme immunoassay (1, 8), radioimmunoassay (5), reverse passive hemagglutination (7), and coagglutination (9), but no methods are yet routinely applied. This paper describes the preliminary development of a rapid and practical reverse passive agglutination (RPA) method based on agglutination of the antibody-sensitized protein A-bearing *Staphylococcus aureus* in the presence of *Legionella*-soluble antigens.

**MATERIALS AND METHODS**

**Urine samples.** Urine specimens (provided by R. B. Kohler, Indiana University Medical Center, Indianapolis, Ind.) were obtained from 15 patients with clinically diagnosed Legionnaires disease and *Legionella* antigenuria detected by radioimmunoassay. The criteria used to select clinically diagnosed Legionnaires disease subjects included at least: indirect fluorescent antibody seroconversion, direct fluorescent antibody-positive result, or culture isolation. Specimens were also collected from 26 healthy adults and 237 patients with or without urinary tract infections.

**Preparation of *Legionella*-soluble antigens.** Soluble antigens of *Legionella pneumophila* serogroups 1 to 6, *L. bozemanii*, *L. micdadei*, *L. dumoffii*, and *L. gormanii* were prepared according to Berdal et al. (1). All *Legionella* spp. were grown on cysteine-yeast extract agar at 35°C for 72 h in 3% CO₂ and harvested in 0.05 M phosphate-buffered saline (PBS), pH 7.4. Cell suspensions were steam autoclaved at 100°C for 1 h and incubated in PBS at 4°C for 7 days to extract soluble antigens. The suspensions were centrifuged at 1,200 × g for 10 min and supernatants were collected. The protein contents of each soluble antigen were determined by the biuret method.

**Preparation of anti-*Legionella* IgG.** Immune sera against *L. pneumophila* serogroups 1 to 6, *L. bozemanii*, *L. micdadei*, *L. dumoffii*, and *L. gormanii* were produced in rabbits with the immunization schedule of Cherry and McKinney (3). Each immune serum was fractionated by protein A sepharose CL-4B chromatography (Pharmacia Fine Chemicals, Sweden) to obtain purified immunoglobulin G (IgG). Protein contents were determined by the biuret method.

**Preparation of RPA reagent.** *S. aureus* Cowan 1 strain was processed according to Kronvall (6). Cultures incubated for 18 h at 37°C were harvested from Casamino-casein-yeast extract agar, washed twice in PBS, and treated for 3 h in 0.5% buffered Formalin, pH 7.4. The suspension was incubated for 1 h at 80°C and adjusted to 10% vol/vol suspension in PBS with 0.01% sodium azide.

For the preparation of RPA reagents, each anti-*Legionella*-purified IgG (0.1 ml of ≥7 mg of IgG per ml) was added to 1 ml of 10% *S. aureus* suspension and incubated at room temperature for 1 h with occasional shaking. The suspensions were washed six times in PBS and diluted to make a 1% (vol/vol) suspension in PBS with 0.01% sodium azide. The RPA reagents were stable at 4°C for at least 4 months.

**RPA.** The RPA method used glass plates, each with 15 wells (14-mm internal diameter; Scientific Products, CanLab, Canada). All specimens were heat inactivated in a 100°C water bath for 15 s before being...
tested. One drop (ca. 40 μl) of test sample, e.g., urine or Legionella-soluble antigen diluted in saline, was added to each well, followed by one drop of RPA reagent. Sample and reagent were mixed with an applicator stick, and plates were rotated at 120 rpm for 10 min. Reactions were determined in indirect light against a black background and graded as follows: 4+, coarse agglutination with clear background; 3+, medium agglutination with clear background; 2+, medium agglutination with turbid background; 1+, fine agglutination with turbid background; nonreactive, identical to nonreactive control consisting of saline and RPA reagent. The endpoint was the highest dilution of soluble antigens giving a 1+ reading.

RESULTS

Optimal IgG concentration. Each of the anti-L. pneumophila IgG preparations of serogroups 1 to 4 were tested as sensitizing agents in concentrations from 0.75 to 20 mg/ml. The concentration of anti-Legionella IgG needed for optimal sensitization of protein A of S. aureus was between 5 and 7 mg/ml for all preparations. Rabbit immune sera prepared as previously described routinely contain IgG at concentrations greater than 7 mg/ml.

Optimal test conditions. The effects of time and speed of rotation were assessed with Legionella RPA reagents for serogroups 1 to 4 with their respective soluble-antigen dilutions. Results were determined at intervals of 1, 2, 4, 6, 8, 10, 15, and 20 min after test samples were mixed and indicated that a rotation period of 8 to 10 min was optimal for all preparations. Rotation periods longer than 10 min did not increase agglutination but resulted in partial drying of samples. Speed of rotation did not affect reactions between 90 and 160 rpm, and a setting of 120 rpm for 10 min was used in subsequent tests.

Subjectivity of results. Twenty titration endpoints for Legionella-soluble antigens were estimated independently by three readers. Of the titrations, 18 (90%) were read identically by all readers. Discordant results agreed within one twofold dilution of each other. To assess subjectivity when untitrated samples were tested, dilutions of soluble antigens were prepared and adjusted to provide RPA scores of 4+, 3+, 2+, 1+, and nonreactive. Forty tests were arranged in random sequence and read independently by three readers. Of 114 semiquantitative readings (1+ to 4+), 86 (75.4%) were in agreement, and the remainder agreed within one score of each other. All readers agreed in all discriminations between nonreactive and 1+ reactive results (33 tests).

Detection of Legionella-soluble antigens from culture extracts. The minimum detection limit of 10 RPA reagents (L. pneumophila serogroup 1 to 6, L. micdadei, L. bozemanii, L. dumoffii, and L. gormanii) for the respective soluble anti-

gens diluted in saline was between 30 and 50 ng/ml of Legionella protein content. A study of intra-genus cross-reactions showed no cross-reactivity except when L. pneumophila serogroup 2 and serogroup 6 RPA reagents were tested against high concentrations (>1,000 ng/ml) of serogroup 3 soluble antigens. Specificity as demonstrated by homologous inhibition studies revealed that preincubation of soluble antigens with 50 ng of homologous IgG per ml completely inhibited agglutination, whereas preincubation with 0.05 ng of specific IgG per ml weakened titr endpoints four-to eightfold. Preincubation of soluble antigens with an excess of heterologous anti-Legionella antisera (100 ng of IgG per ml) did not inhibit RPA titr endpoints despite known cross-reactivity within L. pneumophila serogroups.

Detection of Legionella-soluble antigens in urine specimens. The prevalence of antigenuria detected by RPA in patients with clinically diagnosed Legionnaires disease is shown in Table 1. In all cases the antigen detected was L. pneumophila serogroup 1.

For healthy subjects and non-Legionnaires disease patients, the initial tests showed nonspecific agglutination in 25% of the urine samples. There was no correlation of this nonspecific reactivity with the type of urine specimen (normal or infected) or with urine pH (range, 5.2 to 8.2). Single or repeated preadsorption of urine with 10 to 50% (vol/vol) S. aureus suspensions eliminated some, but not all nonspecific reactions. The nonspecific agglutinator was stable at 56°C for 30 min but was inactivated by incuba-

TABLE 1. Semiquantitative Legionella RPA results in patients with clinically confirmed Legionnaires disease and antigenuria

<table>
<thead>
<tr>
<th>Patient</th>
<th>RPA reactions (L. pneumophila serogroup 1)</th>
<th>Other Legionella spp. reagents&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>1</td>
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<tr>
<td>15</td>
<td>1+</td>
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</tbody>
</table>

<sup>a</sup> As detected by radioimmunoassay (5).

<sup>b</sup> Includes: L. pneumophila serogroups 2 to 6, L. bozemanii, L. micdadei, L. dumoffii, and L. gormanii.
tion in a boiling water bath for 15 s. *Legionella* culture extract antigens detected by RPA were stable at 100°C for 30 min when diluted in saline or urine. All urine specimens were therefore inactivated in a boiling water bath for 15 s before being tested. When heat inactivated, all 26 normal urines and 237 urines from non-Legionnaires disease patients were nonreactive, suggesting a test specificity approaching 100%.

**DISCUSSION**

We applied a RPA method to detect soluble *Legionella* antigens either in urine specimens or in culture extracts. RPA is distinguished in terminology from staphylococcal coagglutination by the detection of soluble rather than particulate antigens (i.e., only one particle type is agglutinated).

RPA is relatively sensitive (as compared with radioimmunoassay), detecting *Legionella* antigenuria in 14 of 15 patients with clinically diagnosed Legionnaires disease who were also positive for *Legionella* antigenuria by radioimmunoassay. Urine specimens did not require concentration before being tested. The non-legionellosis control group, consisting of 263 urine specimens from healthy subjects or patients with urinary tract infections, was nonreactive by RPA, indicating high specificity and lack of interference by whole or soluble antigens from various gram-negative and gram-positive bacteria, other antibody-coated bacteria, and nonspecific antibody. There was no intra-genus cross-reactivity except when high concentrations of *L. pneumophila* serogroup 3-soluble antigens were tested with serogroups 2 and 6 RPA reagents. This cross-reactivity was not evident in heterologous inhibition studies, suggesting that the serogroup 3-soluble antigen preparation contains a low proportion of serogroups 2 and 6 cross-reactive antigens.

Although the RPA method was standardized in its ability to detect soluble *Legionella* antigens at minimum concentrations of 30 to 50 ng of protein content per ml, heat stability studies suggest that the antigen detected by RPA is probably not protein. Antigen characterization studies are in progress. The ease with which the reaction can be inhibited by low concentrations of homologous antibody suggests also that *Legionella*-soluble antigens may have a low valency and that RPA is unlikely to detect *Legionella*-soluble antigens in antibody-excess immune complexes. This necessitates periodic removal by centrifugation of free IgG which may dissociate from the RPA reagents during storage periods longer than 4 months. It is suggested that known soluble antigens be titrated as controls to ensure a consistent level of sensitivity.

Further experience with RPA in clinical laboratories is required to more fully assess its sensitivity and specificity in the rapid diagnosis of Legionnaires disease, especially as related to the dynamics of antigen excretion during acute legionellosis and following treatment. The speed, economy, and practicality of the method should make it useful to both small hospital laboratories as well as reference centers.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**