Broad-Spectrum Enzyme-Linked Immunosorbent Assay for Detection of Legionella Soluble Antigens

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An enzyme-linked immunosorbent assay (ELISA) was developed which detected soluble antigens from culture extracts of Legionella pneumophila serogroups 1 to 8, L. micdadei, L. bozemanii serogroups 1 and 2, L. dumoffii, L. gormanii, L. longbeachae serogroups 1 and 2, L. wadsworthii, L. oakridgensis, L. anisa, L. feeleii serogroup 1, and L. jordanis. The assay was approximately 10-fold more sensitive for the eight L. pneumophila serogroups than for the other Legionella species tested. The ELISA detected Legionella antigens in the urine specimens of 25 of 35 patients with L. pneumophila serogroup 1, 3, 4, 6, and 8; L. micdadei; and L. longbeachae serogroup 1 infections. None of the 334 urine specimens from patients with either non-Legionella pneumonia or urinary tract infections was positive. For 10 patients from whom sequential urine specimens were available, Legionella antigens were not detectable from 7 to 19 days after laboratory diagnosis. Test sensitivity was not affected by heavy bacterial contamination. This ELISA offers the detection of a broad spectrum of Legionella antigens by a single test.

The detection of soluble antigens in urine specimens by immunoassay has proven valuable for the early diagnosis of Legionnaires disease (1, 2, 4, 8, 9, 11–14). Kohler et al. established the time of onset and duration of urinary antigen excretion and noted cross-reactivity with other Legionella or bacterial species (5–7). Soluble antigens have also been detected in serum by enzyme-linked immunosorbent assay (ELISA) with mono- and polyclonal antibodies (2). Except for one assay for Legionella pneumophila serogroup 4, the immunoassays dealt mainly with L. pneumophila serogroup 1 and were highly serogroup specific (6). The lack of a similar test for other Legionella species limits the value of the assays as routine tests for the early diagnosis of Legionnaires disease.

This report describes a broad-spectrum ELISA capable of detecting various Legionella soluble antigens by a single test.

MATERIALS AND METHODS

Soluble antigen preparation. Soluble antigens from the type strains of L. pneumophila serogroups 1 to 8, L. micdadei, L. dumoffii, L. gormanii, L. bozemanii serogroups 1 and 2, L. anisa, L. jordanis, L. wadsworthii, L. feeleii serogroup 1, L. oakridgensis, and L. longbeachae serogroups 1 and 2 were prepared by the method of Berdal et al. (1). Each Legionella species was grown on buffered charcoal-yeast extract agar with α-ketoglutarate (BCYEα; 3) at 35°C for 48 h in 3% CO₂ and harvested in 3 ml of 0.05 M phosphate-buffered saline (PBS; pH 7.4). The cell suspensions were centrifuged, and 0.5 ml of wet packed cells was autoclaved at 100°C for 1 h and then left at 4°C for 10 days to extract soluble antigens. The suspensions were centrifuged at 1,200 × g for 10 min, and the supernatants were collected. Dry weights were measured by heat drying 50 μl of supernatant; weighing was done by analytical balance.

Anti-Legionella IgG preparation. The Bellingham-1 strain of L. pneumophila serogroup 1 was grown on BCYEα at 35°C for 48 h in 3% CO₂ and harvested in PBS with 1% Formalin. After 18 h at 22°C, the cells were washed three times in PBS, and the packed cells were adjusted to a density of 40 IU. The antigens consisted of equal volumes of whole cells and soluble antigens. The immunization of rabbits started with intradermal injections of 1 ml of antigen into 20 sites along the back of each rabbit. After 30 days, 1 ml of antigen was injected intramuscularly. Monthly intravenous injections (1 ml) were begun 7 days later and continued for 8 months. Hyperimmune sera were fractionated by protein A-ephelose CL-4B chromatography (Pharmacia, Uppsala, Sweden) to obtain purified immunoglobulin G (IgG). Protein contents were determined by the biuret method.

Enzyme conjugate preparation. The one-step glutaraldehyde method was used for enzyme conjugate preparation (15). A 5-mg sample of alkaline phosphatase (calf intestine, type VII; Sigma Chemical Co., St. Louis, Mo.) was centrifuged, and the sediment was dissolved in 1 ml of PBS containing 2 mg of anti-Legionella IgG per ml. After extensive dialysis in PBS, glutaraldehyde was added to effect a final concentration of 0.2% and left at 22°C for 2 h. The suspension was dialyzed in PBS and then in 0.05 M Tris hydrochloride buffer (pH 8.0). The conjugate was diluted to a final volume of 5 ml with 0.05 M Tris hydrochloride containing 1% bovine serum albumin, filter sterilized, and stored at 4°C.

ELISA procedures. Polyvinyl chloride U-bottom microtiter plates (Dynatech Laboratories, Inc., McLean, Va.) were used in the ELISA. All reagents were added at 100 μl per well unless otherwise stated. Anti-Legionella IgG at a final concentration of 20 μg/ml in 0.05 M carbonate buffer (pH 9.6) was used to coat the wells. Coating was done either at 4°C for 18 h or 37°C for 1 to 2 h, and then the plates were emptied and washed three times with PBS. Postcoating was done for the same incubation times in 5% bovine serum albumin in carbonate buffer. All test samples were boiled for 1 min in a water bath, centrifuged at 1,200 × g for 10 min, and added to the test wells in duplicates. The plates were then incubated for 1 h at 37°C, emptied, and washed six times with PBS with a 3-min soaking step after wash 3. Enzyme conjugates diluted 1:100 (ca. 4 μg of IgG per ml) in PBS with 2% bovine serum albumin were then added, and

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the plates were incubated at 37°C for 1 h, emptied, and washed as before. A substrate solution of 1 mg of p-nitrophenylphosphate per ml in 10% diethanolamine buffer (pH 9.8) was added and incubated at 37°C for 1 h, and then the reaction was stopped by adding 50 μl of 3 M NaOH. The A_{410} was read with a Microelisa Minireader MR590 (Dynatech). Six negative urine controls were run per plate. The results of the test duplicates were averaged, and the positive threshold was calculated by using twice the mean of the six negative controls.

In preliminary work to standardize the ELISA, combinations of incubation times for antigens (1, 2, and 5 h at 37°C) and conjugates (1, 2, and 4 h at 37°C and 18 h at 4°C) were tested with conjugate concentrations of 2 to 8 μg/ml. The duration of incubation and conjugate concentrations had no effect on the sensitivity of the ELISA, and therefore the described parameters were chosen as optimal conditions. We also tried the Knoxville-1 strain of *L. pneumophila* as an antigen for immunization. The results were not significantly different from those with *Bellingham-1*. An avidin-biotin alkaline phosphatase system was tested, but it had an eightfold lower sensitivity and problems with high background values.

Urine specimens. The urine specimens tested were submitted to our laboratory by hospitals in the Province of Ontario from 1979 to March 1986. Storage was at either 4 or −20°C.

(i) Single or multiple specimens were collected from 35 patients with laboratory diagnosis of Legionnaires disease caused by one of the following: *L. pneumophila* serogroups 1, 3, 4, 6, and 8; *L. micdadei*; and *L. longbeachae* serogroup 1. Diagnosis was made by either indirect fluorescent antibody assay (IFA), direct fluorescent antibody assay (DFA), or culture (10).

(ii) A total of 274 specimens were collected from 191 patients hospitalized with non-Legionella pneumonia. Se-

<table>
<thead>
<tr>
<th>No. of patients</th>
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<th><em>Legionella</em> sp.</th>
<th>No. of results</th>
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* Diagnosis by IFA: at least a fourfold seroconversion to a titer of ≥1:128.
Soluble antigens from culture extracts were detectable for 11 species and 20 serogroups of *Legionella*, with about 10-fold higher sensitivity for *L. pneumophila* serogroups 1 to 8. Cross-reactivity among urinary antigens was also observed in other studies (5, 6), although not so extensively as in our study.

Kohler suggested that the antigens have both shared and distinct epitopes or, alternatively, several types of excreted molecules, some identical among serogroups and others which differ (4). In our preliminary study to assess the most suitable types of IgG for the ELISA, we tried several combinations of immunization routes, in particular an intramuscular and an intravenous scheme as described above. The intramuscular scheme produced IgGs that were serogroup specific with minor cross-reactions similar to those described by Bibb et al. (2). The intravenous scheme produced IgGs that had extensive intragenus cross-reactivity. Therefore, in addition to the antigenic factors suggested by Kohler, the route of immunization may be an important function of broadening the cross-reactivity. Whether our ELISA can detect *Legionella* soluble antigens other than those mentioned has not been determined.

Bacterial contamination and incubation of urine specimens did not affect the titration of soluble antigen; therefore, special collection procedures, transport requirements, and delays in transit may not be critical in the submission of specimens.

Because half of all cases of Legionnaires disease in Canada are caused by *Legionella* species other than *L. pneumophila* serogroup 1, this ELISA is more advantageous than serogroup-specific immunoassays. The drawback of our ELISA lies in the inability to identify which *Legionella* infection occurs. The test would also be ineffective in cases in which urinary antigens are present at very low levels or when the antigenic makeup cannot be detected by the presently used anti- *Legionella* IgG.

The ELISA described here could be a very useful screening test for the early diagnosis of Legionnaires disease. An early diagnosis is of paramount importance in the medical management of patients with Legionnaires disease to significantly decrease the length, severity, and fatality rate of this infection.

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LITERATURE CITED


