

Detection of *Legionella pneumophila* Antigen in Urine by Enzyme-Linked Immunospecific Assay

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An enzyme-linked immunospecific assay "sandwich" technique was developed for detecting soluble antigen from the Legionnaires disease bacterium (*Legionella pneumophila*). With this technique, antigen was detected in urine specimens from guinea pigs inoculated intraperitoneally with heat-killed Legionnaires disease bacteria and in urine specimens from three of four patients who attended the American Legion Convention in Philadelphia in 1976. Urine from a fifth pneumonia patient who attended the Eucharistic Congress (but who was a dubious seroconverter) was negative. Presumably, the test could also be used for detecting antigen in sputum or respiratory aspirates, but this has not been tried to date.

In most cases laboratory confirmation of Legionnaires disease (LD) has been accomplished only retrospectively after recovery by the indirect fluorescent-antibody test on paired sera (8) or postmortem by direct fluorescent-antibody studies on lung tissue (1). In some cases, however, the LD bacterium (LDB) (*Legionella pneumophila*) has been isolated after some delay from lung tissue or pleural fluid (7). There is an urgent need for reliable procedures capable of confirming the diagnosis early enough for therapy to be instituted or modified. To date, attempts to demonstrate the organism in sputum or respiratory aspirates by direct fluorescent-antibody tests (1) have met with only limited success.

Sensitive and specific antigen detection tests such as counterimmunoelectrophoresis, radioimmunoassay, or enzyme-linked immunospecific assay (ELISA) performed on body secretions and fluids have been used in the early laboratory diagnosis of several other infectious diseases (2, 4, 6, 10, 12), and they might logically be useful for early diagnosis of LD. Sputum or respiratory aspirates would probably be ideal specimens, but since none could be obtained for our study, we attempted to detect antigen in urine, an approach which has proved useful in infections with *Streptococcus pneumoniae* and *Haemophilus influenzae* infections (4). Accordingly, we report here an ELISA technique for detection of LDB antigen and preliminary studies on its use with urine. Since this work was completed, investigators conducting a recent independent limited study have shown success-

fully detected antigen in sputum and urine of two patients with LD by an ELISA test (R. C. Tilton, M. Quitadamo, and A. Scala, Ann. Intern. Med., in press).

MATERIALS AND METHODS

A four-layer ELISA "sandwich" technique similar to that described by Yolken et al. (11) for detection of *Escherichia coli* enterotoxin was developed and used for detection of LDB antigen in urine as follows.

Reagents. Goat anti-LDB immunoglobulin G (IgG) was prepared by chromatographic fractionation of serum from a goat hyperimmunized with six serogroup 1 strains of LDB (kindly provided by James C. Feeley, Bureau of Epidemiology, Center for Disease Control) on a diethylaminoethyl-Sephadex A50 column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The IgG fraction thus obtained was concentrated on a Diaflow PM-10 membrane (Amicon Corp., Lexington, Mass.) and adjusted to a protein concentration of 2 mg/ml.

Rabbit hyperimmune antiserum against serogroup 1 (Knoxville) LDB was kindly provided by Patricia Harris, Bacteriology Division, Center for Disease Control, and was used unfractionated in the test.

Alkaline phosphatase-conjugated goat anti-rabbit IgG was prepared by column immunopurification of goat anti-rabbit IgG serum (Antibodies, Inc., Davis, Calif.) on cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Inc.) coated with rabbit IgG to obtain goat IgG with anti-rabbit IgG specificity. The goat IgG thus obtained was labeled with alkaline phosphatase (Sigma type VIII, Sigma Chemical Co., St. Louis, Mo.) by the one-step glutaraldehyde method (10) at a ratio of 5 mg of enzyme to 2 mg of IgG.

ELISA microplate assay. Optimal dilutions of all reagents were determined by checkerboard titrations, and optimal times and temperatures for incubation were determined before assays were carried out. Goat anti-LDB IgG (2 mg/ml) was diluted 1:1,000 in 0.06 M carbonate buffer (pH 9.6), and 0.1 ml was added to

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each well of the polyvinyl microtiter U plates used for the assay (Dynatech Industries, Alexandria, Va.). The plates were incubated in a moist chamber, as with subsequent incubations, at 4°C for at least 14 h (longer intervals up to 6 weeks appear acceptable). After this incubation period, plates were washed four times in phosphate-buffered saline, pH 7.2, and 0.1 ml of 1% bovine serum albumin in phosphate-buffered saline (pH 7.2) was added to each well (to reduce nonspecific binding). Plates were incubated again at 37°C for 30 min and were washed three times in phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (PBST). They were then ready to be used.

Samples of urine to be tested were diluted 1:2 in 1:20 normal goat serum in PBST and 1:2 in 1:20 specific goat anti-LDB serum (unfractionated) in PBST. The latter was for the purpose of binding LDB antigen (if present) with excess free antibody to confirm the immunological specificity of any positive results obtained.

Included in each run were normal negative urine and a series of dilutions (10^{-3} through 10^{-5}) of saline-extracted soluble LDB antigen (3) in normal urine as negativity and sensitivity controls. Samples thus prepared were added in 0.1-ml volumes to wells of the plates, which were then incubated at room temperature for 16 h. Plates were washed four times with PBST, and 0.1 ml of a 1:1,000 dilution of rabbit anti-LDB serum in PBST plus 1% normal goat serum was added to each well. Plates were incubated again at 37°C for 2 h and then washed four times with PBST. Alkaline phosphatase-conjugated goat anti-rabbit IgG, diluted 1:100 in PBST, was added in 0.1-ml amounts to each well and incubated at 37°C for 2 h, and plates were washed six times in PBST. Bound enzyme was detected by adding 0.1 ml of phosphatase substrate (*p*-nitrophenyl phosphate, 1 mg/ml, in diethanolamine buffer; Sigma 104) to each well. After incubation of the plates at room temperature for 30 min, the reaction was terminated by adding 0.025 ml of 3 N NaOH. Reactions were read visually and were considered positive when yellow color developed in wells which had contained urine diluted in normal goat serum and not in wells which had contained urine diluted in goat anti-LDB serum.

RESULTS

Guinea pig urine. The ELISA test was first evaluated for its ability to detect antigen in urine specimens from normal guinea pigs and guinea pigs inoculated intraperitoneally with approximately 10^{10} heat-killed (100°C for 30 min) cells of different strains of LDB. The results indicate that antigen was not detected in normal guinea pig urine or urine from guinea pigs before they were injected with LDB (Table 1). Urine specimens from animals injected with serogroup 1 antigens (Philadelphia 1 and 2; Knoxville) became positive approximately 4 h after injection and remained positive for up to 1 week, the longest time tested thus far. Animals injected with serogroup 2 antigen (Togus 1) (9) were negative, as would be expected because of the

antigenic dissimilarity of this serogroup. Urine of a single animal inoculated with *H. influenzae* type b was negative.

Human urine. Results of tests on urine specimens from human subjects are shown in Table 2. Specimens from 20 normal subjects with no recent history of respiratory disease were negative in the test. Urine specimens from four patients considered to have had LD in association with the American Legion Convention in Philadelphia in 1976 and from one patient who attended the Eucharistic Congress in Philadelphia shortly thereafter were kindly supplied by Renate Kimbrough, Toxicology Branch, Clinical Chemistry Division, Center for Disease Control. These had been collected for toxicological examination before the microbial etiology of the disease was established (5, 8). Antigen was detected in the urine of three of four subjects (cases 1 through 4) who attended the American

TABLE 1. Detection of antigen in urine of guinea pigs inoculated with killed LDB, using the ELISA test

Inoculum ^a	No. positive/no. tested	
	Pre-inoculation	Post-inoculation
Philadelphia 1 & 2	0/2	2/2
Philadelphia 1	0/2	2/2
Knoxville 1	0/2	2/2
Togus 1	0/2	0/2
<i>H. influenzae</i> b	0/1	0/1
None (controls)	0/8	ND ^b

^a Approximately 10^{10} heat-killed (100°C, 30 min) LDB cells injected intraperitoneally.

^b ND, Not determined.

TABLE 2. Detection of antigen in urine from normal humans and patients with LD

Subject	Serum IFA test ^a	ELISA	Collection time (days) ^b
Case 1	SC512	+	<6
Case 2	SC512	-	<2/<13 (2 specimens)
Case 3	NSC	+	<7
Case 4	SC 2048	+	<24
Case 5 ^c	SC? ^d	-	<3
Normals (20)	Not done	- (20/20)	

^a IFA, Indirect fluorescent antibody. SC, Seroconversion (fourfold or greater) to titer stated. NSC, non-seroconverter.

^b Based on interval between date of onset and date of receipt of specimen at Center for Disease Control.

^c Attended Eucharistic Congress; others attended American Legion Convention.

^d Titer rose from 16 to 64 with only one of two antigens used for indirect fluorescent-antibody test.

Legion Convention. Case 3 met the clinical and epidemiological case definition (5) but failed to show seroconversion in the indirect fluorescent-antibody test (8). The present results appear to confirm the diagnosis in spite of the lack of seroconversion later. Case 5 attended the Eucharistic Congress, stayed at the same hotel, and had a clinically compatible and epidemiologically associated illness. Urine of this patient was negative. This case could not be confirmed serologically since an equivocal seroconversion was obtained with only one of two LDB test antigens then in use (8). Unfortunately, precise information on the dates these urines were collected is not available because only the dates of onset and dates of receipt of the specimens are on record. Concentration of urine specimens approximately 10-fold in Amicon B-15 macrosolute concentrators (Amicon Corp.) did not result in positive reactions with either of the two negative specimens from patient 2 or the single specimen from patient 5. When positive urines were concentrated in this fashion, visibly stronger reactions were obtained.

DISCUSSION

The number of cases in this series is small, but the results were sufficiently impressive that early publication of these findings seemed warranted to encourage others to attempt this approach to improve the data base. Another similar recent brief report on two patients is also encouraging (Tilton et al., in press). To date, respiratory secretions and additional urine specimens from patients with pneumonias of undefined etiology have not been available to us for study. A prospective protocol to obtain such specimens from future outbreaks or apparent hyperendemic foci has been developed.

The test described has exquisite sensitivity and is capable of detecting as little as 3 pg of protein present in a purified lipopolysaccharide-protein complex, which is a major antigenic constituent of LDB (K. H. Wong, W. O. Schalla, R. J. Arko, J. C. Bullard, and J. C. Feeley, *Ann. Intern. Med.*, in press).

We have made several attempts to detect circulating antigen in acute and convalescent sera from patients with LD, but as yet unexplained nonspecific reactions (i.e., reactions which cannot be inhibited by excess anti-LD antibody) have been seen in both normal sera and sera from patients with LD. It is likely that detection of antigen in convalescent sera would be difficult, complicated by the patients' rising levels of antibody, but further studies, especially on acute-phase sera, are clearly warranted to define this problem and hopefully to eliminate it.

In the present study, the onset and duration of antigen excretion in the urine could not be defined, and this important parameter must be established by prospective serial urine collection. Most of the specimens from Philadelphia were probably collected in the first few days of illness because they were collected for toxicological studies.

Additional reagents applicable to detection of antigens of other newly recognized serogroups (e.g., Togus 1) which are not detected in the system described here must be developed in conjunction with further studies to better define the role of these serogroups in the etiology of LD.

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LITERATURE CITED

1. Cherry, W. B., B. Pittman, P. P. Harris, G. A. Hebert, B. M. Thomason, L. Thacker, and R. E. Weaver. 1978. Detection of Legionnaires disease bacteria by direct immunofluorescent staining. *J. Clin. Microbiol.* 8:329-338.
2. Edwards, E. A., P. M. Muehl, and R. O. Peckinpugh. 1972. Diagnosis of bacterial meningitis by counterimmunoelectrophoresis. *J. Lab. Clin. Med.* 80:449-454.
3. Farshy, C. E., G. C. Klein, and J. C. Feeley. 1978. Detection of antibodies to legionnaires disease organism by microagglutination and micro-enzyme-linked immunosorbent assay tests. *J. Clin. Microbiol.* 7:327-331.
4. Feigin, R. D., M. Wong, P. G. Shackelford, B. W. Stechenberg, L. M. Dunkle, and S. Kaplan. 1976. Countercurrent immunoelectrophoresis of urine as well as of CSF and blood for diagnosis of bacterial meningitis. *J. Pediatr.* 89:773-775.
5. Fraser, D. W., T. R. Tsai, W. Orenstein, W. E. Parkin, H. J. Beecham, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard, P. S. Brachman, and the Field Investigation Team. 1977. Legionnaires' Disease. Description of an epidemic of pneumonia. *N. Engl. J. Med.* 297:1189-1197.
6. Mathiesen, L. R., S. M. Feinstone, D. C. Wong, P. Skinhoej, and R. H. Purcell. 1978. Enzyme-linked immunosorbent assay for detection of hepatitis A antigen in stool and antibody to hepatitis A antigen in sera: comparison with solid-phase radioimmunoassay, immune electron microscopy, and immune adherence hemagglutination assay. *J. Clin. Microbiol.* 7:184-193.
7. McDade, J. E. 1978. Primary isolation using guinea pigs and embryonated eggs, p. 93-106. *In* G. L. Jones and G. A. Hebert (ed.), "Legionnaires," the disease, the bacterium, and methodology. Center for Disease Control, Atlanta.
8. McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, W. R. Dowdle, and the Laboratory Investigation Team. 1977. Legionnaires' Dis-

- ease. Isolation of a bacterium and demonstration of its role in other respiratory disease. *N. Engl. J. Med.* **297**: 1197-1203.
9. McKinney, R. M., B. M. Thomason, P. P. Harris, L. Thacker, K. R. Lewallen, H. W. Wilkinson, and C. W. Moss. 1978. Recognition of a new serogroup of Legionnaires disease bacterium. *J. Clin. Microbiol.* **9**: 103-107.
 10. Voller, A., D. Bidwell, and A. Bartlett. 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506-512. *In* N. Rose and H. Friedman (ed.), *Manual of clinical immunology*. American Society for Microbiology, Washington, D.C.
 11. Yolken, R. H., H. B. Greenberg, M. H. Merson, R. B. Sack, and A. Z. Kapikian. 1977. Enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. *J. Clin. Microbiol.* **6**:439-444.
 12. Yolken, R. H., H. W. Kim, T. Clem, R. Wyatt, A. Kalica, R. Chanock, and A. Z. Kapikian. 1977. Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. *Lancet* **ii**:263-266.