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Kinetic-Dependent Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to *Legionella pneumophila*

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A semiautomated, kinetic-dependent, enzyme-linked immunosorbent assay (K-ELISA) was adapted to detect serum antibodies to *Legionella pneumophila*. In a comparative study, 158 human serum samples (79 pairs) were tested by K-ELISA and the standard indirect immunofluorescence assay for determination of antibody levels to *L. pneumophila* serogroup 1. K-ELISA determinations were made by using a serogroup-specific antigen or a preparation (unfractionated antigen) which contained both common antigen and serogroup-specific reactivity. There was good correlation between the immunofluorescence assay and the K-ELISA by using either antigen, although greater correlation was achieved with the unfractionated antigen (coefficients of correlation, 0.894 with unfractionated antigen and 0.841 with serogroup-specific antigen). These results indicate that the K-ELISA is a reliable alternative to the immunofluorescence assay for serologically diagnosing legionellosis.

The serological test is often used retrospectively as a diagnostic tool for patients with Legionnaires disease (LD) because, until recently, suitable media for isolating the etiological agent have been unavailable commercially and because invasive procedures for specimen collection are often required for successful culturing or direct immunofluorescence assays. The indirect immunofluorescence assay (IFA) is the standard reference serological test with an estimated specificity of 99% and sensitivity of 78 to 91% (10–14). Alternative tests described so far include the microagglutination test (3), indirect hemagglutination test (15), immunofluorometric assay (1), solid-phase immunofluorescence assay (6), and enzyme-linked immunosorbent assay (ELISA) (3, 14). There are advantages and disadvantages to each method compared with the IFA, which is the only test that has been validated with sufficient numbers of sera from well-documented cases (10). The advantages of the ELISA are the potential for automation, for using purified antigens, and for spectrophotometric readout and computer analysis of test results. The kinetic-dependent ELISA (K-ELISA) as described by Tsang et al. (8, 9), for detection of schistosomal antibodies and in a preliminary report for *Legionella* antibodies, by P. R. B. McMaster et al. (Abstr. Annu. Meet. Fed. Amer. Soc. Exper. Biol., 1982, abstr. no. 2483, p. 695) is especially suitable for these purposes. The unknown ligand (i.e., antibody) is present at a rate-limiting concentration and thus can be quantitatively measured by substrate conversion-rate kinetics, (i.e., Michaelis-Menton kinetics). In this study, we showed that the K-ELISA could be used to detect antibodies which are reactive with *L. pneumophila* serogroup 1 antigens of serogroup or common antigen reactivity.

MATERIALS AND METHODS

Biochemicals and other reagents. Polyoxyethylene sorbitan monolaurate (Tween 20) and *o*-dianisidine dihydrochloride (3,3'-dimethoxybenzidine-2-hydrochloride) were purchased from Sigma Chemical Co., St. Louis, Mo. Tris was obtained from Becton, Dickinson and Co., Orangeburg, N.J. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. All other chemicals were obtained from Mallinkrodt, Inc., St. Louis, Mo.

Sera. The sera used in this study was submitted to the Immunology Section, Centers for Disease Control, Atlanta, Ga., by state health laboratories for LD IFA testing. The study group was composed of 79 blind-coded acute- and convalescent-phase serum pairs (158 sera).

Antigens. IFA antigens were prepared from *L. pneumophila* strains Philadelphia 1 (serogroup 1), Togus 1 (serogroup 2), Bloomington 2 (serogroup 3), Los Angeles 1 (serogroup 4), Dallas 1 (serogroup 5), and Chicago 2 (serogroup 6), as described previously (12).

For each of the K-ELISA test antigens, 15 charcoal-yeast extract agar plates (100 by 15 mm) were inoculat-

ed with the Philadelphia 1 strain which then were incubated in a candle extinction jar for 3 days at 35°C. The bacterial mass was suspended in sterile distilled water (1.5 ml per plate), pooled, and then centrifuged at 5,000 × *g* for 20 min at room temperature. The pellets were suspended in 1.0 M Tris buffer (pH 8.0) at a ratio of 1 to 7.5 (cells to buffer [vol/vol]). After 15 min at room temperature, the suspensions were placed in a boiling water bath. To ensure that both common and serogroup-specific antigens were present, the crude antigen preparation was boiled for only 15 min. For the fractionated antigen, the boiling time was 30 min to maximize destruction of most of the detectable common antigen activity. After being boiled, the suspensions were allowed to cool and then were centrifuged at 45,900 × *g* for 20 min at 4°C. The supernatant fluids were dialyzed overnight against four changes of 0.1 M Tris–0.2 M NaCl buffer (pH 8.0). The unfractionated antigen preparation was stored at −20°C. For preparation of the fractionated antigen, the dialyzed supernatant fluid was concentrated to a volume of 4 ml (concentrated ca. 4×) in a Minicon Macrosolute concentrator (Amicon Corporation, Lexington, Mass.). This concentrated fluid was applied to a Sephadex G-200 column (1.6 by 100 cm) that had been equilibrated with 0.1 M Tris–0.2 M NaCl buffer (pH 8.0). The serogroup 1-specific antigen was eluted and pooled as shown in Fig. 1. Double diffusion in gel analysis (Ouchterlony) with hyperimmune rabbit antisera and the column fractions indicated that serogroup-specific activity was present in fractions 12 to 15 (Fig. 1), whereas the unfractionated antigen contained both serogroup-specific and common antigen reactivity. Protein concentrations of each antigen preparation were determined by the Bradford protein assay (2) with reagents from BioRad Laboratories, Richmond, Calif.

Immune Assays. The IFA was performed as previously described (12) with heat-killed, whole-cell antigens prepared from strain Philadelphia 1. The K-ELISA was adapted from Tsang et al. (9) as follows. Unfractionated or serogroup-specific antigen was used at a concentration of 5 μg of protein per ml of 0.05 M Tris–0.3 M KCl–0.02 M EDTA buffer (pH 8.0). This amount of antigen was determined in preliminary titrations to be in excess of that which is rate limiting. One milliliter of antigen was placed in each tube used in the assay (disposable 1.5-ml polystyrene test tubes, 1-cm light path; Walter Sarstedt, Inc., Princeton, N.J.), which was then wrapped in foil and incubated overnight at 4°C. Tubes then were placed in a 37°C water bath for 1 h. All fluid was aspirated from the tubes which were then washed three times with a jet spray cuvette washer (7, 9). The wash solution was phosphate-buffered saline (PBS) 0.01 M Na_2HPO_4-NaH_2PO_4–0.15 M NaCl (pH 7.2), containing Tween 20 at a final concentration of 0.3% (PBS-Tween). The tubes were aspirated to dryness after the final wash. PBS-Tween (1 ml) containing 5 μl of test or control serum was added to each sensitized tube, which was then incubated in a 37°C water bath for 1 h. Tubes were washed three times as before. One milliliter of peroxidase-conjugated goat anti-human immunoglobulin (reactive with immunoglobulins G, M, and A; Sigma Chemical Co., St. Louis, Mo.), diluted 1:400 in PBS-Tween, was added to each tube. The tubes were again incubated in a 37°C water bath for 1 h, and

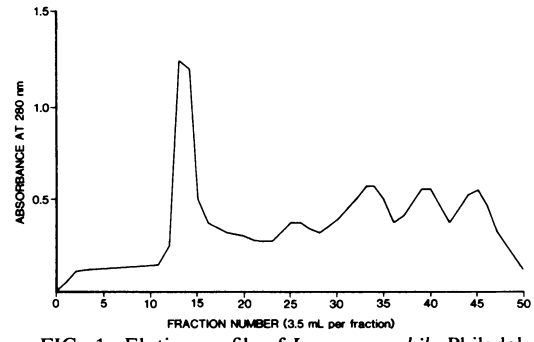

FIG. 1. Elution profile of *L. pneumophila* Philadelphia 1 antigen extract on Sephadex G-200 (column size, 1.6 by 100 cm; elution buffer, 0.1 M Tris–0.2M NaCl [pH 8.0], flow rate, 3 ml/h). Fractions 12 to 15 contained the serogroup-specific antigen and were pooled for use in the K-ELISA. The protein content of the pooled antigen was 1.39 μg/ul.

washed three times as before. Then, 750 μl of 0.05 M sodium acetate–acetic acid buffer (pH 5.0) was added to each tube which was placed in a spectrophotometer (model 635, UV; Varian Associates, Inc., Sunnyvale, Calif.) equipped with a 25°C water jacket surrounding the sample chamber; 250 μl of chromogenic substrate was added to each tube in the spectrophotometer. The substrate was prepared by dissolving *o*-dianisidine-2-hydrochloride in methanol at a concentration of 10 mg/ml. This substrate solution was then diluted 1:2 in 0.05 M sodium acetate–acetic acid buffer (pH 5.0). To make the final solution, 100 μl of H_2O_2 (purchased as a 3% solution) and 1.66 ml of substrate solution were added to 19 ml of 0.05 M sodium acetate buffer (pH 5.0). This was diluted to a final volume of 25 ml with the sodium acetate buffer and used as the K-ELISA substrate solution. Absorbance at 460 nm (A_{460}) was recorded for 1-min reaction periods. Activity was expressed as $\Delta A_{460}/\text{min} \times 10^{-2}$. K-ELISA values were expressed as the mean of duplicate determinations. A total of 158 serum specimens, obtained as paired acute- and convalescent-phase sera from 79 patients with suspected LD, were tested in the IFA and the K-ELISA. K-ELISA values of ≥3 were considered positive because 90% of 62 sera obtained from well individuals whose IFA titers were ≤64 gave K-ELISA values of <3 (results not shown) and because 94% of the sera from the patients with IFA titers of ≤64 had K-ELISA values of <3 (results described below).

RESULTS

When IFA titers of 158 sera were compared with K-ELISA values with the unfractionated antigen (Fig. 2), and with the serogroup-specific antigen (Fig. 3), correlation coefficients were 0.894 ($P < 0.001$) and 0.841 ($P < 0.001$), respectively. Of the sera with IFA titers that were less than 128 (the lowest IFA titer considered to indicate a recent infection), 94% had K-ELISA values that did not exceed 3.0 with the unfractionated or the serogroup-specific antigens, whereas 96% of the sera with IFA titers of ≥128

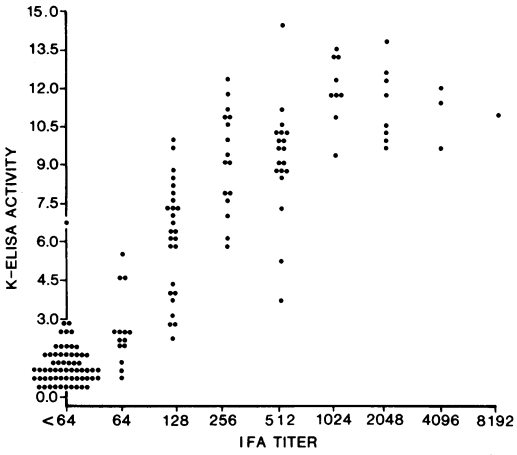

FIG. 2. Scattergram of IFA titer and K-ELISA activity obtained with the unfractionated *L. pneumophila* serogroup 1 antigen, which contains both serogroup-specific and common antigen activity. Correlation coefficient = 0.894, $P < 0.001$. K-ELISA activity is expressed as ΔA_{460} nm $\times 10^{-2}$ per min.

had K-ELISA values of ≥3.0 with the unfractionated antigen and 85% with the serogroup-specific antigen. The higher correlation between the unfractionated antigen K-ELISA and the IFA was expected, as the IFA measures both serogroup-specific and common antigen antibodies (12). Thus, the greater number of sera that gave positive IFA titers but negative K-ELISA values when serogroup-specific antigen was used can partially be attributed to antigen specificity. Nine sera were in this category: 2 were serogroup 4-specific, 1 was serogroup 2-specific, 3 reacted with multiple antigens, and 3 were serogroup 1-specific by IFA testing, although they were nonreactive by K-ELISA tests with serogroup-specific antigen.

Because demonstration of a seroconversion is considered evidence of recent *Legionella* disease in a patient with compatible symptoms, we compared the ability of the K-ELISA to detect increases in antibody concentration with that of the IFA. In the latter test, a seroconversion is defined as a fourfold or greater increase in titer to ≥128. When a 1.5-fold increase to ≥3.0 was considered as the minimum increase in K-ELISA values from the acute- to the convalescent-phase serum specimen that is interpreted as positive, the agreement between IFA and K-ELISA was as follows: 91% when unfractionated antigen was used and 82% when the serogroup-specific antigen was used (Table 1). Decreasing the minimum-fold increase requirement to 1.2 or increasing it to 2.0 resulted in 90% agreement (unfractionated antigen) and 81% (serogroup-specific antigen) or 81 and 74%, respectively.

Between-assay coefficients of variation were 4.8 and 5.9% in seven replicate K-ELISA tests (unfractionated antigen) of two reference sera with IFA titers of <64 and 4096, respectively.

DISCUSSION

In this study, we showed that human antibodies against *L. pneumophila* serogroup 1 antigens could be measured reproducibly (coefficients of variation, 4.8 to 5.8%) with the K-ELISA, a modified ELISA developed by Tsang et al. (7, 8, 9) that measures the rate of substrate conversion by the enzyme when the concentration of antibody is rate limiting. This kinetics-based test is different from the ELISA developed originally for legionellosis serology (3), because the K-ELISA does not require serum titrations, is not based on end-point analysis, and the test result is a spectrophotometric unit which can be converted to quantity of antibody per unit volume by comparison with the appropriate serum standards. By using the assay in this quantitative manner, Tsang et al. (8) reported that the K-ELISA detected as little as 2.0 ng of IgG. The sensitivity and specificity or the predictive values of the K-ELISA as a diagnostic test for legionellosis could not be determined with our data, as we had insufficient numbers of sera to test from culture- or epidemiologically confirmed patients with LD. Therefore, we used the IFA as a standard test for comparison with paired sera from 79 patients with suspected LD. This evaluation is restricted to the performance of the IFA, whereas if sera from well-documented cases were available, the K-ELISA might well prove to be the more sensitive or

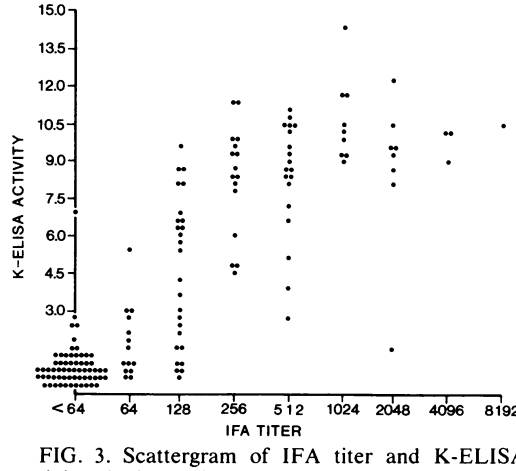

FIG. 3. Scattergram of IFA titer and K-ELISA activity obtained with serogroup-specific *L. pneumophila* serogroup 1 antigen. Correlation coefficient = 0.841, $P < 0.001$. K-ELISA activity is expressed as ΔA_{460} nm $\times 10^{-2}$ per min.

TABLE 1. Number of paired sera from patients with suspected Legionnaires disease with indicated test results

IFA	K-ELISA			
	No. positive[a]		No. negative	
	Unfractionated[b]	Serogroup-specific[b]	Unfractionated	Serogroup-specific
No. positive[c]	62	53	5	14
No. negative	2	0	1	12

[a] At least a 1.5-fold increase in K-ELISA values to ≥3 from the acute- to the convalescent-phase serum.
[b] For antigen preparation, see text.
[c] At least fourfold increase in titer to ≥128.

specific test. Because the IFA detects antibodies that are specific for serogroup-specific antigens and common antigenic determinants (12), the K-ELISA was set up in duplicate with a serogroup 1-specific antigen and an unfractionated extract of *L. pneumophila* serogroup 1 cells that contained common antigens also. As expected, the greater concordance of results was with the latter antigen: 0.894 correlation coefficient ($P < 0.001$) versus 0.841 ($P < 0.001$) and 91% agreement in detecting seroconversions versus 82%. About one-half of the discrepancies could be explained by additional experiments that showed preferential reactivity of the sera in question with IFA antigens other than serogroup 1. Whether the remaining discrepancies were due to differences in antibody avidity, which may affect ELISA values more than IFA titers, cannot be determined with the available data. The use of serogroup-specific antigens may provide a more specific test result that could be advantageous in epidemiologic studies, whereas the use of unfractionated antigen may provide more sensitive test results in the diagnosis of patients with sporadic cases. Further studies are required to determine the relative value of the two antigens.

Further studies should also include increasing the number of antigens in the test to include all known *L. pneumophila* serogroups and *Legionella* species. Using multiple K-ELISA antigens (i.e., antigens from other serogroups and species) may be required only in special circumstances, as the majority of direct immunofluorescence assay-positive specimens tested at the Centers for Disease Control have been *L. pneumophila* serogroup 1 and as the majority of sera from patients infected with other *Legionella* serogroups and species react with common antigens in addition to the serogroup antigen of the infecting strain. Of even greater value would be modifying the reactants used in this study to detect antigen in the urine of patients with LD since theoretically, the K-ELISA should be as sensitive as the ELISA or RIA described by other laboratories for this purpose (4, 5). In either the antibody- or antigen-detection test, the greatest advantage of the K-ELISA is its potential for quantification of test material and for automation in laboratories that test large numbers of specimens.

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