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Serological Evaluation of Thin-Layer Immunoassay–Enzyme-Linked Immunosorbent Assay for Antibody Detection in Human Trichinellosis

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A new immunoenzymatic test, named the thin-layer immunoassay–enzyme-linked immunosorbent assay (TIA-ELISA), was evaluated for antibody detection in human trichinellosis using excretion and secretion products prepared from Trichinella spiralis muscle larvae. Serum samples from people with positive muscle biopsies or symptoms compatible with the disease (n = 8 or 26, respectively), all reactive in enzyme-linked immunoelectrotransfer blot assay (EITB), as well as 67 serum samples from healthy, EITB-negative people, were tested in an ELISA and TIA-ELISA. TIA-ELISA was performed in polystyrene plastic petri dishes by adding dots of 10μl each of antigen (7μg/ml) followed by adding diluted serum and the conjugate. Finally, the substrate mixed with agar was added to develop the reaction. Enzymatic by-products were easily detected by the naked eye as defined dots. Sensitivity and specificity were 76 and 94% for ELISA, and both parameters were 91% for TIA-ELISA. The kappa correlation indices for both tests in relation to EITB were 0.73 and 0.80, respectively. The TIA-ELISA can be carried out with common laboratory equipment in 3 h and uses lower quantities of antigen than EITB and ELISA. Since TIA-ELISA is easy to perform, cheap, sensitive, and specific, the test could be an acceptable alternative to use in clinical laboratories lacking specialized equipment needed for ELISA and EITB and in field studies for antibody detection in human trichinellosis.

Trichinellosis is a worldwide zoonotic infection caused by Trichinella spiralis and also by other Trichinella species (10, 22). In humans, it usually appears as epidemiologically defined outbreaks caused by the ingestion of uncooked mammalian infected meat (11, 17, 25, 27). Clinical symptoms are nonspecific, since they are similar to those present in other infectious diseases, resulting in a number of undetected cases. Furthermore, since parasitoscopical diagnosis is frequently impractical because it requires a microscopic search for larvae in muscle biopsy samples, antibody detection is the best option. Serological tests use surface, somatic, or metabolic T. spiralis antigenic products that are either crude, purified, or semipurified (3, 7, 14, 26). Almost all procedures that detect antibodies (i.e., complement fixation, precipitation, hemagglutination, flocculation, intradermal reactions, and immunofluorescent, radiometric, and immunoenzymatic tests) have been used for the serodiagnosis of this parasitic infection (7–9, 14, 18–20). For a long time, the bentonite flocculation test with excretion and secretion (E/S) antigens, indirect hemagglutination with somatic, semipurified extracts (Melcher’s antigen), and the immunofluorescent-antibody test were considered the most sensitive, specific, and useful assays (20). Nowadays, however, the most broadly accepted procedures for diagnosis are the enzyme-linked immunosorbent assay (ELISA) and the enzyme-linked immunoelectrotransfer blot assay (EITB) used with E/S antigens prepared from T. spiralis muscle larvae (ML). Both tests present good sensitivity and specificity values as demonstrated by an adequate correlation with the presence of ML both in human patients and in naturally or experimentally infected animals (1, 7, 9, 21, 24, 26). Nevertheless, they require delicate, sophisticated, or expensive equipment, such as a microplate reader or electrophoresis and protein transfer equipment. A serological test which does not require such equipment, namely the thin-layer immunoassay (TIA), has been previously reported (12). In this test, the exposed epitopes of the antigen adsorbed on the surface of plastic petri dishes are recognized by specific antibodies in immune serum when spotted onto the dishes and their presence is developed with water vapor. Modifications to this procedure gave rise to the diffusion-in-gel ELISA (DIG-ELISA) technique (13), in which serum antibodies are diffused in a gel layer and their presence is detected with a specific peroxidase conjugate anti-immunoglobulin. DIG-ELISA is a very simple assay which can be carried out with common laboratory equipment and materials, and its results can be easily read by the naked eye. DIG-ELISA has been successfully evaluated and gave good sensitivity and specificity values for serodiagnosis of Chagas’ disease and onchocercosis (4, 15, 16, 23); however, results in DIG-ELISA are obtained after 30 h.

Here we report a modified version of the TIA, named the TIA-ELISA, which provides results as dots in 3 h. We compared it with standard ELISA, using EITB as a reference test.

MATERIALS AND METHODS

Serum samples. One hundred one serum samples previously tested in EITB were used in a double-blind study in ELISA and TIA-ELISA, using T. spiralis E/S antigen obtained from ML. Sera were divided into two groups according to their previous reactivity in EITB. One group (reactive) contained 34 positive samples (8 from patients with positive muscle biopsies and 26 from patients without parasitoscopical studies but with symptoms compatible with trichinellosis). All of them showed the diagnostic bands of 44, 49, and 55 kDa and were classified into three groups according to the intensity of reaction: weak (n = 11), medium (n = 13), and strong (n = 10). The positive criterion is in agreement with the one described before for antibody detection in swine trichinellosis (26) and in human and experimentally infected rats (8, 9). The second group (nonreactive) contained 67 serum samples from EITB-negative individuals having no symp-
were collected, pooled, and clarified at 750 g for 10 min in a refrigerated centrifuge. Protein inhibitor cocktail (N-tosyl-L-phenylalanine-chloromethyl ketone and NaHCO₃, pH 9.6) and incubated at 37°C in a humidiﬁed atmosphere containing 95% air and 5% CO₂ for 48 h. Supernatants were collected, pooled, and clarified at 750 × g for 15 min. After protein precipitation with the Bradford dye reagent (Bio-Rad, Hercules, Calif.), a protein inhibitor cocktail (N-tosyl-L-phenylalanine-chloromethyl ketone and tosyl-L-lysine chloromethyl ketone, 50 μg of each per ml, and phenylmethylsulfonyl fluoride, 100 mM [ﬁnal concentrations]) was added to the E/S antigen. The E/S antigens were kept frozen at −70°C in 1-ml aliquots until used.

ELISA. ELISA was performed as previously described (8). Briefly, E/S antigen (3 μg/ml) was used to coat high-binding polystyrene plates (Costar, Cambridge, Mass.). After washing, serum samples were incubated for 120 min at 37°C. Specific antibodies were detected using a goat anti-human immunoglobulin G-phycoerythrin conjugate (Bio-Rad). Substrate solution containing p-nitrophenyl phosphate (Sigma) was used to develop the reaction by incubation at 37°C for 30 min. The reaction was stopped by adding 1 N NaOH. Absorbance values were obtained in an ELISA plate reader (Bio-Rad) at 405 nm. Samples were considered positive when absorbance values were higher than the cutoff value estimated as the mean plus 3 standard deviations of absorbance values from 15 negative sera (healthy donors) that were separately studied (ΔA₄₀₅ = 0.183).

TIA-ELISA. In order to determine the optimal conditions to perform the TIA-ELISA, several concentrations of the antigen and different solutions (PBS, 0.05% Tween in PBS, distilled water, 0.85% NaCl solution, and 0.1 M carbonate buffer) to coat the plastic surfaces of petri dishes were tested using pooled positive or negative sera. In addition, the optimal time and conditions for incubation of the reagents, the best dilution of serum samples and conjugate, and the usefulness of two chromogenic substances (p-phenylenediamine and 5-aminosalicylic acid) were also determined. The best conditions identiﬁed to perform the test were as follows. Sterile polystyrene petri dishes (Laboratorios Technicare, Mexico City, Mexico) were ﬁrmly positioned on a pattern designed to accommodate 52 samples; the orientation was marked with a soft pen. Dishes were dotted on the marks indicated by the pattern with 10 μl of E/S antigen (7 μg/ml) diluted in 0.1 M carbonate-bicarbonate buffer (30 mM Na₂CO₃ and 70 mM NaHCO₃, pH 9.6) and incubated for 1 h at 37°C in a wet chamber; they were then washed gently three times with 150 mM NaCl solution and three times with distilled water and ﬁnally air dried. Dishes were carefully and appropriately put on the pattern, and 5 μl of a serum sample diluted 1:10 in 0.05% Tween in PBS (PBS-T) was added exactly on the site of one drop of antigen; each serum was tested on duplicate dots. Dishes were incubated for 30 min as described above, washed three times with PBS-T, then washed with PBS, air dried, and positioned again on the pattern. Five microliters of peroxidase-conjugated goat anti-human immunoglobulins (Sigma), diluted 1:40 in PBS-T, was added precisely on the immobilized immune complexes. Dishes were incubated and washed as described above. Agar-Agar (Dibico, Mexico City, Mexico) (0.87 g/100 ml of PBS) was melted and cooled to 45°C; then 10 ml of the agar solution was mixed with the substrate solution prepared with 5 mg of 5-aminosalicylic acid (Sigma), 1 ml of PBS, and 20 μl of 3% H₂O₂. The substrate was poured onto each dish. After 30 min of incubation at room temperature, the colored dots detected by the naked eye were registered. The presence of color reaction zones was the criterion for positivity of the test. The intensity of reaction was arbitrarily classiﬁed as weak, medium, and strong. Finally, dishes were covered with an appropriately sized piece of ﬁlter paper (Whatman no. 4), wetted in distilled water, and left unencapped on the bench for 18 h. Before the ﬁlter paper was removed, dishes were added and incubated with 0.5% glycerol for 5 min, dried, and stored at room temperature.

Serological evaluation. The serological parameters needed to perform the evaluation of TIA-ELISA, i.e., sensitivity, speciﬁcity, and predictive value for a positive and for a negative result, as well as the kappa coefﬁcient, were determined according to previously described procedures (2).

RESULTS

Antigen-antibody reactions were visualized in TIA-ELISA as brown dots formed in the gel-containing substrate (Fig. 1). As is shown, the reaction zones are almost circular and are clearly differentiated from the neighboring clear areas. Color intensity, as well as the size and deﬁnition of the dots, is not uniform. Negative sera did not form colored spots. No changes in results were observed when complete dishes or just the gel-containing results were dried and preserved. Table 1 shows the reactivity of TIA-ELISA and ELISA compared to EITB, as well as the serological parameters analyzed. Sensitivity and speciﬁcity of TIA-ELISA were similar; in contrast, ELISA showed a lower sensitivity but a higher speciﬁcity. The predictive values both for positive and negative results are quite similar between the techniques, although for TIA-ELISA the negative predictive value is higher. The kappa coefﬁcient indica-

<table>
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<th>Test and result</th>
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<th>Specificity (%)</th>
<th>Predictive value (%)</th>
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TABLE 1. Serological evaluation of TIA-ELISA and ELISA in immunodiagnosis of human trichinellosis

FIG. 1. Reactivity of 50 serum samples tested in TIA-ELISA against an E/S product of T. spiralis ML antigen. Colored dots indicate the positions of positive samples (1, 3 through 7, 11 through 15, 24, 30, 35 through 39, and 43 through 47) while clear areas above the numbers indicate the positions of negative serum samples (2, 8 through 10, 16 through 23, 25 through 29, 31 through 34, 40 through 42, and 48 through 50). Positive and negative control serum samples are in positions 51 and 52, respectively.
ces obtained for both tests are considered good. Compared to the long time and high quantities of antigen and conjugate used by EITB (28 h and 2 μg of antigen and 1 μl of conjugate per sample) and ELISA (8 h and 0.63 μg of antigen and 0.1 μl of conjugate per sample), TIA-ELISA is a faster and more economic test, since it requires 3 h and 0.14 μg of antigen and 0.3 μl of conjugate per sample. Regarding the intensity of reaction by TIA-ELISA, 8 samples were classified as having a weak reaction, 10 had a medium reaction, and 19 had a strong reaction. However, no association was found among tests in relation to the intensity of the reaction (data not shown).

DISCUSSION

A novel immunoenzymatic test named TIA-ELISA was developed, standardized, and evaluated for antibody detection in human trichinellosis by using the E/S products of T. spiralis ML as antigens. Results were compared with those obtained with the conventional ELISA and were serologically evaluated using EITB as the reference test (9, 26). During the standardization process it was observed that the antigen could be easily attached to the plastic surface of the petri dish, especially when carbonate buffer was used. Also, the enzyme-substrate reaction took place in the dot area in the gel. Likewise, it was clearly determined that the peroxidase by-products remained stable in the dried gel, provided that 5-aminosalicylic acid was used as the chromogen as previously reported (4, 13, 15, 16).

An ideal serodiagnostic test, especially for developing countries, should be cheap, simple, and easy to perform, in addition to being sensitive and specific (28). As our results show, TIA-ELISA fulfills these requirements. The test could be as useful as EITB and ELISA for antibody detection in human trichinellosis, since the kappa coefficient index was higher than for ELISA and the sensitivity was high, meaning that TIA-ELISA can identify more positive samples than ELISA, which is relevant to selecting a screening test. Furthermore, TIA-ELISA is as fast as possible may be important in order to provide timely medical attention, as required in outbreaks, or to prevent the spread of human infections. Also, TIA-ELISA is a good alternative for clinical laboratories that lack equipment to perform EITB or ELISA. Finally, TIA-ELISA could also be useful for performing a faster diagnosis of T. spiralis infection in abattoirs before or immediately after slaughtering or in rurally reared or free-roaming swine to prevent transmission to humans (5, 6, 17, 24).

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