Whole Blood, Serum, and Tissue Fluids in an Enzyme Immunoassay for Swine Trichinellosis

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ABSTRACT

Two enzyme immunoassays (EIA) for trichinellosis in swine (a research version and a commercial kit obtained from LMD Agro-Vet) were used to test pig whole blood, serum, and tissue fluids as sample sources. Five pigs inoculated with 2,500 Trichinella spiralis were bled weekly and then sacrificed after 42 days. Blood and serum samples from each sampling time and tissue fluids collected at slaughter were tested in both EIA formats. Whether samples were undiluted or diluted, blood could be used as effectively as serum for detecting pigs infected with T. spiralis. Further, it was as effective to use tissue fluids as either blood or serum. With both tests, the research and commercial versions, it is possible to detect infected pigs by 28 days following infection. The results of this study suggest that a rapid EIA test such as the LMD Agro-Vet Trichinella Serology Microwell ELISA can be used as an effective tool for testing pigs for trichinellosis on the farm. Additionally, because it can enable the detection of antibodies in tissue fluids, this test could be used effectively to determine trichinellosis in meat samples following slaughter.

Key words: Trichinella spiralis, pigs, serology, EIA

Trichinella spiralis is a tissue-dwelling nematode found in virtually all warm-blooded carnivores and omnivores. It is the etiologic agent of trichinellosis in humans. Historically, human infections with T. spiralis have been associated with the ingestion of raw or undercooked pork, but more recently infection sources have included bear, walrus meat, and horsemeat (1, 4, 13).

Many countries inspect swine at slaughter for the presence of trichinae. Countries of the European Union inspect in accordance with EU Directives 77/96/EEC and 84/319/EEC (5, 6). A voluntary inspection program for fresh pork is allowed in the U.S. under the Code of Federal Regulations (2).

Numerous reports have documented the efficacy of enzyme immunoassays (EIA) for detecting infection with T. spiralis in pigs, horses, bears, and other species (3, 8, 9, 14). The test is highly sensitive, detecting infections as low as 1 larva per 100 g of tissue (7). Further, since the time following infection when animals become seropositive is inversely proportional to the dose of worms ingested, animals with heavy infections, most likely to cause public health problems, become seropositive coincident with or before the time when larvae reach the infective stage for another host (9). Thus, false-negative results are rare in animals with infection levels high enough to cause human disease. The EIA has been shown to be useful for epidemiological studies (3), for selective depopulation of infected herds (14), and for use in a high-volume slaughterhouse environment (15).

In two recent studies we have shown that the EIA performs as well as artificial digestion methods for the detection of trichinellosis in pigs (7) and horses (9). In the present study, we compare the EIA test as we use it in the laboratory (10) with a rapid commercial test kit (LMD Trichinella Serology Microwell ELISA, LMD Agro-Vet, St. Paul, MN). In addition, both tests use serum at dilutions of 1:100 to 1:200. Because it would be useful to use either whole blood (for field use) or tissue fluids (for postmortem identification) we have performed experiments to compare these two tests using whole blood, serum, and tissue fluids.

MATERIALS AND METHODS

Pig infections

The parasite T. spiralis (Beltsville pig strain, designated T-1 under nomenclature of the Trichinellosis Reference Centre) was maintained by serial passage in female Sprague-Dawley rats. Infective larvae were recovered as previously described (7). A group of 5 pigs (Yorkshire × Duroc) of mixed sex and weighing 25 to 30 kg were inoculated with T. spiralis at a dose of 2,500 larvae per pig. This dose has previously been shown to cause infection in pigs at a level to be considered of public health importance (7). For inoculation, larvae were enumerated, placed into gelatin capsules and administered per os using a bulling gun.

Blood and tissue recovery

Blood samples were collected by jugular venipuncture with a 10-ml syringe fitted with an 18-gauge needle. Two 10-ml samples of blood were collected from each pig before inoculation and on postinoculation days (PID) 7, 14, 21, 28, 35, and 42. One sample...
from each pig was collected in 0.4% sodium citrate to prevent clotting. The second sample was allowed to clot and serum was separated by centrifugation at 10,000 × g for 15 min. Six weeks following inoculation all pigs were killed by stunning with a captive-bolt pistol followed by exsanguination. Tissue samples were collected from the tongue and diaphragm to determine worm burdens (see below). Tissue fluids were collected by grinding small pieces of muscle tissue in a Hobart commercial meat grinder and compressing the ground tissue between glass slides to collect fluid.

**Digestion testing**

Worm burdens of *T. spiralis* larvae in pigs were determined by digestion of 100 g of tongue and diaphragm. Briefly, whole tongue and the criss muscle from the diaphragm were ground together in a commercial meat grinder, and 100 g mixed with 1 liter of artificial digestion fluid (1.0% pepsin, wt/vol [National Standard Formulary 1:10,000] and 1.0% hydrochloric acid, vol/vol). Digests were mixed vigorously on a magnetic stir plate at 37°C for 3 h. Digests were settled for 20 min, the supernatants were decanted, and the sediments were poured through an 80-mesh sieve into round-bottom Filsner glasses. Following settling for another 20 min, the sediments, containing *T. spiralis* larvae, were washed repeatedly by settling in tap water. Recovered larvae were counted on a stereo microscope at a 40× magnification.

**Serology testing**

Serum samples collected from trichinae-inoculated pigs were tested for antibodies to *T. spiralis* using the enzyme immunoassay as described by Gamble et al. (8, 11) (hereafter referred to as the ARS test) and the LMD Agro-Vet Trichinella Serology Microwell ELISA (hereafter referred to as the LMD test).

For the ARS test, flat-bottom microtitration plates (Dynatech #4) were coated with *T. spiralis* excretory-secretory (ES) products (11) (0.5 μg per well diluted in 0.1 M carbonate buffer, pH 9.6) by incubating for 1 h at 37°C. Following coating and all subsequent steps, wells were washed three times with 200 μl of 50 mM Tris buffer (pH 7.4) containing 150 mM sodium chloride, 1.0% Triton X-100 and 5% non-fat dry milk (wash buffer). The following reagents were then added sequentially and incubated for 30 min at 22°C: (i) pig whole blood, serum, or tissue fluids, diluted in wash buffer or undiluted; (ii) goat anti-swine IgG (γ-chain specific) diluted 1:1,000 in wash buffer, and (iii) rabbit anti-goat IgG, conjugated to horseradish peroxidase and diluted 1:1,000 in wash buffer. Following the final wash, substrate (2,2'-azino-di-[3-ethylbenzthiazoline] sulfonic acid and hydrogen peroxide) was added and plates read on an automated microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm.

The LMD test was performed according to the manufacturers instructions. Blood, serum, and tissue fluids were used undiluted and diluted 1:10 and 1:100. Each sample was run in duplicate wells. All plates were run to reach a fixed identical optical density (OD) value (2.0 for the LMD test and 1.0 for the ARS test) for a known set of *T. spiralis*-positive serum standards. A positive cut-off for the EIA was established as 15% of the difference between the positive and negative control ([(positive OD – negative OD) × 0.15] + negative OD).

**Analysis of results**

EIA values for each sample-time-dilution combination were averaged for all 5 pigs and plotted using GraphPad Inplot (GraphPad, San Diego, CA). Significant differences between EIA values obtained with blood and serum were determined for each time point using a paired *t*-test at *P* = 0.05 (GraphPad Instat, San Diego, CA). Variance in EIA results between samples (blood, serum, tissue fluids) at a single time point (42 days after inoculation) was determined by using a one-way analysis of variance at *P* = 0.05.

**RESULTS**

All 5 pigs inoculated with *T. spiralis* had larvae in the musculature 42 days after inoculation (Table 1).

The results of testing blood and serum from these pigs using two EIA tests for the 6 weeks following inoculation with 2,500 *T. spiralis* larvae (L1) are presented in Figures 1 and 2. In all cases, the optical density values were initially higher in undiluted samples; however, the final values were higher in diluted samples (Figure 1). Values obtained for blood and serum, either undiluted or diluted 1:10 or 1:100, were not significantly different, using a paired *t*-test, at any time during the course of infection (Figure 2); the single exception was a slightly, but significantly, lower value as compared with undiluted serum at 42 days after inoculation using the ARS test.

A comparison of tissue fluid samples with blood and serum in the EIA tests is presented in Figure 3. When used undiluted, tissue fluids gave equivalent or higher optical density values in the EIA using either test (Figure 3A). At dilutions of 1:10 or 1:100, tissue fluids resulted in values which were, in most cases, statistically equivalent to those from blood or serum, using a paired *t*-test. Only values from fluids from the diaphragm, diluted 1:10 or 1:100 in the LMD test, were statistically lower than blood or serum values.

The time course of seroconversion for the 5 pigs is presented in Table 2. All pigs were positive, at all dilutions and in both tests, by 28 days after inoculation. Similar numbers of pigs became seropositive at days 14 and 21 when compared between the two tests. However, the LMD test, using undiluted blood or serum, detected 1 positive pig at both days 14 and 21 which was negative using the ARS test.

**DISCUSSION**

Current methods approved for the inspection of swine and other species for *T. spiralis* rely exclusively on variations of artificial digestion methods (2, 5, 6). The availability of a reliable serologic test, which could be used either ante- or postmortem, would be a valuable tool for testing pigs for trichinellosis both on the farm and at the slaughterhouse. An EIA based on the use of excretory-secretory antigens collected from *T. spiralis* muscle larvae maintained in vitro for 24 h has been used extensively in various research investigations.
this type of application, it would be beneficial to have a test which could be used with whole blood rather than requiring serum separation. Another potential application of the EIA is testing carcasses after slaughter or even testing cuts of meat. This would be particularly useful where identification systems make it impossible to match blood or serum samples.

FIGURE 1. Comparison of EIA results measured as optical density at 405 nm (ARS test) or 450 nm (LMD test) for blood or serum samples used undiluted •—•, diluted 1:10 ■—■, or diluted 1:100 ▲—▲. (A) LMD test using blood; (B) LMD test using serum; (C) ARS test using blood; (D) ARS test using serum.

One potential use of the EIA in preharvest control of trichinellosis is testing swine herds prior to slaughter. For laboratories for testing a variety of species and sources of serum (3, 12, 14, 16). This test has recently been adapted by LMD Agro-Vet, to a rapid (20-min) format. Both the research version of the EIA and the commercial test use serum as the test sample.

FIGURE 2. Comparison of EIA results measured as optical density at 405 nm (ARS test) or 450 nm (LMD test) for blood or serum samples. (A) LMD test with undiluted samples; (B) LMD test with samples diluted 1:10; (C) LMD test with samples diluted 1:100; (D) ARS test with undiluted samples; (E) ARS test with samples diluted 1:10; (F) ARS test with samples diluted 1:100. An asterisk indicates time points at which results obtained using the two samples were significantly different.

FIGURE 3. Comparison of EIA results measured as optical density at 405 nm (ARS test) or 450 nm (LMD test) for tissue fluids, whole blood, and serum collected at necropsy. Sample sources are as indicated in the legend. The left portion of each panel shows results using the LMD test and the right portion of each panel shows results using the ARS test. (A) samples undiluted; (B) samples diluted 1:10; (C) samples diluted 1:100. Bars with the same letter superscript are not significantly different as determined by a Duncan multiple range comparison at a P = 0.05. Dia, diaphragm; Ton, tongue.
with carcasses. Thus, the use of tissue fluids in an EIA would abrogate the necessity for collecting blood at the point of slaughter.

In the present study, we have examined the use of blood and tissue fluids in comparison to serum samples in both the ARS and LMD EIA tests. In all cases, undiluted blood gave results which were equivalent to serum as measured by EIA values. Thus, it appears blood can be used in lieu of serum in either test. It is interesting that even undiluted blood or serum performed well in both tests. However, it was noted that in the ARS test, undiluted whole blood had a slight inhibitory effect compared with serum on one occasion. A similar effect using undiluted whole blood was not seen with the LMD test kit.

Tissue fluids were found to be as useful as blood or serum in either version of the EIA test. In fact, at undiluted concentrations, tissue fluids gave higher optical density values in the EIA than blood or serum. When diluted, tissue fluids were still as effective as blood and serum in either test. It would be of interest to determine whether fluids from tissues other than the tongue or diaphragm can be used as effectively in the EIA. If so, commercial cuts of meat could be tested for trichinellosis by EIA at any point prior to reaching the consumer.

Both the ARS EIA and the LMD test kit were effective in detecting T. spiralis-inoculated pigs by 4 weeks postinoculation. Optical density values were initially higher using undiluted samples, but later in the infection, presumably due to rising antibody titers and prozone effects, diluted samples became higher. Worm burdens found in these pigs were high enough to be of public health importance. The mean worm burdens in tongue and diaphragm for the 5 pigs was 212. While it has been shown that commercial cuts of meat (hams, shoulders, ribs, etc.) may harbor five to ten times fewer larvae per gram of tissue than tongue and diaphragm (7), worm burdens in these muscles would still exceed 20 larvae per gram for these 5 animals. This is well above the projected 3 to 5 larvae per gram of tissue which is necessary to cause clinical disease in humans (9). Using undiluted samples or samples diluted 1:10, some pigs were detected as positive as early as 14 days postinoculation. In all cases, the commercial EIA performed as well as the research version of the test.

**REFERENCES**


