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Experimental Swine Trichinellosis: Use of Dot-ELISA and Western Blot with Excretion/Secretion Antigens (ES) from Infective Larvae to Detect Anti-*Trichinella spiralis* Antibodies

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ABSTRACT. Excretion/secretion antigens from *Trichinella spiralis* infective larvae were evaluated in Dot-ELISA and Western blot techniques for the serologic diagnosis of trichinellosis in experimentally infected swine. Three York female pigs were infected with 10,000 *T. spiralis* infective larvae/animal. After infection, weekly blood samples, during 10 weeks, were taken from them to obtain sera. At this time muscle tissue samples were collected from tongue and diaphragm and subjected to artificial digestion to determine the number of larvae per gram of muscle tissue (LPGMT). Animals showed a mean of 10 LPGMT. Positive anti-*T. spiralis* antibody titers (threshold of positivity 1:160) were determined in sera five weeks after infection by Dot-ELISA. When the Western blot method was used a pattern of seven antigenic components of 229, 108, 64, 45, 41, 40 and 33-38.5 kDa were recognized by swine sera at week five after infection. Sera from *Ascaris suum* infected-swine were negative to anti-*Trichinella* antibodies when analyzed by Dot-ELISA and Western blot. Both analysis were similar in discriminating positive from non-positive sera to anti-*T. spiralis* antibodies and showed a sensitivity and specificity of 100%. For this reasons and for its easily to carry out, it is suggested to use Dot-ELISA for the serologic diagnosis of swine trichinellosis.
Key words: *Trichinella spiralis*, Excretion/secretion antigens, Dot-ELISA.

RESUMEN. Los antígenos de excreción/secreción (ES) de larvas infectivas de *Trichinella spiralis* fueron evaluados por las técnicas de Dot-ELISA e Inmunoelectrotransferencia (IET) para el diagnóstico serológico de trichinellosis en cerdos infectados experimentalmente. Tres cerdos York hembra fueron infectados con 10,000 larvas infectivas de *T. spiralis*/animal. Después de la infección, se colectó suero de los cerdos, durante 10 semanas. Los animales fueron sacrificados para colectar muestras de tejido muscular a partir de la lengua y diafragma. Los tejidos fueron sometidos a digestión artificial para determinar el número de larvas por gramo de tejido muscular (LPGTM). El LPGTM promedio fue de 10. Con Dot-ELISA (umbral de positividad 1:160) se detectaron anticuerpos anti-*T. spiralis* a partir de la quinta semana postinfección. Mientras que, por medio de IET, los sueros reconocieron un patrón de siete componentes de 229, 108, 64, 45, 41, 40 y 33-38.5 kDa, también a partir de la semana cinco postinfección. Cuando se examinaron los sueros de cerdos de traspatio infectados con *Ascaris suum* por medio de Dot-ELISA e IET con antígenos de *T. spiralis*, éstos fueron negativos a anticuerpos anti-*Trichinella*. El desempeño de Dot-ELISA e IET fue similar para discriminar sueros positivos de aquellos no positivos a anticuerpos anti-*T. spiralis*. Ambas técnicas mostraron una sensibilidad y especificidad del 100%, por esta razón y por su facilidad de ejecución, se sugiere utilizar Dot-ELISA para el serodiagnostico de trichinellosis porcina.
Palabras clave: *Trichinella spiralis*, Antígenos de Excreción/secreción.

INTRODUCTION

In spite of the established sanitary control programs, human thrichinellosis outbreaks are constantly presented in different parts of the world, including Mexico where the frequency appears to be rising en recent years.[9, 2] This apparent increase may be due in part to the lack of effective diagnostic methods and to the sacrifice of backyard pigs without any sanitary inspection.[14] In Mexico, usually pig meat is inspected by the compression (trichinoscopy)

method, which shows very low sensitivity for detection of larvae in muscle tissue, and sometimes by artificial digestion. Besides, these methods do not detect early and very light infections.[21,17]

For these reasons different immunological tests have been evaluated for detection of anti-*Trichinella spiralis* antibodies in pig sera, such as complement fixation, passive hemagglutination, latex agglutination and indirect fluorescent antibody test (IFAT), among others. In this context, in the last decade the immunoenzymatic tests (ELISA) have displaced other serologic methods due to its sensitivity and capacity for analyzing many samples at the same time.

The first in using ELISA for diagnosing swine trichinellosis were Ruitenberg et al[16] who demonstrated that this assay was more sensible than IFAT and the direct detection of the parasite. However, one problem of ELISA was the high number of false positive reactions observed[16] that probably were caused by the use of crude *T. spiralis* antigens which share common epitopes with other swine parasites such as *Ascaris suum* and *Trichuris suis*.[4]

In this context, the specificity of ELISA is increased by using purified antigens[18] or excretions-secretions antigens from infective larvae.[4]

The use of standard ELISA under field conditions in Mexico is difficult; however, other variant of the test, Dot-ELISA[12] is relatively easy to perform and to read, among other characteristics. It also shows sensitivity and specificity similar to those of standard ELISA and the western blot technique.[20] The sensitivity of Dot-ELISA for diagnosing some parasitic diseases such as acute and chronic esquistosomiasis, quistic hydatid disease and human visceral leishmaniasis has been shown to be higher than 90%.

In this connection, the evaluation of Dot-ELISA for diagnosing swine trichinellosis is of particular interest because due to its characteristics would be an important tool for the seroepidemiological screening of pig sera from different states of the Mexican Republic which in turn would contribute to the control of this disease.

MATERIALS AND METHODS

Animals. Wistar rats and NIH mice were used to maintain the *T. spiralis* strain. Yorkshire pigs, *T. spiralis* free, as determined by ELISA, before the beginning of the experiment were used for the experimental infection with *T. spiralis*.

Sera. Serum samples were obtained from three *T. spiralis* experimentally infected swine, 12 backyard pigs from Jesus María municipality at the Aguascalientes State, and 10 backyard swine from Jilotepec municipality at the Mexico State. Three positive pig control sera to anti-*T. spiralis* antibodies was kindly donated by Dr. Camila Arriaga and Dr. Guadalupe Ortega-Pierres.

Coprological tests. The modified Faust test was used to determine helminth eggs in faeces[10] from pigs used in the study.

Parasites. *T. spiralis spiralis* organisms were used in this study. The parasite has been maintained by serial passage in mice and rats since 1960 at the Department of Parasitology at the Escuela Nacional de Ciencias Biológicas from the Instituto Politécnico Nacional.

T. spiralis larvae were first obtained by artificial digestion from infected Wistar rats. Afterwards each of 30 NIH mice was inoculated by oral route with 250 larvae. At 30 days after infection mice were humanitarianly euthanatized and their muscle tissues were subjected to artificial digestion according to Peña-Piña et al[13] to obtain more infective larvae. Finally, these were counted in a nematode counting chamber.

Infection of swine. Each of three female, two months old, Yorkshire swine was inoculated by oral route with 10,000 infective *T. spiralis*. Blood samples were collected from these to obtain sera before infection and weekly after this during three months. Sera were dispensed in vials and maintained at 70ºC before use. At the end of the study animals were humanitarianly euthanatized and tissues recovered from the tongue and diaphragm which were digested to determine the number of *T. spiralis* larvae per gram of muscle tissue.

Excretion-Secretion antigens (ES). After recovery from mice muscle tissues, *T. spiralis* larvae were washed three times with PBS pH 7.2, containing penicillin (100 IU/ml) and streptomycin (100 µg/ml), and then centrifuged at 800 x g during 5 min. Larvae (10,000 larvae/ml) were transferred to a culture bottle containing Rohrbacher medium[15] and penicillin (100 IU/ml) and streptomycin (100 µg/ml) and incubated for 72 h at 37ºC in a humidified atmosphere containing CO_2 at 5%. Every 24 h of incubation, the medium was replaced and to the collected one a commercial cocktail of protease inhibitors (TPCK, TLCK and PMSF) was added (Lab. Boehringer, Germany) at the concentration suggested by the manufacturer and 0.5 M EDTA was also added (67 µl/10 ml of medium). The supernatant was filtered (22 µm membrane) and kept at 4ºC. Then, the three samples were mixed, concentrated (AMICON 10X with a 25 kDa cutoff membrane) to 2 mg protein/ml. Protein concentration was determined by the Lowry et al method.[8] Finally, the antigen was dispensed into Eppendorf vials and frozen at -70ºC until used.

Western Blot analysis. For Western blot analysis the ES antigen obtained as previously described, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels according to Laemmli.[7] The separated proteins were transferred to nitrocellulose sheets and antigens were revealed by successive incubations with serum samples from experimentally infected pigs and from backyard pig diluted 1:50 and peroxidase conjugated rabbit anti-pig IgG (whole molecule, Sigma, St. Louis, Missouri) diluted 1:250

in PBS. The bands were developed in 1-4-Cl naphtol composed of 50 ml of PBS (pH 7.2), 50µl of hydrogen peroxide, 10 ml of methanol and 30 mg of 1-4-Cl-naphtol.[20] Nitrocellulose paper strips with molecular markers were removed after blotting, blocked and stained with amido black.

Dot-ELISA. The method of Pappas et al[12] was followed. The ES antigen (6µl/dot with a protein concentration of 10µg) was spotted onto nitrocellulose paper (NC, Sigma). The NC paper spotted with antigen was air-dried for 30 min and then blocked with skimmed milk at 5% in PBS for 30 min. After blocking, the NC paper was cut into 5-mm strips and incubated with diluted pig sera (1:160 in blocking solution) and incubated for 30 min at room temperature with gently shaking. The strips were then washed by flushing with PBS and incubated with diluted (1:250 in PBS) rabbit anti-pig IgG peroxidase-conjugated for 30 min at room temperature. After a final washing with PBS the strips were exposed to 3,3-diaminoobenzidine-tetrahydrochloride solution (DAB) composed of 25 ml of PBS (pH 7.2), 25 µl of 3% hydrogen peroxide, and 13 mg of DAB. The reaction was stopped after 5 min by washing with deionized water.

RESULTS

Western blot. Sera from the three infected swine recognized a pattern of seven components from the ES antigen by western blot, and had apparent molecular weights of 229, 108, 64, 45, 41, 40 and 33-38.5 kDa. Recognizing of these seven components by swine sera began in one pig at week four after infection and in the other two pigs one week later (Fig. 1). Taking into account the result, to consider a pig serum as positive to *T. spiralis* antibodies this should recognize that pattern. Sera from four swine naturally infected with *Ascaris suum* (Jilotepec, Mexico State) reacted with two components of 42.5 and 45

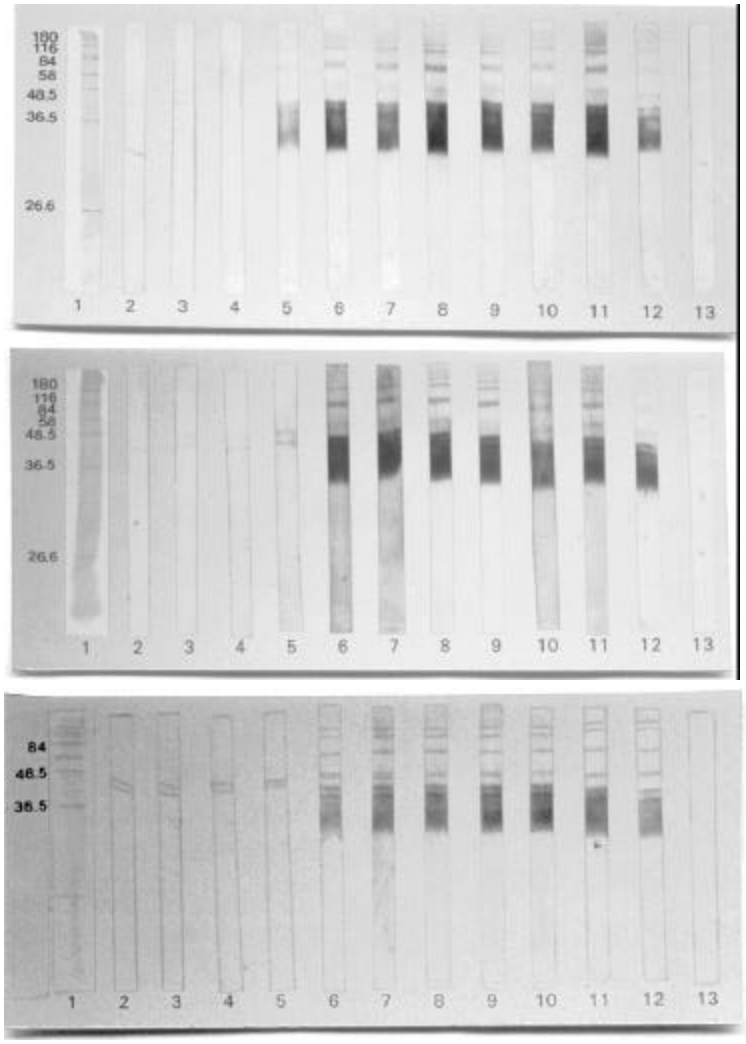

Fig. 1. Recognizing of *Trichinella spiralis* ES larval antigen by sera from three experimentally infected swine detected by Western blot. 1: Molecular weight markers, 2 to 11 samples from 1 to 10th week after infection, 12: Positive control serum, 13: Negative control serum.

kDa from *T. spiralis* ES antigen (Fig. 2) and sera from 12 other backyard swine (Aguascalientes State) showed a similar pattern when analyzed by Western blot (data not shown).

Dot-ELISA. As in the case of Western blot, the serum from one infected pig gave a positive reaction to anti-*T. spiralis* antibodies by Dot-ELISA (Fig. 3A5) at the week four after infection while the sera from the other two pigs showed positive results one week later (data not shown). None of 22 sera from backyard swine (10 from Jilotepec, Mexico State and 12 from Jesus María, Aguascalientes State) were positive to anti-*T. spiralis* antibodies by Dot-ELISA (Table 1). In this context, four out of 10 pigs from Jilotepec, were naturally infected with *A. suum* as determined by coprological examination of feces. Sera from these pigs did not react with ES *T. spiralis* antigen but they reacted with an *A. suum* somatic crude antigen (Fig. 3).

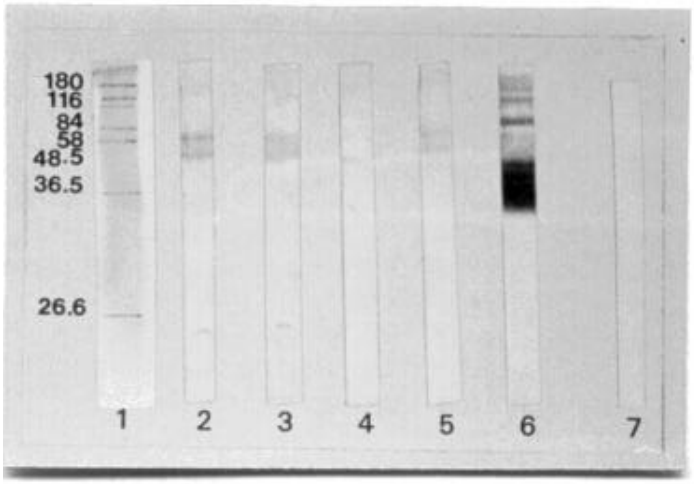

Fig. 2. Recognizing of *Trichinella spiralis* ES larval antigen by sera from pigs naturally infected with *Ascaris suum* detected by Western blot. 1: Molecular weight markers, 2 to 5 samples from *A. suum* infected pigs, 6: Positive control serum, 7: Negative control serum.

DISCUSSION

Differences in time recognition of *T. spiralis* ES antigen in Western blot by sera from the three infected pigs may be related to genetic differences. However, at week five after infection all these three pigs recognized the pattern of seven components in the ES *T. spiralis* antigen.

The explanation to the cross reaction of sera from A. *suum* infected swine with the 45 kDa component in the ES *T. spiralis* antigen observed in Western blot may be related to the 37 kDa component common to nematode antigens subjected to reduced conditions before electrophoresis running and that emigrates along with that 45 kDa.[23]

The components recognized by the sera from *T. spiralis* infected pigs which ranged from 45 to 108 kDa appear to be the same described by Arriaga et al[1] Weiger et al,[23] Gamble *et al*[6] and Despommier *et al*.[3] These researchers indicate that those components are the main antigens of muscle larvae recognized by sera from *T. spiralis* infected swine.

When comparing Western blot (WB) and Dot-ELISA under the conditions of the present study, both tests detected satisfactorily anti-*T. spiralis* antibodies in sera from experimentally *T. spiralis* infected swine; however, sera from backyard pigs reacted in WB with two components of *T. spiralis* ES antigen although these did not filled the criteria of recognizing the pattern of seven components in the *T. spiralis* ES antigen to be considered as positive. When Dot-ELISA was used only the sera from *T. spiralis*-infected pigs gave positive reactions while 22 sera from backyard swine (including four infected with *A. suum*) gave negative reactions. In this context it is worth to mention that the cost of WB is higher than that of Dot-ELISA which, on the other hand. It is easier to apply under field conditions. In this connection, in different studies it has been demonstrated that the larval *T. spiralis* ES antigen

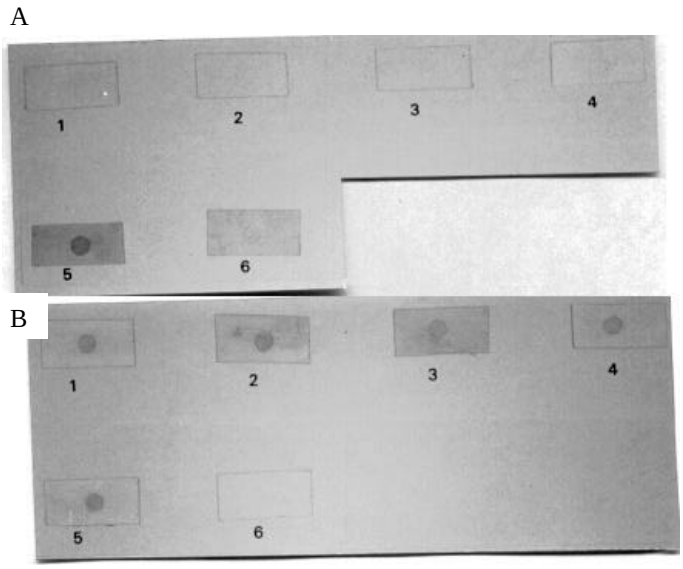

Fig. 3. Detection of anti-*Trichinella spirallis* and anti-*Ascaris suum* antibodies in pig sera by Dot-ELISA. A: Test carried out with *T. spiralis* ES larval antigen; 1 to 4: sera from *A. suum*-infected swine; 5: positive control serum to *T. spiralis*; 6: Negative control serum to *T. spiralis*; B: Test carried out with *A. suum* somatic crude antigen; 1 to 4: Sera from *A. suum*-infected swine; 5: Positive control serum to *A. suum*; 6: Negative control serum to *A. suum.*

Table 1. Number of pig sera with or without anti-*Trichinella spiralis* antibodies as compared by Western blot and Dot-ELISA.

Presence of anti-*T. spiralis* antibodies	+ 21[a]	- 22[b]
Positive serology by		
Western blot[c]	21	0
Dot-ELISA[d]	21	0
% of Sensitivity[e]		
Western blot	100	
Dot-ELISA	100	
% of Specificity[f]		
Western blot	100	
Dot-ELISA	100	

[a] Eighteen sera came from three experimentally infected swine (collected at weeks 5, 6, 7, 8, 9, and 10 after infection. These, have an average of 10.± 1.7 *T. spiralis* larvae as determined by artificial digestion of muscle tissue from tongue and diaphragm. The three other sera came from three pigs previously infected with *T.* spiralis which were used as positive control sera.
[b] Four of these pigs harbored *Ascaris suum* eggs as determined by coprologic examination of feces.
[c] Threshold of positivity = To be considered as positive a serum sample should recognize a pattern of seven components in the E/S *T. spiralis* antigen.
[d] Threshold of positivity = To be considered as positive a serum sample should produce a visible dot at a dilution of 1:160.
[e] Sensitivity = [a/(a + c)] x 100
[f] Specificity = [b/(b + d)] x 100 where: a = truly positive, b= truly negative, c = false negative, d= false positive.

is suitable for using in ELISA for the serodiagnosis of trichinellosis in the domestic pig.[11, 4, 5, 6, 19, 22]

On the other hand, the studies of Shin et al[19] and the results of the present investigation, support the suggestion of using both techniques to detect anti-*T. spiralis* antibodies in swine, particularly in backyard pigs, Dot-ELISA as exploratory test followed by Western blot analysis as confirmatory test.

The ES antigen from *T. spiralis* muscle larvae was suitable for using in both Western Blot and Dot-ELISA tests for detection of anti-*T. spiralis* antibodies in swine. For field purposes the later technique is better than WB due to its relatively simplicity to perform, is cheaper and offers a good sensibility as well as an acceptable specificity.

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