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International ring trial to detect anti-*Trichinella* IgG by ELISA on pig sera

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ABSTRACT

To determine the reproducibility and robustness of an ELISA to detect anti-*Trichinella* IgG in pig sera which was previously validated at the Community Reference Laboratory for Parasites (CRLP), a ring trial was organized involving European and extra-European reference laboratories for *Trichinella*. The sensitivity and specificity of the assay determined by the CRLP validation resulted to be 100% and 98.29%, respectively. The assay was reproducible, moreover, based on the receiver-operator characteristic (ROC) curve, the sensitivity and specificity of the assay reached 97.5% and 96.9%, respectively. The analysis of the differences in optical density (OD) between duplicates indicated a high repeatability of the ELISA with about 95% of the differences between −0.16 and 0.17 absorbance units. The accuracy of the test was determined by calculating the area under the ROC curve (AUC). Overall, the ELISA index (I_E) showed a very high accuracy (AUC = 0.9965) and it performed significantly better than the mean of the duplicated ODs (AUC = 0.9387). Of the 21 participating laboratories, nine performed the test without any modification of the original protocol, and 14 with some modifications. Of the laboratories that followed the protocol exactly, three produced false-negatives; whereas of the laboratories that modified the protocol, five produced false-negatives (differences between these two groups of laboratories were not significant, $p = 0.18$). When comparing these two groups of laboratories, the AUCs were very similar (0.9988 and 0.9955, respectively). Finally, a normal mixed multiple model effect was used to evaluate if the I_E obtained was only related to the serum or to other parameters such as the laboratory, dilution of the serum tested and application of the proposed protocol. The variability found in the test results was mainly due to the serum samples. The assay proposed is robust and reproducible and can be used for monitoring the lack of *Trichinella* infection in domestic pigs.

1. Introduction

Nematode worms of the genus *Trichinella* are zoonotic parasites which circulate in most European countries in both wild and domestic animals (Pozio and Murrell, 2006; Pozio, 2007). Humans acquire *Trichinella* infection by consuming raw or undercooked meat from pigs, horses, wild boars and other game animals (Pozio et al., 2003; Dupouy-Camet and Bruschi, 2007). According to Commission Regulation (EC) No. 2075/2005 (European Community, 2005), all animals that are potential carriers of infective *Trichinella* larvae should be tested at the slaughterhouse, using one of the approved tests. However, for pigs raised in holdings or categories of holdings that the authorities have

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officially recognized as *Trichinella*-free, testing is not mandatory. Nonetheless, to verify the infection-free status, the authorities must implement a monitoring program that covers pigs raised in such holdings and pigs from regions where the risk of *Trichinella* in domestic pigs is considered as negligible. The monitoring program may include serological methods, once a suitable test has been validated by the Community Reference Laboratory for Parasites (CRLP; European Community, 2005).

Since accreditation, according to the International Organization for Standardization (ISO), ensure services such as quality, reliability and efficiency, our laboratory has been accredited according to ISO/IEC 17025:2005 by the Italian accreditation body SINAL (www.sinal.it). The importance and value of Laboratory accreditation has become clearly evident because of a larger diffusion of the quality assurance culture. The objectives of the present study were to determine the reproducibility and robustness of an ELISA to detect anti-*Trichinella* IgG in pig sera which had been previously validated at the CRLP. To this end a ring trial was organized involving European and extra-European reference laboratories for *Trichinella* with experience in serology.

2. Materials and methods

2.1. Antigens

Excretory/secretory antigens (ESA) were prepared from *Trichinella spiralis* muscle larvae collected after HCl-pepsin digestion of infected mouse muscles, according to Gamble (1996). Briefly, *T. spiralis* muscle larvae were washed three times in PBS pH 7.2 with penicillin (500 U/ml) and streptomycin (500 μg/ml). The larvae were then washed four times by allowing them to settle in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (500 U/ml) and streptomycin (500 μg/ml). Five thousand worms per ml were then resuspended in DMEM, supplemented with 1 M HEPES, 200 mM L-glutamine, 100 mM Na-pyruvate, and 5000 units of penicillin/streptomycin (GIBCO, Grand Island, NY, USA), and incubated with 10% CO_2 at 37 °C for 18 h in a 75 cm^2 culture flask (Corning Life Sciences, Pittsburgh, PA, USA). Once the worms settled to the bottom of the flask, the medium was transferred to 50 ml conical tubes. The medium was filtered through a 0.2 μm YM-5 filter, and the supernatant was concentrated 100-fold in an Amicon® pressure concentrating chamber (Amicon Inc. Billerica, MA, USA). To determine the protein concentration and to establish the high quality of the batch (i.e., no bacterial or somatic contamination), the optical density (OD) was evaluated at a 280/260 nm ratio; antigens with a ratio higher than 1.0 were used.

2.2. Pig sera

To validate the assay at the CRLP, single serum samples from 932 pigs were analyzed. Of these, 703 were from pigs raised in modern industrialized holdings known to be *Trichinella*-free, as determined with digestion of 5 g of diaphragm pillars; additional 177 sera were from pigs in the same types of *Trichinella*-free holdings but parasitized with organisms closely related to *Trichinella* sp. (*Oesophagostomum* spp., *Metastrongylus* sp., *Ascaris suum*, *Trichiuris suis*) or protozoa such as *Eimeria* spp., according to stool examinations by standard flotation method using Sheather solution. The panel of positive sera consisted of samples from 17 experimentally infected pigs collected 35 days post-infection (3 pigs with 200 larvae each; 3 pigs with 1000 larvae each; 9 pigs with 20,000 larvae each; and 2 pigs with 30,000 larvae each), and samples from 35 naturally infected pigs from Croazia, Romania (*T. spiralis* infection) and Italy (*Trichinella britovi* infection). Blood samples were collected at slaughtering in 50 ml conical vials. Blood was allowed to clotted and serum samples were collected, distributed in aliquots and frozen at −80 °C. Then each serum sample was tested in duplicate.

2.3. ELISA validation

A standard protocol was optimizated and used according to a previous published protocol (World Organisation for Animal Health, 2008). Briefly, 96-well microtiter plates (Nunc-Immuno Plate Maxisorb, Roskilde, Denmark) were filled with 100 μl/well of *Trichinella* ESA (5 μg/ml) in carbonate buffered saline pH 9.6 ± 0.2. After incubation at 37 °C for 1 h, the plates were washed 3 times with an automatic plate washer (Dynex Technologies, Denkendorf, Germany) with washing solution (0.5% Tween 20 in PBS pH 7.3 ± 0.2), blocked by adding 200 μl/well of blocking solution (0.5% BSA, 0.05% Tween 20), and incubated at 37 °C for 1 h. After another washing, 100 μl/well of each 1/50 diluted serum sample was added in duplicate, and the plates were incubated at 37 °C for 30 min. After washing again, 100 μl/well of the diluted anti-swine IgG peroxidase labeled antibodies (Kierkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added, and the plates were incubated at 37 °C for 1 h. After a final wash, 100 μl/well of the substrate solution containing 3,3′,5,5′-tetramethylbenzidine and 0.02% hydrogen peroxide in a citric acid buffer was added, and the plates were incubated at room temperature (RT). The reaction was stopped by adding 50 μl/well of 1N HCl solution. The OD was obtained by reading the reaction at 450 nm using an ELISA plate microtiter reader (Dynex Technologies, Chantilly, VA, USA). Each plate contained four positive and four negative control serum samples, each of which was tested in duplicate. Since raw OD values are absolute measurements that are influenced by ambient temperature, test parameters, and photometric instruments, the results were expressed as a function of the reactivity of the positive control serum sample with the highest value among the four control sera included in each run of the assay. This control must yield a result that is in the linear range of measurement (World Organisation for Animal Health, 2008). The mean OD values of the control sera, as well as the mean OD values of the duplicate test sera, were then calculated, and for each serum an ELISA index (I_E) expressed as percentage of positivity was calculated according to the following equation:

$$I_E = \frac{\text{mean OD value of duplicate sample} - \text{OD blank}}{\text{mean OD value of the highest positive control} - \text{OD blank}} \times 100$$