Development and evaluation of flow through assay for detection of antibodies against porcine cysticercosis

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Abstract. A flow through assay (FTA) was developed on cellulose acetate membrane for the serodiagnosis of porcine cysticercosis using cyst fluid (CFA) and whole cyst antigens (WCA) of *Taenia solium* metacestode. The assay consisted of antigen of *T. solium* metacestode coated onto membrane, mounted on a flow-through test device to provide assay capture matrix. The optimum concentration of coating antigen was 250 ng. The protein A colloidal gold conjugate served as antigen-antibody detecting reagent. A total of 225 serum samples were tested using two antigens. Results were better with CFA (96.0% sensitivity; 96.0% specificity) compared to WCA (92.0% sensitivity; 96.0% specificity). The test was also compared with enzyme-linked immunosorbent assay. The ELISA showed 96 per cent sensitivity with both the antigens whereas; the specificity was 96 and 92 per cent with CFA and WCA respectively. The sensitivity and specificity of flow through assay agrees closely with those of the ELISA. The cross-reaction was observed in one out of eight hydatidosis positive pigs (12.5%) with CFA by both the assays. The highest diagnostic accuracy (96%) was obtained with CFA-FTA and CFA-ELISA. For its high sensitivity and sporadic cross-reactions, CFA-FTA appears to be suitable for practical use at field level without instrumentation.

INTRODUCTION

Porcine cysticercosis caused by the metacestode of *Taenia solium*, is considered to be a serious public health and economic problem. Cysticerci developing in brain and spinal cord of either human or pig results in neurocysticercosis, which is the major cause of epilepsy in human in developing countries (World Health Organization, 2002). It is considered as emerging and reemerging parasitic disease (Craig & Pawlowski, 2002) and is associated with social and environmental conditions such as poor hygiene and free roaming of pigs. Diagnosis of cysticercosis in pigs is based on antemortem tongue palpation or postmortem meat inspection that is sensitive only in detecting moderate to heavy infections. A reliable antemortem serological test based on detection of specific antibody and antigen has proved very useful in confirming diagnosis (Pathak et al., 1994; Phiri et al., 2002; Dorny et al., 2004). However, currently available diagnostic techniques viz. Ag-ELISA, Ab-ELISA, EITB and tongue inspection were reported to be specific but not sensitive when applied on pigs with low cyst burdens (Sciutto et al., 1998; Boa et al., 2002) and requires access to laboratory with proper instrumentation and trained personnel. Owing to these facts and wide geographical...
distribution of cysticercosis, mostly in developing countries, development of rapid, simple to perform and cost effective diagnostic test for the early detection of cysticercosis using fewer reagents is inevitable.

Eliades et al. (1998) developed a dot immunobinding assay, a simple method conjugating hydatid antigen or protein A with colloidal dye as visualizing agent for the serodiagnosis of human hydatidosis. In the present study, flow through assay (FTA) with a modification employing protein A colloidal gold conjugate was used for the detection of antibodies with cyst fluid and whole cyst antigens of *T. solium* metacestode against porcine cysticercosis. The assay has been validated with meat inspection and compared with ELISA using serum from pigs with cysticercosis and control serum from apparently healthy pigs, pigs affected with hydatidosis.

**MATERIALS AND METHODS**

**Serum samples**

Serum was collected aseptically from 208 native and crossbred pigs at slaughter that were brought to Bacon factory (Dept. livestock products technology, NTR college of Veterinary Science, Gannavaram, AP, India). Natural infection in these animals was confirmed by the presence of metacestode of *T. solium* at meat inspection. Hydatid cysts were found in eight pigs that were not infected with *T. solium* and serum from these were used to test for the cross reactivity to antigens. The negative serum samples (17) were collected from the apparently healthy pigs, pigs affected with hydatidosis.

**Preparation of antigens**

Cyst fluid antigen (CFA) was prepared as per the method of Chung *et al.* (2005) with modifications. Fresh *T. solium* cysticerci without host membrane tissue were collected from naturally infected, slaughtered pigs and washed three times in cold 0.15 M sodium chloride. The cyst fluid was aspirated with sterile syringe needle, centrifuged at 18000 g for 60 min at 4°C in high speed refigerated centrifuge (Sigma – 3K30) and supernatant was sonicated for 1 min at 20 KHz and 1 mA (Sonix – VCX400) in an ice-water bath and used.

Whole cyst antigen (WCA) was prepared as per the method described by Mahajan *et al.* (1974), with modifications. The cysticerci free of host capsules were washed at 4°C in PBS pH 7.4 containing penicillin G (500 IU ml⁻¹) and streptomycin (500 µg ml⁻¹). Ten grams of cysts were mixed with 20 ml of PBS pH 7.4 containing 0.1 mM phenyl methyl sulphonyl fluoride as protease inhibitor. The cysticerci were disrupted in glass tissue homogenizer at 4°C. The homogenate was sonicated four times at 20 KHz, 1 mA for 60 sec on ice. The sonicated material was centrifuged at 14000 g for 60 min at 4°C in refigerated centrifuge and the supernatant was used as the somatic antigen.

The protein content of two antigens was estimated as per the method described by Lowry *et al.* (1951) with bovine plasma globulins as a standard. The antigens were stored at -20°C until used.

**Raising of hyper immune serum**

Hyper immune serum was raised against CFA and WCA in healthy rabbits separately. Five hundred micrograms of antigen was injected subcutaneously after mixing with equal volume of Freund’s complete adjuvant. After four such inoculations at interval of 8 days, the rabbits were test bled 10 days after the last injection and confirmed the presence of antibodies by AGID. Later large amount of blood was collected from the marginal veins of the
ear. Serum was separated under sterile conditions and kept in aliquots at -20°C 0°C until used.

**Development of FTA**
The test was performed in a flow through module, in which the antigen of *T. solium* metacestode coated cellulose acetate membranes were pressed tightly to a water-absorbing pad.

**Test principle**
In the assay, antibodies in the serum sample are captured by antigen of *T. solium* metacestode spotted on to cellulose acetate membrane mounted on a flow through test device that serve as the assay capture matrix. The bound antibodies are visualized by the addition of protein A colloidal gold conjugate, which served as antigen-antibody detecting reagent imparting pink color to the membrane as a dot.

**Detection of anti-cysticercus antibodies by FTA**
The cellulose acetate membrane (M/s mdi, Ambala Cantt, India) was placed above the absorbent pads in a flow through module. One µl (250 ng/µl) of CFA or WCA was placed at one end of the module (T-side) and 1 µl of pig serum was placed at other end (C-side), which acted as reagent control. The membrane was dried in an incubator at 37°C for 1 hour or overnight at room temperature, to which 200 µl of wash buffer (25 mM PBS pH 7.4 containing 1% BSA and 0.05% Triton-x 100) was added and allowed to be absorbed through the membrane. Then 200 µl of test serum diluted in wash buffer (1 in 10) was added and allowed to be absorbed through the membrane. Following this, the membrane was washed with wash buffer. Thereafter, 200 µl of wash buffer diluted (1 in 2) protein A colloidal gold conjugate (mdi Colloidal Au-sPA-300) was added and allowed to be absorbed through the membrane. The wash cycle was repeated. Appearance of two pink dots indicated presence of antibody (Fig. 1).

**Detection of anti-cysticercus antibodies by ELISA**
The ELISA for the detection of cysticercosis antibodies was performed as described by Pathak *et al.* (1994) with minor modification. Microtiter plates (Nunc, Denmark) were coated with 100 µl of CFA (2.5 µg/ml) or WCA (7.5 ng/µl) in carbonate / bicarbonate coating buffer pH 9.6, by overnight incubation at 4°C. Non-coated sites were blocked with 300 µl of 5% skimmed milk in PBS-T for two hours at 37°C. The plates were washed with PBS-T for three times and 100 µl of each serum dilution (1 in 500) was added to wells and incubated for 1 hour at 37°C. After the washing procedure 100 ml of anti-pig IgG HRP conjugate at 1 in 16000 (for CFA) or 1 in 14000 (for WCA) in PBS-T was added and the incubation and washing procedures were repeated. The reaction was developed in 8 min with ABTS and H₂O₂ in sodium citrate buffer and then stopped with 1% SDS. All samples were run in duplicate and the absorbances were read at 405 nm. The mean of the optical densities plus three times standard deviation of the true negative controls were selected as the cut-off value for the positivity in the ELISA.

**Sensitivity and specificity**
Twenty-five serum samples that were positive on meat inspection were used to test the sensitivity of FTA and ELISA, whereas 25 serum samples from control group comprising of 17 from apparently healthy pigs reared in confinement and 8 from pigs with hydatid cyst infection were also used to detect specificity. In pigs, the results of autopsy and enumeration of metacestodes are considered as gold standard and used as a tool for validation of immunodiagnostic tests (Dorny *et al*., 2004). Hence, the results obtained from the test were analyzed for the percentage of agreement with meat inspection test with the use of Cohens’s kappa coefficient. It is a statistical measure of inter-rater agreement for qualitative (categorical) items i.e. between two tests, especially in the absence of a standard and is defined as kappa or k (Fleiss *et al*., 2004).
RESULTS

Optimization of FTA

The optimal concentrations to be coated for two antigens were determined by different dilutions with hyper immune serum raised against respective antigens and known serum positive for cysticercosis antibodies. In preliminary experiments, 1 in 100 serum dilutions used gave clear pink colored dots but color spots with lightly infected pig serum were too faint. Therefore further dilutions of serum samples were made and finally 1 in 10 serum dilutions was found to be optimum even for lightly infected pigs serum samples. The optimum dilution found for protein A colloidal gold conjugate was 1 in 2.

Maintaining these optimal values for the two reagents checkerboard tests were performed on cellulose acetate membrane to determine the optimum concentration of coating antigen. The lowest concentration of antigen that showed clear pink dot was 250 ng/µl and that was chosen as optimum sensitizing dose. The following test conditions were chosen as optimal for FTA with both antigens: 250 ng/µl of coating antigen, 1 in 10 serum dilutions and 1 in 2 dilution of protein A colloidal gold conjugate as the indicator. The assay was performed at room temperature. The whole procedure could be completed within 3 minutes and the results were readily determined with naked eye. Appearance of two pink dots indicated presence of antibody, as positive reaction; only one dot indicated negative reaction. If no pink dot appeared, the test was considered invalid and the sample was retested (Fig. 1).

Thermostability

After blocking the antigen coated cellulose acetate membrane with 5% skimmed milk for 2 hours at room temperature the flow through devices were separated into two groups and stored at room temperature and 4ºC separately. Six positive and six negative control samples were tested at 5-day interval. The results indicated that the test devices were stable for only 25 days at 4ºC and one week at room temperature.

Screening of serum samples

A total of 225 serum samples were tested simultaneously to compare FTA and ELISA using two antigens. The results were summarized in table. The cut off value for the ELISA was 0.101 and 0.125 for CFA and WCA respectively. The mean ODs of cysticercosis and control samples obtained by ELISA were presented in Figure 2. The distribution of ODs among positive and negative samples are also presented in Table 1.

Table 1. Performance of FTA and ELISA with two-cysticercus antigen preparation

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>No. positive in FTA</th>
<th>No. positive in ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CFA</td>
<td>WCA</td>
</tr>
<tr>
<td>Cysticercosis</td>
<td>25</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>Test serum samples</td>
<td>175</td>
<td>73</td>
<td>68</td>
</tr>
<tr>
<td>Hydatidosis</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Healthy pigs</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Sensitivity (%) | 96.0 | 92.0 | 96.0 | 96.0 |
Specificity (%)  | 96.0 | 96.0 | 96.0 | 92.0 |
Accuracy (%)     | 96.0 | 94.0 | 96.0 | 94.0 |

k value          | 0.962 | 0.963 | 0.962 | 0.963 |

FTA: Flow Through Assay, ELISA: Enzyme Linked Immunosorbent Assay,
CFA: Cyst Fluid Antigen, WCA: Whole Cyst Antigen
control samples was well separated (Fig. 3a & 3b). There was no significant difference in sensitivity and specificity between the two assays ($k = 0.962$) with CFA. The sensitivity of the FTA was 96 per cent for CFA and 92 per cent for WCA in 25 cysticercosis positive pigs. ELISA had 96 per cent sensitivity with two antigens. The specificity of the assays was 100 per cent in 17 healthy pigs. The cross-reaction was 12.5 per cent in 8 hydatid positive pigs with CFA. The highest diagnostic accuracy...
(96%) was obtained with CFA-FTA and CFA-ELISA. Better results were obtained with CFA than WCA, with higher sensitivity, in 175 serum samples collected from pigs found negative on meat inspection.

**DISCUSSION**

Porcine cysticercosis imposes a significant economic burden on individual farmers and the meat industry in endemic areas. The annual losses due to pig cysticercosis in India (Pathak & Gaur, 1989), Central America (Garcia-Noval et al., 2002), West and Central African countries (Zoli et al., 2003), and China (Ito et al., 2003) indicate the need to use more sensitive and specific diagnostic test for implementation of effective control measures. Development of user-friendly tests for serological monitoring will be of help at farm level. A semi-quantitative or qualitative test, which simply differentiates positive or negative by the development of dot or change in the colour or development of colored line, will be helpful to the farmer for checking the infection. The assays using colloidal gold, colloidal selenium and latex particles as visible marker, instead of an enzyme as in ELISA, making certain that they are simple to perform and more suitable for field use. The linear flow card test has been widely used in clinical diagnosis of malarial infection (Moody & Chiodini, 2002). But the FTA kit is cheaper when compared with linear flow card test kit. Recently colloidal dye particles were used to label antibody

![Figure 3a. Serum IgG titers of ELISA using CFA in samples from different group of pigs](image)
or antigen for the detection of antigen or antibody in dot immunobinding assay on nitrocellulose membrane (Snowden & Hommel, 1991; Kashiwazaki et al., 1994; Mistrello et al., 1995; Eliades et al., 1998; Xiao et al., 2003). Though colloidal gold labeled protein A was used as a probe for the detection of antibodies against human toxocarosis (Dubinsky et al., 2000) and cysticercosis (Gan et al., 2006), it was not adopted for diagnosis of cysticercosis in pigs. Therefore this study is aimed to evaluate FTA with ELISA for detecting antibodies against porcine cysticercosis based on protein A colloidal gold conjugate.

In the present study, the nitrocellulose membrane (0.22 µm) was used initially. But, this membrane gave background reaction upon the addition of colloidal gold conjugate. Hence, the cellulose acetate membranes were chosen for the test. This membrane gave appreciable results with minimum background colour. After initial coating of antigen to cellulose acetate membrane, the reaction time between the antigen and antibody was very less. The high affinity antibodies present in the test serum could only bind to the antigen strongly and colour development in the form of a dot was noticed within no time after adding protein A colloidal gold conjugate. The positive control in the study was pig serum instead of antigen. Having affinity towards immunoglobulin Fc portion the conjugate bound to it, where as high affinity antibodies only bound to coated antigen on membrane. Perhaps, this is the

Figure 3b. Serum IgG titers of ELISA using WCA in samples from different group of pigs
first report on the use of protein A colloidal gold conjugate as a reagent for the detection of Ig G molecules for porcine cysticercosis in flow through assay.

Literature available on FTA against detection of porcine cysticercosis is scanty and thus literature against other parasitic infections is discussed here. Experiments to determine the sensitivity of the FTA revealed that a 1 in 10 serum dilution was optimum. Whereas the serum dilutions less than 1 in 20 and 1 in 100 showed false positive results in the diagnosis of human cysticercosis and hydatidosis, respectively (Liu et al., 1996; Eliades et al., 1998). In the present study, during preliminary experiments, 1 in 100 serum dilutions provided clear pink colored dots, but lightly infected pig (on meat inspection) serum samples gave very faint dots. Hence, further dilutions were made and finally 1 in 10 serum dilutions was found to be optimum even for lightly infected pig serum samples that makes the test reagent conservative as shown by Dubinsky et al. (2000) and Wan Omar et al. (2001). The assay was performed at room temperature within 3 minutes without any incubation steps. There was an increased colour intensity of the dot with increase in antibody titer as shown by Ali et al. (2005), which was also observed by titrating the hyper immune serum.

In FTA, the highest sensitivity was 96 per cent with cyst fluid antigen followed by WCA (92.0%). The similar higher sensitivity (98.3%) also reported by Gan et al. (2006) in detection of human cysticercosis using crude cyst fluid extracts of T. solium metacestode. On the contrary, lower sensitivity of 84.6 per cent was recorded in immunobinding dot-blot assay for serodiagnosis of human cysticercosis using whole cyst as antigen (Hernandez-Cruz et al., 2009). It is stated that the method of preparation of antigen and the type of antigen had influence on the sensitivity and specificity of the test (Dottorini et al., 1981).

In the present study serum sample from hydatid cysts infected pig showed cross-reaction with CFA and WCA. Similar cross-reactions were also observed in serodiagnosis of human hydatidosis (Eliades et al., 1998), schistosomosis (Xiao et al., 2003) and cysticercosis (Gan et al., 2006; Hernandez-Cruz et al., 2009). The cross reaction in the present study could be attributed to the reaction of low affinity Ig molecules or other adhesive molecules to the antigen, which is spotted on to the cellulose acetate membrane or could be due to the occurrence of transient antibodies in the non-infected animal (Garcia et al., 2001) without establishment of patent infection.

One and two false negative reactions were obtained with CFA and WCA respectively. Among these two serum samples one was from the pig in which the cysts were in brain that gave negative results with both antigens. The two false negative pigs in the present study could be due to inherent non-response of the individual pigs. In some pigs with cysticercosis the host response might be reduced as demonstrated by the absence of inflammatory reactions and / or oedema surrounding metacestodes located in the brain (Flisser, 1988) or could be due to the blood brain barrier that plays a vital role with regards to proinflamatory cytokines and outcome of immune response (Sikasunge et al., 2009). False negative reactions also were reported by Mistrello et al. (1995) in the diagnosis of human hydatidosis using hydatid fluid antigen. In the present study better results were obtained with CFA than WCA, with higher sensitivity, for serum samples collected from pigs found negative on meat inspection. This was probably due to the presence of glycoprotein antigens enriched in the cyst fluid, which are good candidates for diagnosis as reported by Theis et al. (1994).

In comparing two tests, there was no significant difference in sensitivity and specificity (k=0.96) but the FTA was easier to perform and faster than ELISA test. Few reports have simultaneously compared flow through assay (Dot immuno binding assay) with ELISA and reported results that agree to those detected by routine ELISA
during diagnosis of hydatidosis, toxocarosis and schistosomosis (Mistrello et al., 1995; Eliades et al., 1998; Dubinsky et al., 2000; Xiao et al., 2003). The results can be interpreted visually for the detection of antibodies against porcine cysticercosis. However, considering the above results obtained with two antigen preparations, cyst fluid from metacestode of *T. solium* was more sensitive in detecting porcine cysticercosis and may be useful for detecting specific antibodies in serum of pigs with cysticercosis. For its high sensitivity and fewer cross reactions this simple test appears to be suitable for practical use at field level, especially for large-scale antemortem screening of pigs against cysticercosis.

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