Immunodiagnostic potency of homologous antigens for natural *Paramphistomum epiclitum* infection in small ruminants in plate and paper enzyme linked immunosorbent assay

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**ABSTRACT**

The objective of the present work was to standardize and evaluate indirect plate and dot-enzyme linked immunosorbent assay (ELISA) using purified *Paramphistomum epiclitum* homologous antigens in the small ruminants. Electrophoretic separation of somatic antigen (PeSAg) in reducing condition on 15% polyacrylamide gel resolved into 16 proteins of the molecular weight ranging from 14-100 kDa. Two step ethanolic precipitation of supernatant of *in-vitro* culture of the fluke yielded *P. epiclitum* excretory-secretory antigen (PeESAg) of molecular weight 28 kDa. The animals (Goats=123; Sheep=91) were broadly kept into post-mortem and faecal examined groups. At many occasion the PeSAg found to cross reacts with other helminths parasites thus minimizing the specificity of the tests and antigens. There was no any direct correlation between the parasites load and ELISA reactivity pattern. The noted prevalence rate after combining the results of post-mortem examination and PeESAg based ELISA (plate and paper/ dot) was 30.08% (37/123) in goats and 28.57% (26/91) in sheep. While using PeESAg, the calculated overall sensitivity% was 92.86 (goats)/ 100 (sheep) in both plate and dot-ELISA, specificity% was 91.58 (goats)/ 91.55 (sheep) in plate ELISA while 88.42 (goats)/ 92.96 (sheep) in dot-ELISA, positive predictive value% was 76.47 (goats)/ 76.92 (sheep) in plate ELISA while 70.27 (goats)/ 80 (sheep) in dot-ELISA and negative predictive value% was 97.75 (goats)/ 100 (sheep) in plate ELISA while 97.67 (goats)/ 100 (sheep) in dot-ELISA, these values were optimum for the field sera sample so the tests and PeESAg can be recommended for the detection *P. epiclitum* infection in the small ruminants.

**Key words:** Antigens, *Paramphistomum epiclitum*, Paper/ dot-ELISA, Plate-ELISA, Sheep.

**INTRODUCTION**

Amphistomes are group of digenetic trematodes commonly known as stomach or rumen flukes, adult localized in rumen and reticulum and immature stage in duodenum of ruminants including goats and sheep. In India, 20 species were described and classified by Dutt (1980) in ruminants but the predominant species are *Paramphistomum epiclitum*, *Gigantocotyle explanatum*, *Gastrothylax crumenifer*, *Cotylophoron cotylophorum* and *Fischoederius elongatus*, with prevalence rate of 3-70% in different states (Hassan et al., 2005; Tariq et al., 2008; Swarnakar et al., 2014). The adult stages generally have low pathogenicity while the pathological changes induced by the immature stage in the gastro-intestinal (GI) tract of the ruminant are responsible for severe economic losses (Singla et al., 1998; Silvestre et al., 2000).

Coproscopical analyses in case of amphistomosis often results in misdiagnosis and could not be used during prepatent period of 6-12 weeks (Salib et al., 2015). Thus, immunological diagnosis especially by indirect enzyme linked immunosorbent assay (ELISA) (plate and paper based) can prove to be an important tool for early diagnosis of amphistomosis which is essential for prompt treatment before irreparable damage to the GI tract (Díaz et al., 2006; Saifullah et al., 2013). The paper based ELISA can stand as pen side diagnostic test for field condition or in the laboratories with limited resources (Pappas et al., 1983). The potential of immunodiagnostic assays based on whole/semi-purified crude antigens for early detection of helminth infections is marred by low specificity and cross-reactions due to presence of common antigenic epitopes in several trematodes (Meshgi et al., 2009; Kaur et al., 2013). Hence, it necessitates the use of purified immunochemically characterized antigens to improve the sensitive and specificity of the test and minimize the chances of cross-reactivity (Saifullah et al., 2011).

**MATERIALS AND METHODS**

**Collection of parasite:** The compound stomach along with intestines of slaughtered goat and sheep’s was collected from the local abattoir of Navsari, Gujarat in the ice box and

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carried to the departmental laboratory for further processing. Adequate amount of tap water was added into the collected gut content to make a homogenous mixture and then filtered through sieve of 250 µm aperture. The worms were collected after examining the small amount of the filtrate into a petri-dish, washed in phosphate buffered saline (PBS; pH = 7.4) having ampicillin (100 mg/ml) and cloxacillin (50 mg/ml) antibiotics and identified using the important morphological features followed by molecular identification.

**Collection of biological materials:** The sera were separated as per the set protocol from 5 ml of blood taken from the jugular vein of small ruminants from animals slaughtered at slaughter houses of Navsari and stored at -20°C for further use. Faeces of the small ruminants were collected directly from the rectum into a sealable plastic bag to minimize dehydration. The faeces were screened for the presence/absence of parasites as per the standard protocol.

**Animal’s grouping:** The slaughtered animals were grouped as per their parasitic fauna recorded during post-mortem (PM) or faecal examination. The animals were broadly grouped into PM (group P) and faecal (group F) (PM examination not done) examined group. The group P animals were further sub-grouped into PM positive (+ve) (group PP) and negative (-ve) (group PN) animals. The group PP animals were further sub-grouped into P. *epiclithum* +ve (group PE), P. *epiclithum* and *Haemonchus contortus* +ve (group PH) and P. *epiclithum* and other helminths +ve (group PO) animals. The group F animals were further sub-grouped into faecal examination +ve (group FP) and –ve (group FN) animals.

**Preparation of antigens:** The *P. epiclithum* somatic antigen (PeSAg) was prepared as per the protocol mentioned by Kumar *et al.* (2008a). Four gram dried fluke powder was suspended in 100 ml of chilled phosphate buffered saline (PBS; pH 7.4) containing a cocktail of protease inhibitors and stirred for extraction. The extracted antigen was centrifuged at 5000 rpm for 30 min at 4°C. Supernatant was collected and again centrifuged at 10000 rpm for 45 min. The collected supernatant was equilibrated against 20 mM carbonate coating buffer (pH=9.6) containing 1.0 and 4.0 µg/ml of PeSAg and PeESAg, respectively. After examining the small amount of the filtrate into a petri-dish containing ampicillin (100 mg/ml) and cloxacillin (50 mg/ml) proteins precipitated overnight at -20°C. Finally, the solution was centrifuged at 14000 rpm for 30 minutes to harvest the precipitate containing PeESAg. The retrieved pellet representing regurgitated PeESAg / cathepsin L cysteine proteinase was washed in absolute ethanol, air dried and resuspended in 50 µl of sterile PBS (pH=7.4). The several batches of pooled E-S proteins were dialyzed overnight at 4°C in PBS (pH=7.4) using 10 kDa molecular weight cut-off dialysis bag. The dialyzed PeESAg was aliquoted and concentrated up to 2 times using vacuum lyopholiser (Heto, Germany). The protein concentration of the antigens were estimated in Nanodrop (Stratagene, USA) and stored at -20°C for further use.

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**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):** It was performed as per the method described by Laemmli (1970) to know the polypeptide profile of somatic and E-S antigens.

**Indirect enzyme linked immunosorbent assay (ELISA):** The flat bottomed polystyrene microtitre plates (Nunc, Denmark) were coated with 100 µl of carbonate and bi-carbonate coating buffer (pH=9.6) containing 1.0 and 4.0 µg/ml of PeSAg and PeESAg, respectively in each well by incubating overnight at 4°C. The nitro cellulose paper (NCP) (Thermo Scientific, USA) strip was coated with 100 and 200 ng of PeSAg and PeESAg, respectively by incubating at 37°C for 1 hour and then at 4°C for overnight. Further, steps of indirect plate and paper/ dot ELISA were performed as per the methods described by Kumar *et al.* (2008a and b). Depending upon the intensity of the developed colour each dot was assigned with a score 1, 2, 3 and 4 in paper ELISA. Negative cut-off O.D. value/ cut off dot score for the selected tests and antigens was calculated using the formula “mean ELISA OD at 450 nm/ mean dot score of uninfected sera + 2 standard deviation”.

**Statistical analysis:** The obtained data was analysed using SPSS 20.00 for Windows (SPSS, 2016) to perform chi-square tests and/or student t-test and/or one-way ANOVA using Dunkan t (2 sided) for determination of statistical significance. The p value ≤0.05 was considered as significant. The PM and faecal data was used as fixed factors. The sensitivity% = [true positive / (true positive + false negative)] x 100; specificity% = [true negative / (true negative + false positive)] x 100; positive predictive value (PPV) % = [true positive / (true positive + false positive)] x 100; negative predictive value (NPV) % = [true negative / (true negative + false negative)] x 100 of the assays and antigens were decided for fluke infection.
The targeted amphistome was on the basis of common.

... SDS-PAGE showing purified protein (Lane M: Protein
ese to the method of antigens preparation.

In the present experiment, the electrophoretic pattern of PeSAg in 15% SDS-PAGE gel revealed 16 polypeptide bands (14, 16, 21, 23, 25.8, 27, 29, 32, 37, 39, 42, 43, 47, 52, 57 and 76 KDa) in molecular range of 14-76 kDa (Fig. 1). However, Maji et al. (1997) identified 21 polypeptides in the molecular range of 19.9-125.8 kDa of whole worm extract and TritonX100 solubilised antigen of P. epiclitum. While Saifullah et al. (2001) revealed 44 protein bands (14.4-205 kDa) in soluble egg fraction of P. epiclitum. Recently, Salib et al. (2015) observed 14 (11.5-174 kDa) distinct protein bands in the adult Paramphistomum somatic antigens. The variation observed in polypeptide profile, by each worker, could be attributed to the method of antigens preparation.

Fig. 1: SDS-PAGE showing purified protein (Lane M: Protein marker, Lane 1: Purified PeESAg, Lane 2: PeSAg)

Purification of P. epiclitum functional antigens by specific methods is expected to remove cross reacting components, which if present are liable to cross react and elicit false results in ELISA (Maji et al., 1999; Yadav et al., 2003; Pal and Dasgupta, 2007; Kaur et al., 2013). The phosphoryl-choline epitope is the main element of crossreaction, an important conserved epitope of trematodes (Sloan et al., 1991). Two steps alcoholic precipitates of the P. epiclitum RPMI-1640 culture supernatant resolved as a doublet at 28 kDa in 15% SDS-PAGE (Fig. 1). Many researchers identified this fraction of E-S protein as Cathepsin L cysteine proteinase and suggested to use it specific diagnostic antigen for prepatent diagnosis of trematodes infection in the animals (Coles and Rubano, 1988; Dixit et al., 2003). By two step alcoholic precipitation technique Dixit et al. (2003) and Varghese et al. (2012).
isolated native 28 kDa cathepsin-L cysteine proteinase from the regurgitants of Fasciola gigantica of bubalian origin.

Indirect plate and dot-ELISA of animals belonging to different groups: To test the diagnostic potentiality of the antigens indirect plate and dot-ELISA were standardized, since indirect plate-ELISA has been found to be very suitable for the diagnosis of parasitosis owing to its high sensitivity and possibility of processing many sera samples simultaneously. In the cases of diagnosis of parasitic infection in ruminants, it should comply the recommendation issued by the Office International des Epizooties (OIE), in the sense that it is necessary to develop diagnostic methods for parasitic diseases, which should be cheap, simple and useful under field conditions, for the reason, dot-ELISA was further standardized (Pappas et al., 1983) (Fig. 6). The estimated cut-off values for plate/paper-ELISA were 0.522/3.7 (PeSAg) and 0.363/1.89 (PeESAg) for goats and 0.552/3.71 (PeSAg) and 0.389/1.81 (PeESAg) for sheep (Fig. 2-5 and 7-10). Group-wise plate and paper ELISA reactivity pattern was presented in Table 2-4 and Fig. 2-10.

It was observed that in most of the cases the antigens and tests could detect *P. epiclitum* infection even when the infection was at the low level (Table 2). The parasites load independent reactivity of the test and antigen was also claimed by Mezo et al. (2004), who developed an ultrasensitive monoclonal antibody MM3 capture ELISA for the detection of *F. hepatica* coproantigen in sheep and cattle. The assay detected the coproantigen by 1st-5th week post
Table 2: Parasites load and ELISA reactivity patterns of group PP animals

<table>
<thead>
<tr>
<th>Host Group</th>
<th>Range of parasites</th>
<th>No. of parasites (Mean=±S.E.)</th>
<th>Plate ELISA</th>
<th>Dot-ELISA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PeSAg</td>
<td>PeESAg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Mean O.D.±S.E.)</td>
<td>(Mean O.D.±S.E.)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>p value</td>
<td>p value</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(S./ N.S.)</td>
<td>(S./ N.S.)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.7 (PeSAg)</td>
<td>1.89 (PeESAg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.71 (PeSAg)</td>
<td>1.81 (PeESAg)</td>
</tr>
</tbody>
</table>

**Note:** Group PP: Post-mortem +ve animals, Group PE: *P. epiclitum* +ve animals, Group PH: *P. epiclitum* and *H. contortus* +ve animals, Group PO: *P. epiclitum* and other helminths +ve animals.

Estimated cut-off optical density (O.D.) values were 0.522 [P. epiclitum somatic antigen (PeSAg)] and 0.363 [P. epiclitum excretory-secretory antigen (PeESAg)] for goats and 0.552 (PeSAg) and 0.389 (PeESAg) for sheep in indirect plate ELISA. The estimated cut-off scores were 3.7 (PeSAg) and 1.89 (PeESAg) for goats and 3.71 (PeSAg) and 1.81 (PeESAg) for sheep in indirect dot-ELISA.

No. (Number); S. (significant), N.S. (non-significant).

infection (WPI) even at the lower liver fluke burden of 1-36 parasite(s). Although in the current study, the lowest time period required by the used tests and antigens to detect the infection had not been determined but many works agreed with the facts that the ELISA/ western blot based assays with somatic, E-S, semi-purified or recombinants antigens can detect the trematodes infection as early as 1 st WPI (Kaur et al., 2004; Ram et al., 2014). It was quite interesting to note that in the present study in group PE, PH and PO goats and sheep infected with different grade of *P. epiclitum* a weak to strong antibody response was detected in both plate and dot-ELISA (Table 2; Fig. 2-5, 7-10). Likewise, Kumar et al. (2008a and b) also failed to draw any correlation between the parasites load and the plate-ELISA OD values/ intensity of dot in dot-ELISA. While Almazan et al. (2001) noticed the correlation between absorbance of E-S antigen in sera with *F. hepatica* burden up to 0.77 and in faces up to 0.76 by 12 th WPI. The plate/ dot-ELISA gave high level of sensitivity (100%) in group PE, PH and PO animals with both the antigens. Cornelissen et al. (2001) observed 100% sensitivity of the recombinant *F. hepatica* cathepsin L-3 ELISA for cattle in experimental infection. While Srivney et al. (2006) observed 100% sensitivity of native cathepsin L cysteine proteinase of *F. gigantica* using ELISA, dipstick ELISA and western blotting in bovine. The high level of sensitivity of the PeESAg observed in the present investigation was due to the isolation of the antigen from E-S product of the *P. epiclitum* having functional epitopes of the parasite.
There were few animal belonging to group PN, showed positive reaction in plate and dot-ELISA with both the selected antigens (Table 3; Fig. 2-5, 7-10). Positivity of the test was observed more with PeSAg than the PeESAg.

The extra positivity observed in this group might be due to prepatent infection or cross reactivity with other closely related helminths parasites (Maji et al., 1999; Yadav et al., 2003; Salib et al., 2015). The observed specificity of the group PN in the plate-ELISA in the goats was 89.83 (PeSAg) and 91.53% (PeESAg) and in sheep was 89.23 (PeSAg) and 93.85% (PeESAg). The specificity of the dot-ELISA with somatic and E-S antigens in the goats was 88.14 and 89.83% while it was 89.23 and 93.85% in sheep. Previously, Kumar et al. (2008a) noted 97, 95, and 98% (in plate)/ 92.3, 94.7, and 90% (in dot) specificity against G. crumenifer, G. explanatum and mixed infection of G. crumenifer and G. explanatum, respectively in ELISA using immunoaffinity column chromatography purified antigen from the somatic antigen F. gigantica in buffaloes. Kumar et al. (2008b) observed 97-98% specificity of the indirect ELISA test and affinity column chromatography purified 27 kDa glycoprotein from the somatic antigen of F. gigantica against G. crumenifer, G. explanatum, or a mixed infection with both parasites in buffaloes.

The low level of sensitivity (28.57%) in group FP animals with both tests and antigens might be due to different antigenic pattern observed among the stages of the flukes or observed trematodes eggs belong to different genus or species of the amphistomes other than the P. epiclitum (Saifullah et al., 2001; Pal and Dasgupta, 2007; Kaur et al., 2009; Salib et al., 2015).

There was high level of specificity in group FN animals in plate-ELISA (86.11% for somatic and 91.67% for E-S antigens in goats, 66.67% against both antigens in sheep) and dot-ELISA (83.33% for somatic and 86.11% for E-S antigens in goats, 83.33% against both antigens in sheep). Comparatively high level of specificity in negative group animals was also observed Kumar et al. (2008 a and b).
Table 3: ELISA based distribution of *P. epiclitum* +ve animals in different group

<table>
<thead>
<tr>
<th>Group</th>
<th>Plate-ELISA PeSAg Mean OD±S.E</th>
<th>Plate-ELISA PeESAg Mean OD±S.E</th>
<th>Dot-ELISA PeSAg Mean dot score±S.E</th>
<th>Dot-ELISA PeESAg Mean dot score±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of +ve animals</td>
<td>No. of +ve animals</td>
<td>No. of +ve animals</td>
<td>No. of +ve animals</td>
</tr>
<tr>
<td>PP (PE, PH and PO)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PN</td>
<td>6</td>
<td>0.577±0.01</td>
<td>0.239±0.01</td>
<td>0.613±0.01</td>
</tr>
<tr>
<td>FP</td>
<td>4</td>
<td>0.66±0.03</td>
<td>0.559±0.06</td>
<td>0.61±0.01</td>
</tr>
<tr>
<td>FN</td>
<td>5</td>
<td>0.537±0.03</td>
<td>0.33±0.02</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>PP (PE, PH and PO)</td>
<td>19</td>
<td>0.651±0.02</td>
<td>0.651±0.02</td>
<td>0.651±0.02</td>
</tr>
<tr>
<td>PN</td>
<td>7</td>
<td>0.587±0.02</td>
<td>0.252±0.02</td>
<td>0.61±0.01</td>
</tr>
<tr>
<td>FP</td>
<td>1</td>
<td>0.75</td>
<td>0.75</td>
<td>0.64</td>
</tr>
<tr>
<td>FN</td>
<td>2</td>
<td>0.593±0.02</td>
<td>0.387±0.05</td>
<td>0.33±0.02</td>
</tr>
</tbody>
</table>

*Note:* Group PP: Post-mortem (PM) positive (+ve) animals; Group PE: *P. epiclitum* +ve animals; Group PH: *P. epiclitum* and *H. contortus* +ve animals; Group PO: *P. epiclitum* and other helminths +ve animals; Group PN: PM negative (-ve) animals; Group FP: Faecal examination +ve animals; Group FN: Faecal examination –ve animals. Estimated cut-off optical density (O.D.) values were 0.522 [*P. epiclitum* somatic antigen (PeSAg)] and 0.363 [*P. epiclitum* excretory-secretory antigen (PeESAg)] for goats and 0.552 (PeSAg) and 0.389 (PeESAg) for sheep in indirect plate ELISA. The estimated cut-off scores were 3.7 (PeSAg) and 1.89 (PeESAg) for goats and 3.71 (PeSAg) and 1.81 (PeESAg) for sheep in indirect dot-ELISA. No. (number).
In the current study, after combining the PM examination and PeESAg based indirect ELISA (plate and dot) results, a increased level of prevalence rate was noted in both goats (37/123 = 30.08%) and sheep (26/91 = 28.57%), indicative of *P. epithilum* either in the stage of active (pre-patency and patency) or passive infection (Table 1 & 3). This trend of finding was in agreement with the observation of Kumar et al. (2008 a and b). Kaur et al. (2008) recorded 57, 25, 20 and 20% prevalence rate of amphistomosis in cattle, buffalo, sheep and goat, respectively in dot-ELISA using somatic whole adult antigen of *P. epithilum* while 10% in coprological examination. Hafeezi et al. (2006) recorded 8.42% prevalence rate of *P. epithilum* infection in sheep by dot-ELISA.

The overall sensitivity, specificity, positive predictive values and negative predictive values (Table 4) observed for the tests and antigens in the present study was compromised due to utilization of field sera, these parameters can be optimized with sera of experimentally infected animals. The significant decrease in the level of sensitivity and specificity in field conditions were noted by many workers. For example: Cornelissen et al. (2001) using recombinant cathepsin-L-like protease in ELISA reported 100% sensitivity and specificity of 98.5% (cattle) and 96.5% (sheep) with *F. hepatica* experimentally infected sera but the sensitivity was as low as 90.2% and specificity of 75.3% were recorded when the test was conducted using sera collected from naturally infected cattle.

While using PeESAg, the calculated overall sensitivity% was 92.86 (goats)/ 100 (sheep) in both plate and dot-ELISA, specificity% was 91.58 (goats)/ 91.55 (sheep) in plate ELISA while 88.42 (goats)/ 92.96 (sheep) in dot-ELISA, positive predictive value% was 76.47 (goats)/ 76.92 (sheep) in plate ELISA while 70.27 (goats)/ 80 (sheep) in dot-ELISA and negative predictive value% was 97.75 (goats)/ 100 (sheep) in plate ELISA while 97.67 (goats)/ 100 (sheep) in dot-ELISA (Table 4), these values were optimum for the field sera sample so the tests and PeESAg can be recommended for the detection *P. epithilum* infection in the small ruminants.

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