Evaluation of Indirect ELISA and Western Blotting for the Diagnosis of Amphiistomes Infection in Cattle and Buffaloes

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Abstract
Amphiistomes are snail-borne trematodes infect rumens and reticulums causing acute and chronic diseases in cattle and buffaloes. The economic losses caused by Amphiistomes resulted from drop in milk and meat yield in addition to mortalities. Eighty four serum samples (50 from cattle and 34 from buffaloes, 30 from amphiistomes infected and 54 from amphiistomes free cattle and buffaloes) collected from Giza and Garbia governourates, Egypt. The collected Amphiistomes were morphologically and histologically classified. The identified worms of Par amplistomum and Carmyerius were used for the preparation of somatic antigens separately. The collected 84 serum sample were tested twice by indirect ELISA, firstly with Par amplistomum somatic antigen and secondly with Carmyerius somatic antigen. The antigenic profiles of adult Par amplistomum spp somatic antigen and Carmyerius gergaeirus somatic antigen were analysed by (SDS-PAGE). Four male Rabbit were used for the preparation of hyper-immune serum against Par amplistomum and Carmyerius somatic antigens. Nine serum samples (two rabbit hyper immune sera; one for Par amplistomum and the another for Carmyerius , 3 serum sample from Par amplistomum infected cattle and 3 serum samples from Carmyerius infected cattle and one serum sample from non-infected cattle as negative control) were blotted and tested twice at the same time on the nitrocellulose membrane by Western blotting techniques., firstly by using Par amplistomum somatic antigen and secondly with Carmyerius antigen. The results of both ELISA and Western blotting were statistically analysed. The sensitivity, specificity and accuracy for ELISA and Western blotting were (74% and 100%),(82.4% and 33.3%) and (79.76% and77.78%) respectively . The antigenic profile of adult Par amplistomum somatic antigen showed 14 distinct protein bands of protein molecular weights ranging from11.5 to 174 KDa. While Carmyerius somatic antigen showed 13 distinct protein bands ranging from 11.5 to 166KDa. One distinct immunogenic band at 63 KDa was found to react with all sera from infected cattle and buffaloes with Par amplistomum somatic antigen while the same serum samples gave one distinct immunogenic band at 71 KDa with Carmyerius somatic antigen. It is concluded that ELISA is more reliable test for early diagnosis of amphistiomiasis. There is a strong cross immune reaction between Par amplistomum and Carmyerius.

Key words: Par amplistomum, Carmyerius, bovine, ELISA, Western blotting

Introduction
Amphiistomes had been a neglected trematode infectious disease in ruminants, but has recently emerged as one of the most important trematodes of ruminants which produce high economic losses to the
livestock industries through morbidity and mortality particularly in the young stock. Older animals can develop resistance but may still harbor numerous adult flukes in the rumen and reticulum without showing overt disease, however damage to the rumen due to heavy infection has been recorded and may be responsible for unthrifitness, emaciation, lower feed conversion rate, decrease milk yield and reduction of fertility ((Meshgi et al., 2009; Kamaraj et al., 2010; Sanchis et al., 2012). The adult fluke that lives in rumen and reticulum of ruminants does not cause serious problem, but massive number of immature paramphistomes can migrate through intestinal tract causing acute gastroenteritis in the small intestine with high morbidity and mortality rate especially in young animals. Mixed infection is common in ruminant. In Iran , 3 species were identified in cattle, sheep and goats. *Paramphistomum cervi* and *Paramphistomum microbothriuim* are considered to be one of the most common species of *Paramphistomum* since they have a wide –host range including cattle and buffaloes with cosmopolitan distribution (Rangel- Ruiz et al., 2003 and Magdy et al., 2009). In Egypt, the fresh water snails including *Bulinus truncates* and *Bulinus forskali* are prevalent and play role in the distribution of Amphistomes (Elsokkary et al., 2009) .The intermediate hosts (fresh water snails) living under some particular conditions including the presence of vegetation, humidity, frequent rainfall and mild temperatures (Pinedo et al., 2010).

Coproscopic examination often results in misdiagnosis so it is not wholly reliable, and could not be used for the early diagnosis of Amphistomes which is essential for prompt treatment before irreparable damage to the rumen and bile duct (Meshgi et al., 2009).Therefore, the present study was conducted to evaluate indirect ELISA and Western blotting as serodiagnostic tests for Amphistomes infection in cattle and buffaloes in Giza and Gharbia governorates, Egypt.

**Material and methods**

**Serum samples**

Eighty four serum samples (50 from cattle and 34 from buffaloes collected from Giza and Garbia gouvernourates, Egypt) were tested twice at the same time, firstly with Paramphistomum somatic antigen and secondly with Carmyerius somatic antigen. Nine serum samples (two rabbit hyper immune sera; one for Paramphistomum and the other for Carmyerius , 3 serum sample from Paramphistomum infected cattle and 3 serum samples from Carmyerius infected cattle and one serum sample from non-infected cattle as negative control) were tested twice at the same time, firstly by using Paramphistomum somatic antigen and secondly with Carmyerius antigen.
Flukes burdens

Paramphistomum and Carmyerius adult flukes were collected from rumens and reticulums of slaughtered cattle and buffaloes at Warak and Alsanta abattoirs for preparation of antigens (Meshgi et al., 2009).

Antigens

**A-** A whole worm somatic antigen of Paramphistomum was prepared from adult worms according to Shivjot et al. (2009), it was used for the preparation of hyper – immune serum and as a diagnostic antigen for ELISA and Western blotting.

**B-** A whole worm somatic antigen of Carmyerius was prepared from adult worms according to Shivjot et al. (2009), it was used for the preparation of hyper –immune serum and as a diagnostic antigen for ELISA and Western blotting.

Identification and classification of collected Amphistomum flukes

The flukes were identified and classified according to the classification of Eduardo (1982, 1983) and Nasmark (1937). Protein determination of somatic antigen of Paramphistomum and Carmyerius The protein content of somatic antigen solution was measured using the modified Lowry method (Lowry et al., 1951).

Indirect ELISA

Each serum sample was tested twice, firstly against *Paramphistomum* somatic antigen and secondly against *Carmyerius* somatic antigen. It was carried out according to Shivjot et al.(2009) with some modifications.

The optimal reactant concentrations for working diluents were determined by checkerboard titration according to Sanchis et al. (2012). Prepared somatic antigen had protein content of 3.3 mg / ml was diluted in coating buffer at their optimal dilution (10μg/ml coating buffer). Each well was filled with 200μl of the corresponding antigen concentration and then the plates were incubated overnight at 4°C. The plates were washed three times with PBS-T 0.05% to get rid of excess unbound antigen and the remaining free binding sites were then blocked with BSA for blocking buffer (200μl/well) and kept for one hour then washed three times with PBS- T 0.05%. Sera of different groups were diluted 1/20 in PBS-T then added to the plates (100μl/well) and incubated at 37°C for 90 minutes. The plates were then washed 3times with PBS-T 0.05% and 100μl/well of the conjugate (Anti-bovine IgG whole molecule-horse radish peroxidase conjugate, antibody developed in the donkey, Sigma-Aldrich, USA), 1:2000 dilution in PBS-T 0.05%, were added to all wells and incubated at 37C° for one hour. After incubation, plates were washed 5 times with PBS-T and twice with the substrate buffer then
substrate was added at 100µl/well and incubated in dark place for 5 minutes at room temperature. Readings were recorded by using ELISA reader at 492 nm. The cutoff point was O.D= 0.211, serum sample had O.D higher than 0.211 was considered as positive and serum sample had O.D less than 0.211 was considered as negative.

**Preparation of anti-Paramhistomum and ant-Carmyerius rabbit polyclonal IgG** Rabbit hyper-immune serum against *anti-Paramhistomum and anti-Carmyerius somatic* antigen was prepared according to Hassan et al (2005). 4 males of about 2 Kg, each New-Zealand White rabbits were inoculated 4 times (1\textsuperscript{st}, 14\textsuperscript{th}, 21\textsuperscript{th}. 28\textsuperscript{th}) 2 rabbits with Paramhistomum somatic antigen and the other 2 rabbits with Carmyerius somatic antigen In the first injection, eight hundred µg of somatic antigen were emulsified with 0.5ml of Mantonid oil -ISA-206 adjuvant and subcutaneously inoculated in each rabbit. In the preceding 3 injections 400 µg of somatic antigen were emulsified with 0.5ml of Mantonid oil -ISA-206 adjuvant and subcutaneously inoculated in each rabbit. Blood samples were collected from the ear vein of each rabbit before injection of the antigen (zero day) and used as negative control, then before each injection, rabbits slaughtered at day 35 and blood collected then sera separated, divided and stored in small ependofl tubes at –20 C° until used. The polyclonal hyper-immune serum was evaluated against somatic antigens of *Paramhistomum* and *Carmyerius* isolated from cattle and buffaloes by indirect ELISA and Western blotting.

**Western blotting**

Western blotting was applied according to Anuracpreeda et al. (2008) with modifications. Somatic antigens was denatured in boiling water, diluted as 1:2 with sample buffer and was added as 15µl per well in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). High molecular weight marker (Amersham pharmacia biotech.) was used. The samples were run at 200 volt then 150 volt in electrophoresis buffer. The antigen was transferred from gel into nitrocellulose membrane (sigma, nitrocellulose membrane, electrophoresis grade 0.2 µm pore size, 20x20 cm). Ice was added in a set during transfer to avoid high temperature. Gel was faced the side of black plate of electrodes. Transfer was continued for 1 hour. Nitrocellulose membrane containing antigen was blocked with 1% bovine serum albumin in PBS 1X. Nitrocellulose membrane stripes were cut then each stripe was put in plastic tube of 5 ml capacity. In each tube was added 2.5ml of PBS1X contain serum sample (rabbit hyper-immune serum was diluted 1:500, bovine serum was diluted as 1:50) then was put in refrigerator at 4 C° overnight. Washing with 2.5 ml of PBS-Tween in each tube was applied twice with shaking. A 2.5 ml of anti-rabbit and anti-bovine conjugate peroxidase was added to stripes in dilution of 1:2000 with shaking for 1.5 hour. Washing with PBS-Tween was applied twice. A substrate 0.5 ml of AEC (1 tablet in 2.5 ml diethyl formamide) was added in 9.5 ml
acetate buffer with 10μl H2O2, the stripes were incubated for 10 minutes in dark place then the reaction was stopped with distilled water and the reaction was detected, positive result was seen as coloured bands on nitrocellulose membrane.

Statistical analysis

According to Wayne et al. (1987), Dawson and Trapp (1990) and Riegelman and Hirsch (1989)

Experiment and results

ELISA and Western blotting were evaluated for the sero-diagnosis of *Amphistomases* infection in cattle and buffaloes by screening the collected serum samples as follows: Eighty four serum samples (50 from cattle and 34 from buffaloes) were tested twice at the same time, firstly with *Paramphistomum* somatic antigen and secondly with *Carmyerius* somatic antigen. The results of ELISA for the *Amphistomes* infected cattle and buffaloes and the normal control ones are shown in table (1).

<table>
<thead>
<tr>
<th>Gold Standard <em>Paramphistomum</em> infected cattle &amp; buffaloes</th>
<th>Indirect ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Positive</td>
<td>30</td>
</tr>
<tr>
<td>Negative</td>
<td>54</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
</tr>
</tbody>
</table>

Table 2: The results of Western blotting the reaction of antigenic component and serum samples from hyper immune sera of rabbit, individual infected cattle and normal control cattle

<table>
<thead>
<tr>
<th>Gold Standard <em>Paramphistomum</em> infected cattle &amp; buffaloes</th>
<th>Western blotting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Positive</td>
<td>16</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 3: The statistical analysis results of ELISA and Western blotting

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>Western blotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (%)</td>
<td>74</td>
<td>100</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>82.4</td>
<td>33.3</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>79.6</td>
<td>77.8</td>
</tr>
</tbody>
</table>

The prepared *Paramphistomum* somatic and *Carmyerius* somatic antigens were run on SDS- gel with protein marker (6.5-200 KD), then it was stained by Commassie Brilliant Blue stain to compare the protein profiles of both antigen as illustrated in figure(1).
Nine serum samples (two rabbit hyper immune sera; one for Paramphistomum and the other for Carmyerius, 3 serum sample from Paramphistomum infected cattle and 3 serum samples from Carmyerius infected cattle and one serum sample from non-infected cattle as negative control) were tested twice at the same time, firstly by using Paramphistomum somatic antigen and secondly with Carmyerius antigen.

Figure-1: Commassie Brilliant Blue stained protein profiles from somatic antigens of adult Paramphistomum and Carmyerius separated by SDS-PAGE. (M: molecular weight protein marker, P: somatic antigen of adult Paramphistomum, C: somatic antigen of adult Carmyerius).

Figure-2: Immunoblotting pattern of serum samples with Paramphistomum somatic antigen showing common specific band at 63 KDa
The results of Western blotting for the tested serum samples from the *Amphistomes* infected and non-infected cattle are shown in table (2) and figures (2 and3).

Statistical analysis of ELISA and Western blotting were carried out by comparison of their results against the gold standard (presence of *Amphistomes* in rumen and reticulum) and the results are presented in table (3).

**Results**

The results of statistical analysis of indirect ELISA and Western blotting were presented in tables (1-3). By using SDS-PAGE and Commassie Brilliant Blue R-250 staining method *Paramphistomum* somatic antigen showed 14 distinct protein bands of protein molecular weights ranging from 11.5 to 174 KDa. The bands appeared at 11.5,13.5,19,25,29,46,52,63,66,72,87,105,120 and 174 KDa. While the *Carmyerius* somatic antigen showed 13 distinct protein bands ranging from 11.5 to 166 KDa. The bands appeared at 11.5, 15,19,25,29,46,52,63,68,72,78,110 and 166 KDa as illustrated in figure (1).

The Western blotting analysis of *Paramphistomum* and *Carmyerius* somatic antigens with Serum samples from hyper immune rabbit and individual cattle and buffaloes with *Amphistomes* infection revealed immune-dominant polypeptides of molecular weights 27, 39, 58, 63, 71 and 87 KDa. One distinct immunogenic band at 63 KDa was found due to the reaction of *Paramphistomum* somatic antigen with all sera from infected cattle and buffaloes as shown in figure (2). While the same serum sample give one distinct immunogenic band at 71 KDa with *Carmyerius* antigen as presented in figure (3).
Discussion

As coprological positive results reflect the existence of adult fluke in definite host, the need of an urgent test for early detection of the acute phase of the disease in which maximum pathogenicity is caused by the juvenile stages of *Amphistomes* is essential. Confirmation of juvenile stages of *Amphistomes* is difficult because it depends on demonstration of immature flukes in feces or at necropsy. The Death usually occurs 15-20 day after the first signs appear. The mortality rate in heavily infested animals may be high (Radostitis et al., 2007).

The IgG humoral response against the gastric flukes was analyzed by using *Paramphistomum* somatic antigen and *Carmerius* somatic antigen (for detection the cross reaction between the two species). Somatic antigen of *Carmerius gregarius* was used in this study as this amphistome parasite was found to be concurrently present along with *Paramphistomum* species in the rumen and reticulum of infected host in Egypt. This is fulfilled by immunodiagnosis, so, this experiment was under taken.

A study for evaluation of *Paramphistomum* and *Carmerius* somatic antigens of bovine origin was carried out for serodiagnosis of amphistomum infection in cattle and buffaloes using ELISA and western blotting with the use of postmortem and fecal examinations as reference tests.

Eighty four serum samples were tested twice, one time with *Paramphistomum* somatic antigen and the other one with *Carmerius* somatic antigen, out of 84 serum samples tested 30 samples gave positive ELISA results with both antigens (20 were true positive and 10 serum samples were false positive) while the other 54 serum samples gave negative ELISA results (47 serum samples were true negative and 7 serum samples were false negative).

False positive results may be attributed to migration of early immature larvae through the wall of small intestine, cross reactivity with antibodies from other infections especially trematodes as *Fasciola gigantica* and also may be due to treated cases Sanchis et al. (2012). On the contrary, false negative results may be attributed to low levels of specific IgG, variant Ig antibody expression and/or formation of circulating immune complexes.

In the present study the sensitivity, specificity and accuracy of somatic antigens of *Paramphistomum* and *Carmerius* was 74%, 82.4 % and 79.76 % respectively. This finding was in agreement with Sanchis et al. (2012) who recorded that ELISA sensitivity and specificity using secretory excretory antigen of Paramphistomum were 82 % and 79 % respectively. Also Hussan et al. (2005) recorded 82 % sensitivity of *Paramphistomum somatic* antigen but they recorded higher specificity of 90%. On the other hand these
results disagree with Shiv jot et al. (2009) who recorded higher sensitivity percent 85.71% and a lower specificity percent 23.65% using somatic antigen of Paramphistomum and Tariq et al. (2011) who reported sensitivity of dot ELISA as 100 % for detection of Paramphistomum cervi antibodies in hyper immune rabbit using crude antigen of Paramphistomum.

Thirty serum samples give positive results with both Paramphistomum and Carmyerius somatic antigens, two samples give positive result with Paramphistomum and give negative result with Carmyerius and vice versa which indicate presence of strong cross reactivity between the two species.

The electrophoretic analysis of Paramphistomum somatic antigen gave 14 distinct protein bands (ranging from 11.5 KDa to 174 KDa) of protein molecular weight 11.5,13.5,19,25,29,46,52,63,66,72,87,105,120 and 174 KDa. While Carmyerius somatic antigen separated into 13 distinct protein band (ranging from 11.5 to 166 KDa) of protein molecular weight 11.5,15,19,25,29,46,52,63,68,72,78,110 and 166 KDa. There were eight polypeptides of molecular weight 11.5,19,25,29,46,52,63,72 KDa found common between both antigens. These results are relatively similar with that obtained by Meshgi et al. (2009) who found that electrophoresis analysis of somatic antigens revealed the presence of 10 protein bands of molecular weights ranging from 25-120 also Arora et al. (2010) who recorded that Paramphistomum epiclitum somatic antigen contained protein bands ranging from 14.9-95.5 KDa. and Anuracpreeda et al. (2008) who reported that analysis of components of adult Paramphistomum cervi excretion-secretion (ES) fraction using SDS-PAGE revealed 13 distinct protein bands These antigenic proteins had molecular weights ranging from 10-170 KDa.

The somatic antigens of Paramphistomum and Carmyerius were analyzed using SDS- PAGE then transferred to nitrocellulose sheet. The strips of the blotted Paramphistomum and Carmyerius somatic antigens were tested against sera from hyper immune rabbit, naturally infected cattle and buffaloes and negative control bovine sera. There were 6 protein bands corresponding to molecular weight slandered at 27, 39, 58, 63,71 and 87 KDa react with sera of animals infected with either Paramphistomum or Carmyerius and didn’t react with negative sera.

One antigenic protein of Paramphistomum antigen with a molecular weight of 63 KDa exhibited a consistent reaction with most sera from infected cattle, while one antigenic protein of Carmyerius antigen with a molecular weight of 71 KDa exhibited a consistent reaction with most sera from infected cattle. These results were nearly similar to that recorded by Anuracpreeda et al. (2008) who found that five major antigenic bands of molecular weights ranging from 23 to 116 KDa were recognized by serum of cattle infected with P.cervi and reported that 52 KDa antigenic protein band is most specific band for the
diagnosis of *Paramphistomum cervi* and Meshgi *et al.* (2009) who demonstrated five major proteins of molecular weight ranging from 50 to 100 kDa which were recognized by serum of cattle naturally infected with mixed amphistomes. They concluded that ninety-kDa protein can be specific diagnostic antigen for mixed amphistomes in Iran. However, these results are in agreement with Arora *et al.* (2010) who reported two lower molecular weight protein bands of molecular weight 37.6 and 39.8 as a most common specific antigen for *P. epiclitum*.

The statistical evaluation of both ELISA and Western blotting as shown in Table (3) revealed that the sensitivity, specificity and accuracy for ELISA and Western blotting were (74% and 100%), (82.4% and 33.3%) and (79.76% and 77.78%) respectively. So from the previous it is clear that ELISA is more specific and accurate but less sensitive than Western blotting for the diagnosis of *Amphistomes* infection in cattle and buffaloes.

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