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Full Length Research Paper

Electrophoretic behaviour of proteins of *Ostertagia ostertagi* found in India sheep

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Economic losses due to helminth parasites in sheep throughout the world are considerable. *Ostertagia ostertagi* is a parasitic cattle nematode belonging to the superfamily of Strongyloidea and the family of Strongylidae. *Ostertagia* engenders immunity more slowly and is therefore the most important species in older cattle. These are able to induce structural, biochemical and immunological changes in the host like inappetence, diarrhoea, dull hair coat and weight loss, that is, ostertagiosis. At present, control is almost exclusively based on anthelmintics. For isolation of the proteins of the parasite, a well defined methodology was adopted. The abomasae of sheep in which this parasite resides were collected from abattoirs of various districts and were then carried to the laboratory for screening. The parasites were collected in normal saline, washed and stored in 0.05M PBS with pH of 7.4 at 0°C. After refrigeration, frozen nematodes were thawed, homogenized and centrifuged at 1000-15000 rpm for 15 min. The supernatant was thus collected as a protein mixture and stored at -20°C. Protein concentration of the samples was estimated by Lowry method. The samples were then analyzed through Polyacrylamide Gel Electrophoresis (PAGE) and then through Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein estimation of the samples was estimated to be 3.9 mg/ml. The processed parasite samples were then subjected to PAGE and SDS-PAGE to determine the presence of the proteins. The proteins were seen as continuous bands intermixing with each other in PAGE analysis. The present study revealed two bands of molecular weights - 67 and 20 kDa in PAGE analysis. The proteins when analyzed through SDS-PAGE were mostly found in the range of 20-70 kDa. The SDS-PAGE analysis showed seven prominent bands of molecular weights of 60, 50, 45, 40, 32, 28 and 20 kDa. The present work was a challenging one since only a single study was conducted in this region on this aspect and thus obviously was a big task to peep into the field where scanty input was available.

Keywords: *Ostertagia ostertagi*, polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), PBS.

INTRODUCTION

Gastro-intestinal (GI) parasitism represents a severe health problem in small ruminant production system especially sheep and its consequences are extensive, which may range from reduced productivity to mortality (Skykes, 1994). *Ostertagia ostertagi* is a parasitic cattle nematode belonging to the superfamily of Strongyloidea and the family of Strongylidae (de Ley and Blaxter, 2004). The organism has a direct life-cycle consisting of a free-living phase on pasture and a parasitic phase in the host. At present, control is almost exclusively based on preventive treatment with synthetic chemotherapeutic drugs, that is, anthelmintics. There are several non-chemical alternative strategies for controlling ostertagiosis in ruminant livestock. One possibility is to optimize grazing management by introducing pasture resting, late turn-out, mowing, reduction of the livestock density, stock rotation onto clean pasture and interchange of grazing between different species e.g. sheep and cattle (reviewed by Waller, 2006). A second strategy would be to develop cattle lines or breeds with an enhanced disease resistance.

A third proposal focuses on the use of natural compounds. While the results obtained so far look promising, this area of research is still in its infancy.

In order to have an idea about the previous work done on the said research topic, an attempt to review the available literature was made. Some of the earlier workers investigated the prevalence of gastro-intestinal nematodes of sheep, some studied ideal diagnostic methods for the detection of the parasite surface antigens to accurately determine the level and intensity of the infection, while some others studied the protective effect of nematode antigens. A voluminous literature related to the subject is available. From this region, that is, J&K, no work on the isolation of proteins from *Ostertagia ostertagi* had been carried out till now which somehow shows the need and importance of the present study.

MATERIALS AND METHODS

Parasite collection

Naturally infected guts were obtained from slaughtered sheep on the day of slaughter from local slaughterhouses in particularly three districts namely Anantnag, Pulwama and Srinagar of Jammu and Kashmir. Guts were examined thoroughly especially the abomasums part, and nematode particularly *O. ostertagi* was collected and placed in petridish containing 0.05M PBS (pH 7.4) for initial washing to remove host material and allow regurgitation of gut contents. The nematode was stored in collection vials containing PBS and transported to the Parasitology Lab, Centre of Research for Development (C.O.R.D), University of Kashmir, Srinagar. The length and width was measured. The nematodes were segregated into *O. ostertagi* based on standard body lengths: *O. ostertagi*: (~10 mm) and general morphology (Soulsby, 1982). Moreover, the nematode was kept in deep freezer throughout the procedure to prevent protein degradation.

Processing of the material for protein analysis

Procedure for preparation of sample

For extraction of proteins, nematode species were homogenized separately in 10 ml of cooled 0.05M PBS in a glass tissue homogenizer. The disintegrated parasite extract was centrifuged at 4°C at 10000-15000 rpm for 15 min and the supernatant was collected and stored at -20°C till use.

Protein estimation by Lowry method

The protein concentration of samples was assessed using Lowry Assay (Lowry et al., 1951). It is a highly sensitive method and can detect proteins as low as 5 µl/ml. This is the most widely used method for protein estimation (Zargar et al., 2000).

Preparation of reagents

Copper reagent: This reagent was prepared by dissolving 4% Sodium carbonate (4 gm of Sodium carbonate dissolved in 100 ml of distilled water), 4% Sodium potassium tartrate (0.5 gm of Sodium potassium tartrate in 12.5 ml of distilled water) and 2% Copper sulphate (0.25 gm of Copper Sulphate in 12.5 ml of distilled water). The aforementioned components were mixed in the ratio of 100:1:1 at the time of experiment. In order to avoid precipitation, the solution, “4% Sodium potassium tartrate”, was added to solution “4% Sodium carbonate” followed by “2% Copper sulphate”.

Folin-Ciocalteau reagent solution: The Stock solution was diluted in the ratio of 1:4 by distilled water, that is, 4 ml dissolved in 16 ml of distilled water.

BSA stock solution: This solution was prepared by dissolving 50 mg of BSA in 100 ml of distilled water. BSA was used as reference protein for estimation of concentration of unknown protein in the sample.

Analysis of the material for proteins

The analysis of the materials for the proteins present was done by the method of Polyacrylamide Gel Electrophoresis (PAGE) and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis [SDS-PAGE (Laemmli, 1970)]. The total protein profile of the parasite was carried out using PAGE. In SDS-PAGE, the separation of proteins was done on the basis of molecular size of the proteins. SDS, a denaturing agent along with β-mercaptoethanol (reducing agent) was applied to the protein mixture in order to disrupt the secondary, tertiary and quaternary structure attached to it.

When the polymer- polyacrylamide was formed from the acrylamide, it turned into gel and then electricity was used to pull the proteins through this gel. It works on the principle that if all the proteins enter the gel at the same time and have same force pulling them towards other end, the smaller ones will be able to move through polyacrylamide gel faster and reach near the other end first. While running SDS, the proteins were left to electrophorese, run so long that they actually reached the other end (side) of the gel. The current was turned off and then the proteins were stained with Coomassie brilliant blue stain to analyze how far they moved through the gel.

The molecular weight marker of known weight was also run in a separate lane in the gel and was used to determine the weight of unknown proteins by comparing the distance travelled by individual proteins relative to the marker. SDS-PAGE was carried out to observe the polypeptide pattern of proteins to find out their molecular weight in 10% gels. Discontinuous Polyacrylamide Gel Electrophoresis was carried out with a stacking gel and

separating gel having different pH and acrylamide concentrations.

Preparation of reagents

I. 30% Acrylamide:

Acrylamide : 29 mg
Bis-acrylamide : 1 mg

These reagents were dissolved in 70 ml distilled water to make final volume (100 ml).
Note: - *Acrylamide is a potent neurotoxin and can be absorbed through the skin. Therefore, one is expected to wear gloves and a mask when weighing the reagents and while handling the solutions containing these reagents.*

II. 1.5M Tris (pH=8.8):

18.17 gms of Tris buffer (Molecular Weight-121.14 gms) was dissolved in double distilled water and final volume was made (100 ml). pH of the solution was adjusted at 8.8.

III. 1M Tris (pH=6.8):

15.76 gms of Tris-HCl (Molecular Weight-157.6 gms) was dissolved in double distilled water and final volume was made (100 ml). pH of the solution was adjusted at 6.8.

IV. 10% SDS:

10 gms of sodium dodecyl sulfate was dissolved in 100 ml of distilled water.

V. 10% Ammonium persulfate (APS):

10% freshly prepared ammonium persulfate was used. This was prepared by dissolving 10 gms APS in 100 ml of distilled water. It was then covered with aluminium foil and stored at -20°C.

VI. TEMED (N-N-N’-N’-Tetramethylethylenediamine):

TEMED was added at the time of loading the gel.

VII. Preparation of running buffer:

2 mM Tris was mixed in 490 ml of distilled water. After that, glycine was added and mixed thoroughly. Finally, SDS was added and mixed carefully until all the reagents got thoroughly mixed with each other.

VIII. Preparation of sample buffer:

1M Tris was mixed in 80 ml of distilled water. 1.5 g of SDS and 0.002 g of Bromophenol blue was then added and mixed thoroughly. Glycerol was added to make the final volume of 12.5 ml and then 150 µl of β- beta mercaptoethanol was added to 1 ml aliquote. Sample buffer was stored at -20°C in 1 ml aliquot.

IX. Preparation of staining solution:

0.2 g of Coomassie brilliant blue was added to a mixture of 45 ml of methanol and 10 ml of acetic acid. 45 ml of distilled water was then added to make the final volume up to 100 ml.

X. Preparation of destaining solution:

10 ml of acetic acid was added to 10 ml of methanol and 80 ml of distilled water was added to make the final volume up to 100 ml.

Gels prepared

1.5 M Tris buffer was used in Resolving gel and 1 M Tris buffer was used in stacking gel. The 10% resolving gel and 5% stacking gel for both PAGE and SDS-PAGE were used during present study.
Note: - *10% SDS and β-mercaptoethanol were used only in the case of SDS-PAGE.*

Loading of the gels

After setting the apparatus, the sealing was done with 1% agar. 10% resolving gel prepared for PAGE and SDS-PAGE, respectively was then poured in between the plates immediately. The top of the Resolving gel was overlaid with 1 ml of distilled water in order to reduce the surface tension. The gel slab was left for one hour for polymerization. After polymerization, water was removed and the stacking gel prepared for PAGE and SDS-PAGE, respectively was poured over the Resolving gel. The plastic comb was inserted immediately in the stacking gel to form the wells and the gel slab was kept undisturbed for polymerization.
Note: - *Gloves should be worn at all times while performing SDS-PAGE. To insure proper alignment and casting, the glass plates, spacers, combs and casting stand gaskets must be clean and dry. The glass plates should be cleaned with 70% ethanol.*

Loading of samples

Sample buffer and protein solution were mixed in the ratio of 1:4 in an appendorf tube. The tube was then placed on thermostat for about 3 min. The micro pipette to be used for loading samples was rinsed a few times with distilled water. The micro pipette was inserted to about 1-2 mm from the well bottom before delivery. The micro pipette was rinsed a few times with distilled water after loading each well so as to prevent contamination.

30 µl of protein samples was loaded in each well.15 µl molecular weight marker (sigma) was also loaded in separate lane.

Electrophoresis run

After loading, the gel was run at room temperature. A constant voltage of 200 volts was maintained during the migration of proteins through the stacking and separating gels. When the tracking dye (Bromophenol blue) reached approximately up to the end of gel, the run was stopped.

Staining and de-staining of gels

Since proteins are transparent and cannot be seen as such, therefore staining process was carried to make proteins visible in the gel. Hence, after the electrophoresis, the gel was removed carefully and placed in a petridish containing staining solution. The staining was done at room temperature for two hours. The gel was then removed from staining solution, rinsed with distilled water and placed in de-staining solution in order to remove the excess stain. After the completion of de-staining of gel, it was compared with known Molecular Weight Marker (proteins of known molecular weight) to analyze the molecular weight of protein bands of the parasites.

Statistical Analysis

The statistical analysis was done by feeding data into statistical software Primer. Chi Square test was used for the analytic assessment. The differences were considered significant when the P-value obtained was less than 0.05.

RESULTS AND DISCUSSION

The present study was carried out to isolate the proteins of *O. ostertagi*. For this purpose, abomasums collected from the sheep of different districts of the Kashmir valley were scanned and the parasites were collected and then passed through different laboratory techniques and processing steps in order to isolate the proteins. The whole work was carried from November, 2011 to December, 2012.

The protein concentration of the parasites which was estimated by Lowry method (Lowry et al., 1951) came out to be 3.9 mg/ml (Table 1 and Figure 1).

Arunkumar and Sangaran (2009) estimated total protein concentration of adult somatic antigens of *Haemonchus contortus* and *Ostertagia columbianum* to be 1.4 and 3.6 mg/ml, respectively. Also Prasad et al. (2007) estimated the protein concentration of *H. contortus* adult somatic antigen as 6.3 mg/ml. The difference in the concentrations of the proteins may be attributed to the difference in the processing steps utilized

Table 1. Table showing concentration and relative absorbance of *Ostertagia ostertagi.*

Concentration	Absorbance
0.05	0.181
0.1	0.216
0.15	0.291
0.2	0.357
0.25	0.401
0.3	0.461
0.35	0.631

for the isolation of the proteins from the parasites. For instance, in the present study, parasite samples were homogenized and no sonication was done, while Prasad et al. (2007) homogenized the parasite sample and then sonicated it. Also the use of buffers used for the isolation of the proteins may have a significant impact on the net outcome of the total protein concentration of the parasites, for instance in the present study, lytic buffer and PBS were used for the estimation of the total protein concentration.

The electrophoretic behaviour of *O. ostertagi* was observed both through Polyacrylamide Gel Electrophoresis (PAGE) as well as Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The PAGE analysis showed a high concentration of the total proteins of the parasite. The proteins were seen as continuous bands intermixing with each other, and as such, no prominent bands were observed. As in case of SDS-PAGE, distinct bands were observed which were later compared with molecular markers to determine their exact molecular weight.

The PAGE analysis showed a high concentration of the total proteins of the parasite. The proteins were seen intermixing with each other. Two distinct bands were however observed which were approximately of the molecular weights of 67 and 20 kDa, and this is shown in Figure 2 where lane-1 shows the marker protein – 66, 43, 29 and 14 kDa and lane-2 and lane-3 show the parasite protein.

SDS-PAGE of whole worm extracts were performed and subsequent gels stained. Electrophoresis of soluble proteins of *O. ostertagi* revealed the presence of 7 prominent protein bands with molecular weights ranging from 20-70 kDa. These bands when compared with molecular markers were found having molecular weights of 60, 50, 45, 40, 32, 28 and 20 kDa (Figure 3).

Maere et al. (2005) detected a specific band of approximately 28 kDa from somatic extracts of L_3, L_4 and adult nematodes of *O. ostertagi*. Bahrami et al. (2010) identified 29 protein fractions in the sera of sheep infected with *O. ostertagi* which ranged from 14 to 130 kDa of which prominent fractions were 130, 116, 106, 98 69, 52, 36 and 21 kDa. Vercauteren et al. (2004) identified

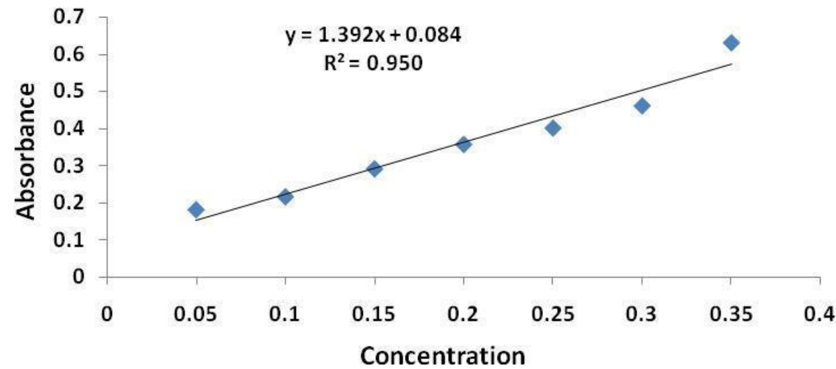

Figure 1. Graph showing vol. of BSA plotted on X-axis against absorbance at 620nm plotted on Y-axis used for estimating unknown protein concentration of *O. ostertagi*.

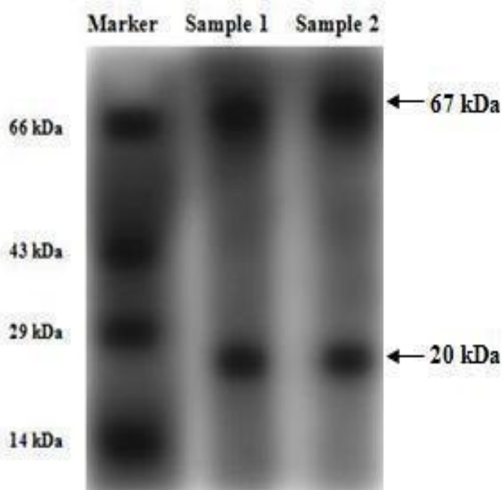

Figure 2. PAGE analysis of *O. ostertagi.* Note: - Lane 1 shows marker protein, Lane 2 and 3 show parasite proteins.

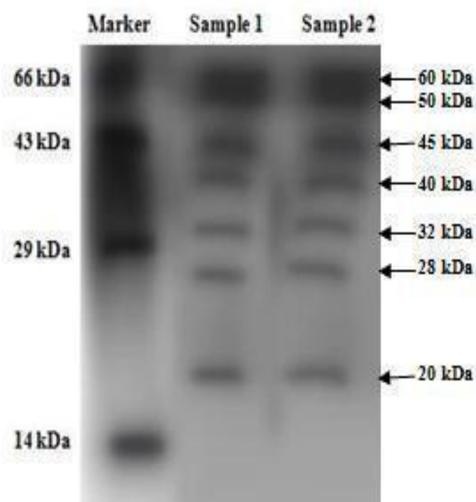

Figure 3. SDS-PAGE analysis of *O. ostertagi.*

one main protein band at 14 kDa in nOPA fractions of L_3, L_4 and adult *O. osertagi* in SDS PAGE and 45 kDa protein in rOPA fractions. Maere et al. (2002) identified a protein of 32 kDa from *O. ostertagi* specifically detected on Western blot by mucus antibodies from immunized animals.

The present results are in accordance with Maere et al. (2005), Vercauteren et al. (2004), Maere et al. (2002), who purified proteins from *O. ostertagi* and their molecular weights were almost similar to that reported in the present study. Here also, it is important to mention that the results obtained may vary which may be due to difference in preparing the solutions, chemical reagents of different quality and quantity or application procedures (Norouzi et al., 2007). Therefore this also needs further investigation.

In a nutshell, it is believed that the proteins derived from the *O. ostertagi* can be used as good immunogens and hence can be exploited for mounting the protective immune response against them. The results of the present study suggest that low molecular weight proteins deserve further investigation and could be isolated in the future as vaccinating agents.

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