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Analysis of excretory–secretory and somatic antigens of *Gastrothylax crumenifer*

M.K. Saifullah, Gul Ahmad* and W.A. Nizami

Section of Parasitology, Department of Zoology, Aligarh Muslim University, Aligarh 202 002, India

Abstract

The excretory/secretory (ES) metabolic products released by *Gastrothylax crumenifer* (Trematoda: Digenea) during *in vitro* incubations and the somatic extract of the adult parasite were analysed using polyacrylamide gel electrophoresis (PAGE). Immunogenicity of ES and somatic extracts were evaluated by immunoblotting and ELISA using sera raised against ES and somatic antigens in rabbits. The electropherograms of ES and somatic extracts have been resolved into 38 and 41 polypeptides, respectively. The apparent molecular weights of these polypeptides range from <29 to >205 kDa. A total of 14 polypeptides were found to be common to both of the samples. The analysis of immunoblot results revealed 22 and 27 antigenic polypeptides in ES and somatic extracts respectively. Only 11 and 13 antigenic polypeptides were found specific to ES and tissue extract respectively. The molecular weights of these specific polypeptides were calculated to be <14.4, 16, 20, 25, 33, 42, 119, 125 and >205 kDa for metabolic products and <14.4, 25, 30, 35, 78, 84 and >205 kDa for the tissue extracts, respectively. Analysis of ELISA results revealed that a dilution of up to 1:3200 of the test sera could react with the ES product. Further, when the ES antigens were allowed to react with antisomatic extracts in hyperimmune sera the titre of IgG increased up to a dilution of 1:12800. The potential importance of these antigens in the immunodiagnosis of amphistomiasis is discussed.

Introduction

Paramphistomiasis is one of the most common diseases of ruminants causing high morbidity and mortality in tropical and subtropical areas. Adult parasites, especially those inhabiting the rumen, have low pathogenicity. However, migrating immature stages cause severe pathological disturbances including haemorrhagic inflammation in the alimentary tract, oedema and anaemia (reviewed by Horak, 1971). In India, numerous outbreaks of acute paramphistomiasis associated with high mortality among young sheep, goats, cattle and buffaloes have occurred (Pande, 1935; Bawa, 1939; Katiyar & Varshney, 1963; Panda & Misra, 1980). Various species of amphistomes infecting domestic ruminants have been reported and most of the literature is confined to taxonomic and histomorphological descriptions (Stiles & Goldberger, 1910; Näsmark, 1937; Thapar, 1956; Sey, 1979; Dutt, 1980; Mehra, 1980; Eduardo, 1985; Mattison *et al.*, 1994).

Despite the enormous economic importance of amphistomes, no comprehensive attempt has been made to analyse the antigens of this group of trematode parasites. The identification, isolation, purification and characterization of parasite antigens are required for vaccination, immunological diagnosis, analysis of immunopathology and for the quantitation of various immune responses of the host. Considering the importance of immunological diagnosis of amphistomes, particularly during the premonsoon period when the rumen parasites stop shedding

*Author for correspondence.
Fax +91 0571 400637
E-mail zyr015@amu.up.nic.in

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eggs (Hanna *et al*., 1988), the present investigation was carried out to identify *Gastrothylax crumenifer* excretory–secretory and somatic antigens which may be useful for the immunodiagnosis of rumen amphistomiasis.

Materials and methods

Collection of parasites

Active and mature *Gastrothylax crumenifer* were collected from the rumen of Indian water buffaloes (*Bubalus bubalis*) slaughtered at the local abattoir. Worms were washed with Hanks' Balanced Salt Solutions (HBSS) without glucose, pH 7.4, premaintained at 37°C. The worms were divided into two groups, one for collection of excretory–secretory (metabolic) antigens and a second group for total worm homogenates.

Collection of excretory–secretory (ES) antigens

Mature worms were washed in 0.01 M sterilized phosphate buffered saline (PBS) pH 7.2. A total of 15 worms per 20 ml saline were incubated for 5 h at 37°C. After incubation, worms were removed and the suspension containing ES proteins centrifuged at $16000 \times g$ for 30 min at 4°C. The supernatant obtained was dialysed against double distilled water for 24 h at 4°C and then lyophilized in Freeze Dryer DC 41 (Yamato Scientific Co., Ltd., Tokyo, Japan). Finally, the freeze dried material was stored at 4°C for further studies.

Homogenization

Worms were homogenized in 0.1 M Tris HCl, pH 7.4, sonicated in a Ralsonic disintegrator for a few seconds and centrifuged at $10{,}000 \times g$. The resultant pellet was discarded and the supernatant stored at 4°C. Protein concentration of somatic extracts as well as ES proteins were determined by the dye binding method of Spector (1978).

Collection of hyperimmune sera

Hyperimmune sera were raised in rabbits as described by Harlow & Lane (1988). One millilitre of metabolic and somatic antigens (containing 4 mg ml^{-1} protein) was mixed with Freund's Complete Adjuvant and injected intramuscularly in rabbits at four sites. Subsequently, two boosters each consisting of 2 mg ml^{-1} protein and Freund's Incomplete Adjuvant were given at an interval of 10 days. Thereafter the rabbit was bled and the sera isolated.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

For analyses of protein profiles, SDS-PAGE was performed using the method of Laemmli (1970) in a separating 7–15% gradient slab gel and 4% stacking gel using a vertical slab gel system (Pharmacia, LKB, Sweden). The soluble protein sample containing about 70 μg protein was mixed with an equal volume of Laemmli's sample buffer (0.625 M Tris-HCl, pH 6.8), containing 20% SDS and 5% (v/v) β-mercaptoethanol, and the sample was boiled for 8 min at 100°C. Standard high molecular weight marker proteins ranging from 29 kDa to 205 kDa (carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66 kDa; phosphorylase-b, 97.4 kDa; β-galactosidase, 116 kDa and myosin, 205 kDa) were purchased from Pharmacia (LKB). Electrophoresis was carried out at 6°C using a Multitemp II, thermostatic circulator (Pharmacia, LKB, Sweden) by applying a constant current of 30 mA/slab gel until the marker tracking dye reached 1 cm above the gel before the end of the electrophoretic run.

Silver staining

Silver staining of gels was performed using the method of Oakley *et al*. (1980). Gels were prefixed in 50% (v/v) methanol, 10% (v/v) acetic acid, 40% DDW for 30 min and finally fixed in 10% glutaraldehyde for 45 min. Thereafter the gel was rinsed overnight in large volumes of DDW. After thorough washing, the gel was treated with dithiothreitol (0.5 mg per 100 ml) for 30 min and then with 0.1% aqueous silver nitrate (w/v). The colour was developed in 3% $Na_2CO_3 + 50\,\mu l$ of 37% formaldehyde (per 100 ml of solution). After visualizing an appropriate colour of polypeptides, the gel was quickly washed in DDW and the reaction was stopped with 2.3 M citric acid.

Immunoblotting

Excretory–secretory and somatic extracts of *G. crumenifer* were solubilized in SDS-reducing buffer and applied to 7–15% gradient polyacrylamide separating gels with 4% stacking gel as described earlier. After electrophoresis for 3 h proteins were transferred onto 0.45 μm pore size Immobilon P membrane (Millipore, USA) by the semidry technique using the Nova Blot assembly (Pharmacia, LKB) as described by Towbin *et al*. (1979). Non-specific sites were blocked with blotto (i.e. 5% skim milk, Anikspray, India), and blots were incubated in hyperimmune sera with blotto in a ratio of 1:100 for 3 h. Subsequently the strips were thoroughly washed with Tris buffered saline at pH 7.4 containing 0.02% Tween 20. The strips were then incubated in antirabbit IgG conjugated with alkaline phosphatase at a dilution of 1:2000 in blotto for 3 h. Finally, antigenic polypeptides were visualized by bromochloro-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) chromogen. All the steps were carried out at room temperature with continuous shaking. After the reaction the strips were rinsed in distilled water and photographed using black and white film.

Enzyme linked immunosorbent assay (ELISA)

Flat-bottomed polystyrene microtitre plates (Dynatech, USA) were coated with 50 μl per well of antigens in coating buffer (protein concentration of 10 μg ml^{-1}) and left overnight at 4°C. The wells were then washed three times with PBS containing 0.1% Tween, blocked with 150 μl of 5% skim milk in PBS and incubated at room temperature for 1 h. The plates were again washed three times with PBS containing 0.1% Tween-20 (washing buffer), 100 μl of serially diluted test sera were added to each well and incubated for 3 h at room temperature in a humid chamber. The plates were washed again three

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times for 10 min with washing buffer. Thereafter, 50 μl of 1:1000 diluted antirabbit IgG conjugated with alkaline phosphatase was added to each well and again incubated for 3 h at room temperature. After incubation the plates were washed three times for 10 min with the washing buffer. The reaction was developed by adding 100 μl of substrate, p-nitrophenyl phosphate (Sigma, USA) in a concentration of 1 mg ml^{-1} in 10% diethanolamine (DEA) buffer and incubated at room temperature for 20 min. Finally, the reaction was stopped by the addition of 50 μl of 3 N NaOH solution and optical density (OD) was read at 405 nm on an ELISA Reader (SLT Instruments, Austria).

Results

SDS-PAGE

The results of gradient SDS-PAGE profiles of somatic as well as metabolic products (ES) of *G. crumenifer* revealed a total of 41 and 38 polypeptides, respectively. The apparent molecular weight (Mr) of these polypeptides ranged from < 29 kDa to > 205 kDa (fig. 1). Further analysis revealed some common as well as a number of unique polypeptides in both somatic and metabolic antigens, with a total of 14 polypeptides being common in both types of antigens. Somatic extracts of the worm revealed 11 characteristic polypeptides with molecular weights of 190, 105, 95, 70, 67, 65, 56, 54, 52 and 40 kDa while metabolic products of the parasite showed nine characteristic polypeptides of 160, 100, 64, 53, 51, 45, 43, 39 and 29 kDa.

Immunoblotting

In order to find out the antigenicity of somatic and metabolic antigens of *G. crumenifer*, SDS-PAGE was performed and the proteins electrotransferred onto a nitrocellulose membrane. The transblotted polypeptides were then allowed to react with homologous (ES antigen vs. anti-ES antisera or somatic antigen vs. antisomatic antisera) and heterologous (ES antigen vs. antisomatic antisera or somatic antigen vs. anti-ES antisera) hyperimmune antisera. The results revealed 27 and 22 antigenic polypeptides when somatic extracts and metabolic products respectively were treated with hyperimmune sera raised against metabolic antigens. The apparent molecular weights ranged from < 14.4 to > 205 kDa. A total of 11 and 13 antigens were found specific to ES and somatic extracts respectively (fig. 2). The apparent molecular weights of these individual specific antigens were > 205, 125, 119, 42, 33, 25, 20, 16 and < 14.4 and > 205, 89, 78, 35, 30, 27 and 14.4 kDa for ES and somatic extracts respectively. The prominent antigens of ES and somatic extracts are indicated by arrows in fig. 2.

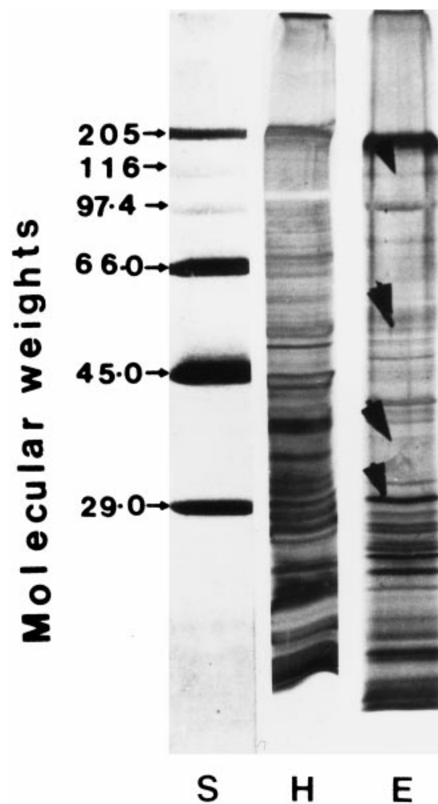

Fig. 1. SDS–PAGE profile of somatic and ES proteins of *Gastrothylax crumenifer*. S, standard; H, somatic protein; E, ES protein.

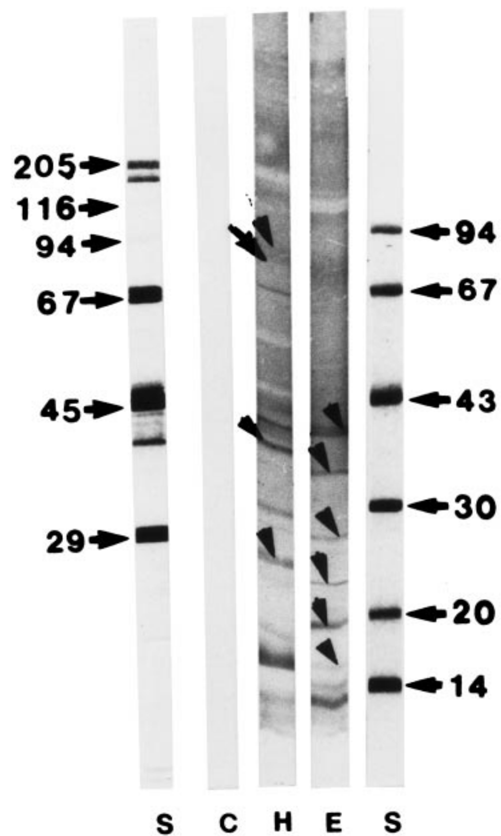

Fig. 2. Immunoblot analysis of excretory–secretory and somatic antigens of *Gastrothylax crumenifer*. S, standard; C, control; H, somatic antigens; E, ES antigens.

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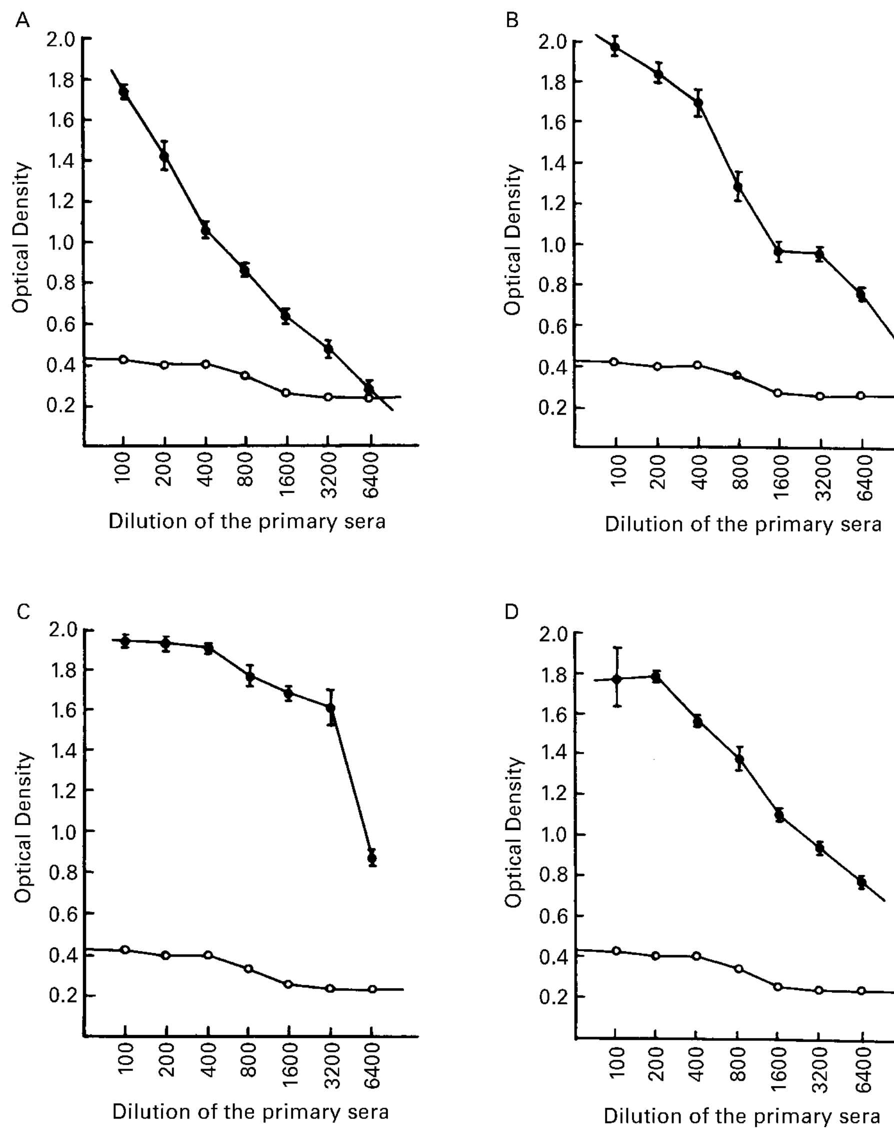

Fig. 3. Mean ELISA absorbance values (nm) of ES and somatic extract antigens of *Gastrothylax crumenifer* as detected by various dilutions of rabbit hyperimmune sera. A: ES antigen vs. anti-ES antisera; B: ES antigen vs. anti-somatic antisera; C: somatic antigens vs. antisomatic antisera; D: somatic antigen vs. anti-ES antisera. ○, control; ●, experimental.

ELISA

When ES antigens treated with anti-ES hyperimmune sera it was found that a dilution as high as 1:3200 of test sera could react well with the ES antigens. Further dilution of the primary sera could not detect ES antigens in the ELISA. In the present experiment, a cut-off point of 0.420 OD was identified, below which reaction was considered to be negative (fig. 3). When ES antigens reacted with anti-somatic extracts in the antisera, it was observed that a 1:12800 dilution of primary sera can detect ES antigens by ELISA. This revealed a two-fold increase in the detection ability of anti-somatic antisera (fig. 3). The titre of IgG was found to be very high when the reaction was carried out with somatic antigens and antisomatic primary sera as a homologous reaction. An absorbance value as high as 1.949 ± 0.24 was detected when the dilution of the primary sera was 1:100. The absorbance value at 1:12800 dilution was 0.463 ± 0.20 which was well above the discriminant point.

Discussion

Many workers have emphasized the importance of ES antigens in host–parasite interactions and it is not surprising that antibodies to these antigens arise in natural infections. In the present study we have demonstrated a number of antigens in ES and somatic extracts of *G. crumenifer*. Although the SDS-gradient PAGE of both

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ES and somatic extracts separated into several polypeptides, some of which were common to both ES and somatic extracts, only a small number of polypeptides was found to be antigenic in nature. Among 11 specific polypeptides of ES antigens, 16, 20, 25, 33, 42, 119 and 125 kDa polypeptides were found to be highly immunodominant based on Western blot analysis. Similarly, Paron *et al*. (1995) demonstrated about 40 polypeptides using SDS-PAGE in the somatic extracts of *Paragonimus heterotremus* but only nine of these polypeptides were shown to be antigenic in Western blots. The molecular weights of these polypeptides ranged from 35 to 16 kDa. Of these only three polypeptides of Mr 35, 33 and 32.5 kDa were reported to be specific to *Paragonimus* which did not cross-react with any other antiparasite sera.

Our interest has been to study both metabolic (ES) and somatic extracts of *G. crumenifer* to compare the antigenic profiles in the two samples. Both somatic extracts and ES products of this parasite are highly complex yet some common and specific polypeptides were demonstrated. Specific antigenic polypeptides of the somatic extracts were <14.4, 27, 30, 35, 78 and 84 kDa, respectively. Unfortunately, there is no previous report on the antigenic polypeptides of any amphistome parasite, therefore, the present investigation is a preliminary attempt to analyse antigenic polypeptides in this neglected parasite. Several investigators have attempted to characterize the metabolic antigens of the other trematodes, including Maleewong *et al*. (1992) who analysed the metabolic products of *P. heterotremus* by SDS-PAGE and immunoblotting. A total of 11 antigenic polypeptides of *P. heterotremus* were identified by Western blot, in the molecular weight range of 12.3–144 kDa.

In the present study, some antigenic polypeptides in the range of 25–42 kDa molecular weight reacted strongly with hyperimmune rabbit sera but it is premature to assign any specific immunodiagnostic role for these polypeptides unless their antigenicity is tested using sera from naturally infected buffaloes. Although ES and somatic preparations showed some similarity, the distinct electrophoretic patterns in SDS-PAGE, the unique and strongly reactive bands in the immunoblots and the high titre of IgG in ELISA indicate the potential importance and strong immunogenicity of the metabolic products.

The enzyme linked immunosorbent assay (ELISA) has been used extensively in the immunodiagnosis of parasitic infections for the last two decades, but not in the diagnosis of *G. crumenifer* infections. In the present study when the ES and somatic extracts of *G. crumenifer* were used to detect IgG antibodies in hyperimmunized rabbit sera, the titre of IgG was very high. A dilution of 1:3200 of primary sera could comfortably detect the IgG antibodies using ES antigen in homologous reactions. However there were some variations in IgG titres when heterologous reactions were performed (i.e. somatic antigens were used to detect the anti-ES antibodies). Other workers have observed that crude antigens prepared, for example, from *Schistosoma mansoni* gave strong cross reactions with *Fasciola hepatica* as well as *P. westermani* (Hillyer & Serrano, 1983) but in the present study the cross reactivity of *G. crumenifer* ES antigens with other parasites have not been checked. However, the partial characterization of these polypeptides reported here is an important first step towards the goal of obtaining recombinant homologues which can be exploited for immunodiagnosis or immunoprotection of amphistomiasis in buffaloes.

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