Preparation and characterization of excretory and secretory antigen of *Cotylophoron cotylophorum* and *Gastrothylax crumenifer*

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Abstract Excretory and secretory antigen of adult *Cotylophoron cotylophorum* and *Gastrothylax crumenifer* was prepared and characterized by SDS-PAGE. Adult paramphistomes were collected in PBS from rumen of sheep slaughtered at Perambur and Villivakkam slaughter houses, Chennai, Tamil Nadu. Excretory/Secretory (E/S) antigens of *G. crumenifer* and *C. cotylophorum* were prepared by incubating in PBS at 28.3 °C overnight in a shaking incubator and centrifuged. The culture supernatant was used as antigen. The protein concentration of E/S antigens of *C. cotylophorum* and *G. crumenifer* ranged from 0.4 to 7.1 mg/ml and 3.3 to 5.9 mg/ml, respectively. SDS-PAGE analysis of E/S antigen of *C. cotylophorum* revealed two polypeptide bands from the range of 32 and 40 kDa. Whereas, SDS-PAGE analysis of E/S antigen of *G. crumenifer* revealed only one polypeptide band of 35 kDa. Excretory/secretory antigen may be used for serodiagnosis of paramphistomosis infection in sheep.

Keywords Paramphistomes · *Cotylophoron cotylophorum* · *Gastrothylax crumenifer* · E/S antigen sheep · Tamil Nadu

Introduction

Paramphistomosis is considered to be one of the most important diseases in cattle, buffalo, sheep and goats of tropical and subtropical regions (Godora et al. 2014). Immature flukes in the duodenum are plug feeders and cause haemorrhage, resulting in anaemia, acute gastroenteritis with profuse, foetid diarrhoea, weight loss, decreased production and mortality in young animals (Horak 1971; Soulsby 1982; Singh et al. 1984). The prevalence of paramphistomosis in India was ranging 7.25–36.20% (Soundararajan and Iyue 2003; Shahnawaz et al. 2011; Chaudhary et al. 2014; Godora et al. 2014).

In helminthic infections, diagnosis is done mostly through demonstration of eggs in the dung of the infected animal. However, in case of paramphistomosis, the death is caused by immature flukes and hence the identification of eggs in the dung could not be recommended to save the animals (Wang et al. 2006). Immunological diagnosis is an important tool for early diagnosis of paramphistomosis which helps in early treatment (Sanabria and Romero 2008). Hence, the study was undertaken to prepare and characterize the excretory/secretory antigens of *Cotylophoron cotylophorum* and *Gastrothylax crumenifer* which can be used in serological tests for the early diagnosis of paramphistomes in sheep.

Materials and methods

Collection of paramphistomes

Adult paramphistomes viz., *Cotylophoron cotylophorum* and *Gastrothylax crumenifer* were collected from rumen of sheep slaughtered at Perambur and Villivakkam slaughter

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houses, Chennai, Tamil Nadu. The collected flukes kept in Phosphate buffered saline (PBS) for antigen preparation.

Preparation of Excretory/Secretory (E/S) antigen from paramphistomes

The flukes collected in PBS were washed thrice in normal saline and twice in phosphate buffered saline (PBS, pH 7.2). After thorough washing, the flukes were used for preparation of antigen. Excretory/Secretory (E/S) antigens of *C. cotylophorum* and *G. crumenifer* were prepared with necessary modifications (Saifullah et al. 2000). Fresh and motile *G. crumenifer* and *C. cotylophorum* flukes were incubated at the concentration of 10 flukes per 6 ml of PBS (pH 7.4) in a six well plate at 28.3 °C overnight in a shaking incubator. After the incubation period, the culture medium (PBS) was collected and the suspension containing E/S protein was centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was dialyzed against double distilled water for 24 h at 4 °C and then concentrated with polyethylene glycol (PEG-6000 - Sigma, USA). The concentrated antigens were stored at – 20 °C as 1.5 ml aliquots. Likewise, 4 batches of antigens were prepared.

Estimation of protein concentrations

The protein concentration of E/S antigen was determined by using Bradford assay (1976). A protein standard was made by dissolving 2 mg of Bovine serum albumin (BSA) in 1 mL of distilled water to make a final concentration of 2 mg/mL of BSA. This protein standard solution was further diluted with distilled water to 1 and 0.5 mg/mL and so on up to 0.015625 mg/mL concentration of BSA by serial dilution. A flat bottom cell culture plate (96 well) was taken and used for the estimation of E/S antigen concentration. Ten microliters (10 µL) of each dilution BSA standard/sample was taken in duplicates in wells and 200 µL of Bradford reagent (Bio-Rad) was added into each well. So the final volume in each well would be 210 µL. The plate was incubated at room temperature for 10–20 min and then the absorbance was measured at 595 nm in a spectrophotometer (Bio-Rad, USA). A standard curve for BSA was generated based on the readings. The concentration of E/S antigen was calculated by using Bradford protein determination protocol table and standard curve.

Characterization of E/S antigen from paramphistomes by SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) was carried out to observe the polypeptide patterns of E/S products of *C.*

cotylophorum and *G. crumenifer*. The total E/S antigen fractions were electrophoretically resolved on 1 mm thick gel using discontinuous system. The stacking gel constituted 12% acrylamide in 0.5 M Tris, pH 6.8 with 0.4% SDS and resolving gel was 12% acrylamide in 1.5 M Tris, pH 8.8 with 0.4% SDS. Gel was stained with Coomassie Brilliant Blue R-250 to identify the marker proteins in the range of 20–205 kDa (Bangalore Genei, India). The gels were photographed with gel documentation system (Bio-Rad Gel Documentation System XR⁺ with Image Lab Software version 3.0, USA).

Results and discussion

In the present study 4 batches of E/S antigens were prepared from 850 adult live and motile *C. cotylophorum* and 320 adult live and motile *G. crumenifer* flukes in 1× PBS, pH 7.2 at 37 °C for overnight, which is similar to that of Vijayashanthi (2013) incubated adult *C. cotylophorum* and adult *G. crumenifer* in 1× PBS, pH 7.2 at 37 °C for overnight, Saifullah et al. (2011) incubated adult *G. crumenifer* in 10 mM PBS, pH 7.2 and Saifullah et al. (2013) incubated adult *G. explanatum* and *G. crumenifer* in 0.1 M PBS, pH 7.2 at 37 °C for 5 h. Tariq et al. (2014) prepared E/S antigen of *G. crumenifer* from sheep by incubating 30 flukes/10 mL of PBS 30 °C for 3–6 h.

On the contrary, the excretory/secretory antigen of adult *P. epiclitum* and *G. crumenifer* were obtained by incubation in 0.01 M PBS (pH 7.4) at 37 °C for overnight Arora et al. (2007) and adult *F. gigantica* and *F. hepatica* were incubated in 0.01 M PBS (pH 7.2) at 37 °C for 1 h (Meshgi et al. 2009). Diaz et al. (2005) prepared E/S antigen of *Calicophoron daubneyi* by incubating the flukes in RPMI-1640 medium containing 0.5 mM PMSF at 37 °C for 24 h. The adult *F. gigantica* were incubated (1 fluke/ml) in RPMI-1640 (pH 7.2) containing 2% w/v glucose, 25 mg/l Gentamycin and 30 mM HEPES for 3 h at 37 °C (Sriveny et al. 2006; Anuracpreeda et al. 2008; Latchumikanthan et al. 2012) and 3 h at room temperature by Zhang et al. (2006), 4 h at 37 °C by El-Ghaysh et al. (1999), 6 h at 37 °C by Wijffels et al. (1994) and Awad et al. (2009). Arunkumar et al. (2014) prepared E/S antigen of *G. crumenifer* from buffalo by incubating the fluke in DPBS (pH 7.2) 37 °C for 8 h.

The protein concentration of E/S antigens of *C. cotylophorum* were 0.4, 5.6, 3.9, 7.1 mg/ml in batch I, II, III and IV respectively, whereas, the protein concentrations of E/S antigen of *G. crumenifer* was 3.8, 3.3, 5.9 and 4.9 mg/ml in batches I, II, III and IV, respectively.

In the present study, the protein concentration of E/S antigens of *C. cotylophorum* and *G. crumenifer* ranged from 0.4 to 7.1 mg/ml and 3.3 to 5.9 mg/ml, respectively.

Vijayashanthi (2013) reported the protein concentration of E/S antigens of *C. cotylophorum* and *G. crumenifer* ranged from 0.312 to 0.392 mg/ml and 0.368 to 0.423 mg/ml, respectively. Arora et al. (2007) also reported that the protein concentration of 0.387 and 0.372 mg/ml for the E/S antigen of adult *G. crumenifer* and *P. epiclitum*, respectively.

In the present study, E/S products of *C. cotylophorum* and *G. crumenifer* from the rumen of sheep were collected in vitro and fractionated using membrane dialysis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) analysis of E/S antigen of *C. cotylophorum* revealed two polypeptide bands from the range of 32 and 40 kDa. Whereas, SDS-PAGE analysis of E/S antigen of *G. crumenifer* revealed only one polypeptide band of 35 kDa (Fig. 1). Vijayashanthi (2013) obtained many polypeptide bands from the range of 10–100 kDa from total E/S antigen of *C. cotylophorum* and several peptide bands ranging from 10 to 110 kDa from *G. crumenifer* revealed by using 12% SDS-PAGE analysis and also few faint bands at the range of 10–50 kDa were common for the total E/S antigen of *C. cotylophorum* and *G. crumenifer*. Arora et al. (2007) reported 10 bands in the range of 19.9–84.1 kDa using 10% SDS-PAGE from the E/S product of *G. crumenifer* obtained from buffaloes.

Vijayashanthi (2013) obtained 6 polypeptide bands within the range of 10–30 kDa from purified E/S antigen of *C. cotylophorum* and the purified E/S antigen of *G. crumenifer* revealed hazy peptide bands between the range of 50–100 kDa. Saifullah et al. (2011) observed 8 bands for F₁ fraction, while 12 and 8 bands for F₂ and F₃ fraction of purified E/S antigen of *G. crumenifer* by chromatographic separation using Sephadex G-200. with the range of < 14–165 kDa using 7–15% gradient. Arunkumar et al. (2014) observed seven major bands ranging from 30 to

240 kDa and minor bands with 30 kDa using 10% SDS-PAGE from E/S product of *G. crumenifer* from buffaloes.

Conclusion

From the present study the excretory/secretory antigen may be used as easily available, safe and inexpensive antigen in serodiagnosis and seroepidemiological survey of paramphistome infection which commonly found in sheep.

Author's contribution GA Collection, preparation and characterization of ES antigen of paramphistomes; CS collection of literature and preparation of manuscript; KN Assistance in literature collection, typing and preparation of manuscript; SG Assistance in preparation of manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval Since, the study was carried out by collection of paramphistomes from the slaughtered sheep and there is no involvement of experimental animals in this study.

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Fig. 1 SDS-PAGE (12%) analysis of E/S antigen of *Cotylophoron cotylophorum* and *Gastrothylax crumenifer*

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