



Isolation and immunoblotting of somatic antigens of *Cotylophoron cotylophorum* and *Gastrothylax crumenifer*

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Paramphistomosis is the neglected digenetic trematode infection which causes high mortality and morbidity (Anuracpreeda *et al.* 2008). In Asia, *Paramphistomum cervi*, *P. explanatum*, *G. crumenifer*, *C. cotylophorum*, *Fischoederius elongates* and *F. cobboldi* have been reported from India, Pakistan, Ceylon and China (Maqbool *et al.* 2003, Wang *et al.* 2006). Paramphistomosis in young sheep has been reported from many states in India like Punjab (Hassan *et al.* 2005), Andhra Pradesh (Sivajothi and Sudhakar Reddy 2014) and Uttarakhand (Maitra and Sanjukta 2014). Few immunological works has been carried out to characterize the somatic antigens of *Gastrothylax crumenifer* (Saifullah *et al.* 2000; Hassan and Juyal 2006; Meshgi *et al.* 2009; Arora *et al.* 2010) and *Cotylophoron cotylophorum* (Hassan and Juyal 2006; Meshgi *et al.* 2009) from various domestic animals. Early diagnosis of infection using faecal examination is not a reliable tool as immature flukes are more pathogenic than mature flukes. Therefore, immunodiagnostic methods are more preferable and ideal method for early diagnosis of paramphistomes to treat infected animals. Hence, the study was carried out to detect the sharing antigens among *Cotylophoron cotylophorum* and *Gastrothylax crumenifer*.

Collection of flukes: Adult paramphistomes were collected from the rumen of sheep slaughtered at Corporation slaughter house, Perambur and Department of Meat Science, Madras Veterinary College, Chennai. The flukes were identified as per the keys provided by Soulsby (1982).

Preparation of Somatic antigen: The somatic antigens of *C. cotylophorum* and *G. crumenifer* were prepared as described by Arora *et al.* (2007) with few modifications. The flukes (20 flukes/10 ml of 10 mM PBS) were triturated using sterilised mortar and pestle. After sonication, the suspensions were centrifuged at 12,000 rpm for 20 min at 4°C.

Purification of somatic antigens using DEAE Sephadex

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A-25: The somatic antigens of flukes were purified by anion exchange chromatography as described by Sriveny *et al.* (2006). The bound proteins were eluted using elution buffer 1× PBS with the pH of 7.0, 7.2 and 7.4, subsequently and the fractions were collected as 1.5 ml aliquots in microfuge tubes. OD values were taken using spectrophotometer at 260 nm and 280 nm and the peak fractions were pooled. The protein concentrations were determined. The purified antigens were dialyzed with double distilled water for overnight at 4°C and concentrated with polyethylene glycol-6,000 (Sigma, USA). The protein concentration was determined by BCA method (Smith *et al.* 1985) using protein estimation kit (Bangalore Genei, India). The antigens were lyophilized in freeze dryer at –80°C.

Raising hyper immune serum: The hyper immune serum was raised using 6 to 7 months old New Zealand white rabbits (IAEC Approval No. 2182/DFBS/B/2012 dated 1.10.2012) against somatic antigens of *C. cotylophorum* and *G. crumenifer* as described by Saifullah *et al.* (2011). The freeze dried antigens were suspended in 70 µl of 1× PBS per vial and mixed thoroughly. Rabbit was injected with 500 µg of purified protein with equal volume of Freund's complete adjuvant followed by two boosters of 500 µg of protein with equal volume of Freund's incomplete adjuvant at 10 days intervals, intramuscularly. The pre-immune and post-immune serum was collected before and after immunization.

Characterization and immunoblotting of somatic antigens: 12% SDS-PAGE was carried out to determine polypeptide patterns of somatic antigen of *C. cotylophorum* and *G. crumenifer* under reducing gel condition as described by Laemmli (1970) using vertical slab gel electrophoresis system (Bio-Rad Ltd, USA) and broad range molecular weight marker (Bangalore Genei, India). The gel was electrophoresed at a constant voltage of 120 V for 45 minutes and stained with Coomassie Brilliant Blue stain. The separated proteins were transferred electrophoretically onto PVDF membrane. The rabbit hyper immune serum (1:100 dilution with PBST) was used as a primary antibody. Anti-sheep IgG (1:1000 dilution with 1× PBST) used as the secondary antibodies. The membrane and the gel was scanned using Gel documentation system model DP-001

FDC (Bio-Rad, USA) with image Lab Software Version 3.0, USA.

Paramphistomosis due to immature flukes are more pathogenic than adult flukes, early diagnosis of infection is most important to treat the infected animals. But early diagnosis of infection by coprological methods is not possible because of long patent period. In such cases, the immunodiagnostic methods are more ideal and reliable techniques.

In this present study, the protein concentrations of *C. cotylophorum* varied from 1.237 to 2.172 mg/ml, whereas, the protein concentrations of *G. crumenifer* were in the range of 1.987 to 2.587 mg/ml (Table 1). A total of 50.2 mg and 80.67 mg of protein were extracted from 480 adult *C. cotylophorum* and 300 adult *G. crumenifer*. Similarly to that of Arora *et al.* (2007) who extracted 6.04 mg/ml of protein from 500 adult *G. crumenifer*. Jadhav *et al.* (2018) prepared somatic antigen of *Paramphistomum epiclitum* using PBS (pH 7.4) with protease inhibitors with the protein concentration of 6 mg/ml.

Table 1. Estimation of protein concentration of somatic antigens of paramphistomes

| Batch no. | <i>C. cotylophorum</i> (mg/ml) | <i>G. crumenifer</i> (mg/ml) |
|-----------|-----------------------------------|---------------------------------|
| I | 1.237 | 1.987 |
| II | 2.172 | 2.001 |
| III | 1.561 | 2.370 |
| IV | 1.421 | 2.587 |
| V | 1.371 | – |
| VI | 1.489 | – |

SDS-PAGE analysis (12%) of total somatic antigen of *C. cotylophorum* and *G. crumenifer* revealed several bands in the range of 10 to 100 kDa and 10 to 250 kDa, respectively. Whereas, the purified fraction of *C. cotylophorum* resolved many interlinked bands in the range of 10 to 70 kDa. The most prominent polypeptides were noticed in the range of 10 to 15 kDa, 20 to 30 kDa and 30 to 40 kDa. Whereas, the purified somatic antigen of *G. crumenifer* revealed many prominent bands from 10 to 90 kDa and one sharp band at 150 kDa. These results were comparable with previous research findings: Salib *et al.* (2015) reported that the somatic antigen of *Paramphistomum sp.* and *Carmyerius sp.* resolved into 14 bands ranging from 11.5 kDa to 174 kDa and 13 peptide bands ranging from 11.5 to 166 kDa, respectively. Ahmad *et al.* (2004) reported that the Sephadex G-200 purified fraction of somatic antigen of *Gigantocotyle explanatum* revealed many polypeptides ranging from < 29 kDa to >205 kDa, in the 7 to 15% gradient electrophoretic analysis. The total somatic antigen of *G. crumenifer* revealed 15 polypeptides in the range of 9.4 to 100 kDa (Kaur *et al.* 2009).

Western blot analysis of purified somatic antigen of *C. cotylophorum* using anti-*C. cotylophorum* rabbit hyper immune serum showed 6 polypeptides ranging from 25 to 30 kDa (3), 35 to 40 kDa (1) and 45 to 100 kDa (2). Whereas,

the purified somatic antigens of *G. crumenifer* revealed 9 bands at the range of 15 kDa (1), 15 to 25 kDa (1), 25 to 35 kDa (2), 35 to 55 kDa (1), 55 to 70 kDa (1), 70 to 100 kDa (1) and 100 to 130 kDa (2), using anti-*G. crumenifer* rabbit hyperimmune serum. Of which, 25 to 30 kDa (2), 55 to 60 kDa (1) were common for both purified somatic antigen.

This result partially coincides with Hassan and Juyal (2006) who observed 8 and 4 immunodominant polypeptides using anti-*Paramphistomum epiclitum* rabbit hyperimmune serum for the somatic antigen of *C. cotylophorum* and *G. crumenifer*, respectively. They obtained two polypeptides at the molecular weight of 56.2 kDa and 50.1 kDa were common between *C. cotylophorum* and *G. crumenifer*. Meshgi *et al.* (2009) reported that 5 major polypeptides from 50 to 100 kDa using serum of naturally infected cattle with mixed amphistomes. Whereas, the immunoblot analysis of somatic antigens of *G. crumenifer* using experimental serum of *P. epiclitum* revealed two polypeptides at the range of 21.8 kDa and 35.5 kDa (Arora *et al.* 2007). Ahmad *et al.* (2004) reported that immunoblot analysis of column purified somatic antigen of *Gigantocotyle explanatum* using *G. explanatum* infected cattle sera showed the polypeptide at the range of < 14 to > 94 kDa. Among that polypeptides < 14, 14, 18, 21 to 25 and 34 to 36 kDa appeared to be dominant.

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SUMMARY

Paramphistomosis is the emerging fluke infection in young ruminants which results in high morbidity and mortality in tropics and sub-tropics. The present study was carried out to detect the sharing antigens among the purified somatic antigens of *G. crumenifer* and *C. cotylophorum*, in order to use as a candidate antigen for diagnosis of paramphistomosis in sheep. Immunoblot analysis of the purified somatic antigens of *C. cotylophorum* and *G. crumenifer* using their specific rabbit hyper immune serum revealed 3 common polypeptides at the range of 27, 29 and 60 kDa. Hence these three immunodominant peptides can be used candidate antigens in development of vaccines against ovine paramphistomosis and also for early diagnosis of infection.

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