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

R VIJAYASHANTHI1, C SOUNDARARAJAN2, M RAMAN3 and T M A SENTHILKUMAR4

Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu 600 007 India

Received: 16 August 2018; Accepted: 10 May 2019

Key words: Paramphistomes, Somatic antigen, SDS-PAGE, Western blot

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. coboldi 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

Present address: 1Junior Research Fellow (vijayashanthi.ram@gmail.com), 2Professor and Project Director (raman.tanuvaz@gmail.com), Translational Research Platform for Veterinary Biologics, 3Professor (dsoundarapara@gmail.com), Department of Veterinary Parasitology; 4Professor (maskumar@yahoo.com), Department of Animal Biotechnology, Madras Veterinary College, Chennai.

A-25: The somatic antigens of flukes were purified by anion exchange chromatography as described by Srinveny 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 PBS) 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. | C. cotylophorum (mg/ml) | G. crumenifer (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 Carnyvrius 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 immuodominant 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.

ACKNOWLEDGEMENTS

The authors are thankful to the Department of Veterinary Parasitology, Madras Veterinary College, Vepery, Chennai, for the support and facilities provided.

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.

REFERENCES

Ahmad G, Saifullah M K and Nizami W A. 2004. Partial purification and characterization of Gigantocotyle explanatum somatic antigens. Journal of Helminthology 78: 95–99.

Anuracpreeda P, Wanichanon C and Sobhon P. 2008. Paramphistomum cervi: Antigenic profile of adults as recognized by infected cattle sera. Experimental Parasitology 118: 203–207.

Arora R, Singh N K, Hassan S S and Juyal P D. 2007. Identi-
fication of immunodominant antigens of *Paramphistomum epiclitum*. *Journal Veterinary Parasitology* **21**: 117–20.

Arora R, Singh N K, Juyal P D, Jyoti and Ghosh S. 2010. Immunoaffinity chromatographic analysis for purification of specific diagnostic antigens of *Paramphistomum epiclitum*. *Journal Parasitic Diseases* **34**: 57–61.

Hassan S S and Juyal P D. 2006. *Diagnosis of paramphistomosis in domestic ruminants in Punjab (INDIA)*, 11th International Symposium on Veterinary Epidemiology and Economics.

Hassan S S, Kaur K, Joshi K and Juyal P D. 2005. Epidemiology of paramphistomosis in domestic ruminants in different districts of Punjab and other adjoining areas. *Journal of Veterinary Parasitology* **19**: 43–46.

Jadhav M, Niranjan K, Bhupamani D and Solanki J B. 2018. Immunodiagnostic potency of homologous antigens for natural *Paramphistomum epiclitum* infection in small ruminants in plate and paper enzyme linked immunosorbent assay. *Indian Journal of Animal Research* **52**: 83–91.

Kaur S, Singla L D, Hassan S S and Juyal P D. 2009. Standardization and application of indirect plate ELISA for immunodiagnosis of paramphistomosis in ruminants. *Journal of Parasitic Diseases* **33**: 70–76.

Laemmli U K. 1970. Cleavage of structural proteins during assembly of the head of T4 bacteriophage. *Nature* **227**: 630–84.

Maitra A, Yadav C L and Sanjukta R K. 2014. Seasonal prevalence of paramphistomosis in domestic ruminants in different agro-climatic zones of Uttarakhand, India. *Asian Pacific Journal of Tropical Diseases* **4**: 748–53.

Maqbool A, Hayat C S, Tanveer A and Ahmad I. 2003. Prevalence and ecology of *Lymnaea* snails in Punjab. *Iranian Journal of Veterinary Research* **4**: 132.

Meshgi E A, Eslami A and Halajian A. 2009. Determination of diagnostic antigens in cattle amphistomiasis using Western Blotting. *Iranian Journal Parasitology* **4**: 32–37.

Saifullah M K, Ahmad G and Abidi S M A. 2011. Isolation and partial characterization of excretory/secretory antigens of *Gastrothylax crumenifer*. *Veterinary Parasitology* **180**: 232–36.

Saifullah M K, Ahmad G, Nizami W A and Abidi S M A. 2000. Partial purification and characterization of *Gastrothylax crumenifer* somatic antigens. *Veterinary Parasitology* **89**: 23–29.

Salih F A, Halium M M A, Mousa W M and Abdel Massieh E S. 2015. Evaluation of Indirect ELISA and Western blotting for diagnosis of amphistomes infection in cattle and buffaloes. *International Journal of Livestock Research* **5**: 71–81.

Sivajothi S and Sudhakara Reddy B. 2014. Immature paramphistomosis in a sheep herd. *International Journal of Biological Research* **2**: 140–42.

Smith P K, Krohn R I, Hermanson G T, Mallia A V, Gartner F H, Provenzano M D, Fujimoto E K, Goke N M, Olson B J and Klenk D C. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**: 76–85.

Soulsby E J L. 1982. *Helminths, Arthropods and Protozoa of Domesticated Animals*. 7th ed. pp. 40–51. Bailliere Tindal Ltd. London.

Sriveny D, Raina O K, Yadav S C, Chandra D, Jayraw A K, Sigh M, Velusamy R and Singh B P. 2006. Cathepsin L cysteine proteinase in the diagnosis of bovine *Fasciola gigantica* infection. *Veterinary Parasitology* **135**: 25–31.

Wang C R, Qiu J H, Zhu X Q, Han X H, Ni H B, Zhao J P, Zhou Q M, Zhang H W and Lun Z R. 2006. Survey of helminthes in adult sheep in Heilongjiang province, peoples Republic of China. *Veterinary Parasitology* **140**: 378–82.