

## An immunological analysis on sharing of antigens amongst gastro-intestinal nematodes using inhibition ELISA

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**Abstract:** Antigen sharing amongst gastro-intestinal nematodes viz., *Haemonchus contortus*, *Oesophagostomum columbianum* and *Bunostomum trigonocephalum* was evaluated by inhibition ELISA. Two antigens namely soluble extract antigen (SEA) and gut integral membrane antigen (GIMA) were prepared and hyper immune sera were raised in rabbits against SEA of three referral nematodes. Based on inhibition ELISA, it was observed that the SEA of *H. contortus* had 36.4 and 28.87% cross-reactivity with inhibited sera of *B. trigonocephalum* and *O. columbianum* respectively whereas, the GIMA showed 29.2 and 25.1% cross-reactivity with *B. trigonocephalum* and *O. columbianum*. The SEA of *O. columbianum* exhibited 34.3 and 22.0% and the GIMA revealed 27.3 and 22.8% cross-reactivity with *B. trigonocephalum* and *H. contortus* respectively. The SEA of *B. trigonocephalum* showed 24.8 and 34.2% and the GIMA exhibited 21.48 and 30.25% cross-reactivity with *O. columbianum* and *H. contortus* respectively. Further, it was concluded that *H. contortus* showed a high degree of antigen sharing with *B. trigonocephalum* than *O. columbianum*.

**Keywords:** antigen sharing, nematodes, ELISA, Sheep.

### Introduction

Parasitic nematodes belonging to the order strongylida represent diverse groups of helminthes with variable economic significance in small ruminants (Jasmer & Mc Guire, 1996). Gastrointestinal nematode infections of livestock animals are prevalent and costly problems worldwide (Markus Kiel *et al.*, 2007). Parasitic gastro-enteritis caused by *H. contortus*, *O. columbianum* and *B. trigonocephalum* constitute an important group of nematodes and they adversely affect both wool and milk production in animals (Sood, 1981; Knox, 1998). In heavy infections, mortality may arise as an important cause of economic loss, while moderate infection frequently causes stunted growth leading to premature culling of affected animals (Sykes 1994; Sykes & Coop, 2001). Despite the increasing evidence of cross-reactivity among helminths has been reported, the information on strongylid nematodes is, however limited (Cuquerella *et al.*, 1993, 1994; Molina *et al.*, 1999). Hence, the present investigation was undertaken to elucidate the extent of antigens shared amongst the three gastro-intestinal nematodes namely *H. contortus*, *O. columbianum* and *B. trigonocephalum* using inhibition ELISA.

### Materials and methods

Three species of nematodes viz., *Haemonchus contortus*, *Oesophagostomum columbianum* and *Bunostomum trigonocephalum* were collected from local abattoir. The worms were recovered from their respective sites of predilection at necropsy following standard technique (Sahu & Misra, 1988). They were washed repeatedly with distilled water followed by physiological saline and phosphate buffered saline (PBS, pH 7.4). Then, the worms were identified up to species level using standard keys (Soulsby, 1982).

#### *Preparation of antigens*

**Soluble extract antigen (SEA):** Soluble extract antigen for each species of the referral nematodes was obtained by processing adult parasites of *H. contortus*, *O. columbianum* and *B. trigonocephalum* separately using standard technique (Klesius *et al.*, 1986).

One gram of freshly collected adult nematodes was suspended into homogenizing buffer (0.1 M PBS, pH 7.4, supplemented with 1mM PMSF and 10% Triton X-100). The mixture was subjected to repeated freeze-thawing cycle (approximately 8-10 times). Finally, the worms were homogenized using ground glass homogenizer and the suspension was subjected to high speed centrifugation at 10,000 Xg for 1 hr at 4°C. The supernatant was designated as soluble extract antigen (SEA) and was stored at -20°C until use.

**Gut integral membrane antigen (GIMA):** The gut integral membrane antigen for each referral nematodes was obtained from dissected out worm intestines following the procedures described earlier (Smith, 1993; Knox & Smith, 2001).

About 10 worms of mixed sex were placed on a microscopic slide in a few drops of cold homogenizing buffer (0.1M PBS, pH 7.4, 1mM Na- EDTA and 1mM PMSF) and were transected 2 or 3 times with a scalpel blade. By applying a gentle finger pressure after placing a second slide on top of the microscopic slide, the organs were allowed to extrude out of the dissected worms. Under stereoscopic binocular microscope, the pieces of intestines were picked out of the debris manually into the homogenizing buffer and stored at -20°C. The worm intestines previously stored -20°C were thawed at room temperature and centrifuged (10,000xg for 10 minutes) in microcentrifuge and the resulting pellet was weighed. After adding sufficient homogenizing buffer to create at 10% (w/v) suspension, the preparation was subjected to homogenization manually in a glass homogenizer. The pellet collected on centrifugation (10,000xg) was

resuspended in 10%(w/v) homogenizing buffer containing 0.1% Tween-20. This membrane suspension was centrifuged again and the pellet was washed with Tween-20 buffer. Finally, the washed pellet was resuspended in homogenizing buffer containing 2% (w/v) Triton X-100 and allowed incubation for 2 hours at 4°C. The integral gut membrane proteins were extracted as a supernatant (10,000xg for 10 min) and stored - 20°C till further use.

#### Protein estimation

The protein concentration of the referral antigens viz., SEA and GIMA was estimated by the method of Lowry *et al.*, (1951) using bovine serum albumin fraction V as the standard.

#### Raising of hyper immune sera

Rabbit hyper immune sera (RHIS) were raised against SEA of *H. contortus*, *O. columbianum* and *B. trigonocephalum* using standard immunization protocol to serve as reference sera.

#### Analysis of antigen sharing

Analysis of serologically relevant common antigens amongst the referral nematodes was attempted using inhibition enzyme linked immuno sorbent assay.

#### Standardization of enzyme linked immuno sorbent assay (ELISA)

An ELISA was performed to assay the antibody independently with each antigen using homologous and heterologous sera (Engvall & Perlman, 1971). An indirect-ELISA for the detection of parasite specific antibodies in serum was standardized on the basis of block titration at 10.0, 5.0, 2.5 and 1.25 µg/ml concentration as coating antigen against serially diluted (double fold) reference test sera. On the basis of block titration, an optimum concentration of antigen (5 µg/ml) was standardised and used in all subsequent assays.

#### Inhibition ELISA

The precision of ELISA was also further examined to assay the extent of antigen sharing amongst the referral nematodes by an inhibition ELISA. The test was designed on the basis of absorption/ inhibition pattern of parasite specific antibody to a common antigenic (cross-reacting) determinants present in variable amounts on different antigens by prior *in vitro* incubation. The test was set-up as follows:

Preparation of particulate antigens of the referral nematodes under study was achieved by partial homogenization of freshly collected live intact worms. Care was taken to ensure that partial homogenization of the parasites in PBS is sufficient to get clean particulate matter, which was later separated by centrifugation at 1000 xg for 10 mins. Each antigen preparation from referral nematodes was adjusted to a common concentration at 660nm visible range by colorimetry, finally suspended in 1ml PBS (pH 7.4). Following microcentrifugation of the above said particulate antigen preparation, the supernatant was removed and the pellet was further reconstituted in 20 µl of PBS in eppendorf tube. The antigen was incubated overnight at 4°C with

200 µl of heterologous sera (for e.g. 20 µl of particulate antigen preparation of *H. contortus* with 200 µl of rabbit hyper immune serum raised against *O. columbianum* and *B. trigonocephalum* separately). The incubation was carried out in duplicate for each antigen preparations separately. At the end of the incubation, the incubated mixture was centrifuged in a microcentrifuge at 10,000xg for 10 min and the supernatant was designate as "inhibited test sera".

Each antigen of referral nematodes was tested for immunoreactivity against both parasite specific uninhibited homologous serum (primary antibody), as well as, homologous inhibited test sera by prior *in vitro* incubation with other nematode antigens using appropriate controls in ELISA.

The assay was designed as indirect non-competitive ELISA using 96 well flat bottom microtitre plate (Nunc, Denmark). The wells were coated with 100 µl of antigen diluted in 0.1M carbonate-bicarbonate buffer (protein concentration 5 µg/ml) and incubated at 4°C for overnight. The plate was washed five times with wash buffer and then blocked with 300 µl of blocking buffer (1% gelatine in PBS) for one hour at room temperature to prevent non-specific binding. After blocking, the plate was again washed five times with PBS-T. Then, the plate was added with 100 µl of diluted serum (1:100) in duplicate wells over double fold dilutions and incubated at 37°C for one hour. After that, the plate was washed five times with PBS-T. Then, the wells were added with 100 µl of enzyme labeled secondary antibody (goat anti-rabbit IgG -HRPO, dilution 1:5000) and incubated at 37°C for one hour. Then, the plate was washed five times with PBS-T. The plate was added with 100 µl of freshly prepared substrate solution (orthophenylenediamine, OPD 1mg/ml in 0.1M citrate buffer pH, 5.0 containing H<sub>2</sub>O<sub>2</sub>) and incubated at 37°C in dark room for 15 minutes to observe the colour development. The excess colour development was stopped by addition of 100 µl of 0.1M sulphuric acid to each well. Finally, the colour reaction was read in ELISA reader at an absorbance of 492nm.

## Results and discussion

The soluble extract antigen (SEA) of *H. contortus*, *O. columbianum* and *B. trigonocephalum* had a protein concentration of 1.4, 3.6 and 1.8 mg/ml respectively. The gut integral membrane antigen (GIMA) of *H. contortus*, *O. columbianum* and *B. trigonocephalum* had a protein content of 2.3, 2.4 and 2.0 mg/ml respectively.

Assessment of serological cross reactivity amongst referral gastro-intestinal nematodes was made by inhibition ELISA, to elucidate the reactivity patterns of homologous antigen and antibody system modified (inhibited) in the interface of a cross-reacting heterologous parasite moiety. Prior *in vitro* incubation of a parasite-specific hyper immune serum with a defined particulate moiety of the potentially cross-reacting

parasite was expected to result in altered seroreactivity due to the blocking of cross-reacting antibodies during the incubation. The altered seroreactivity thus achieved by this version of ELISA was successfully used in examining the serological cross-reactivity amongst referral nematodes selected for study.

The ELISA O. D. values representing seroreactivity of a parasite specific antigen with antibody containing sera (uninhibited) were taken as cent percent reactivity (zero percent inhibition). The altered seroreactivity exhibited as lowered ELISA O.D. values of the homologous sera subjected to prior *in vitro* incubation with the heterologous parasite antigen was accordingly noted and expressed as percentage inhibition in relation to uninhibited serum reactivity.

Based on ELISA reactivity, it was observed that the SEA of *H. contortus* showed 36.4 and 28.87% cross-reactivity with inhibited sera of *B. trigonocephalum* and *O. columbianum*. Whereas, the GIMA of *H. contortus* revealed 29.2 and 25.1% cross-reactivity with inhibited sera of *B. trigonocephalum* and *O. columbianum*. The SEA of *O. columbianum* exhibited 34.3 and 22.0% cross-reactivity with inhibited sera of *B. trigonocephalum* and *H. contortus* while, the GIMA of *O. columbianum* showed 27.3 and 22.8% cross-reactivity with inhibited sera of *B. trigonocephalum* and *H. contortus*. The SEA of *B. trigonocephalum* showed 24.8 and 34.2% cross-reactivity with *O. columbianum* and *H. contortus* whereas, the GIMA of *B. trigonocephalum* exhibited 21.48 and 30.25% cross-reactivity with inhibited sera of *O. columbianum* and *H. contortus*.

It was also reported that the cross-reactivity potency was high with SEA than GIMA of the referral nematodes. Cuquerella *et al.* (1994) also demonstrated that the soluble extract antigen of *H. contortus* had a cross-antigenicity with sera of *T. colubriformis* and *T. circumcincta* in ELISA. Molina *et al.* (1999) reported that the SEA of *H. contortus* showed a cross-reactivity with serum of *T. circumcincta* in ELISA.

### Conclusion

On the basis of inhibition ELISA findings, it was concluded that *H. contortus* showed a greater degree of antigen sharing with *B. trigonocephalum* than *O. columbianum*. On the other hand, *H. contortus* was antigenically more closer to *B. trigonocephalum*. Hence, the present study will also be useful for understanding the evolutionary conservation of antigens and for designing effective immunodiagnostic / immunoprophylactic method. Further studies warranted for identifying species specific antigenic components of these referral nematodes.

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