

## Prepatent detection of *Fasciola gigantica* infection in bovine calves using metacercarial antigen

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Received 31 March 2006; revised 30 May 2006

Metacercarial antigen of *Fasciola gigantica* was evaluated for early immunodiagnosis of experimental bovine fasciolosis using ELISA and Western blot. In ELISA, the experimental *F. gigantica* infection was detected as early as 2 weeks post-infection (WPI). The gradual increasing trend of antibody level was observed from 2 to 7 WPI, followed by a plateau, which was maintained up to 14 WPI. In Western blot, sera from experimentally infected calves recognized one distinct polypeptide of 21 kDa in fractionated metacercarial antigen as early as 10<sup>th</sup> day post infection. From 2 WPI, more polypeptide bands were reacting. Recognition of these protein bands persisted till the end of the experiment (14 WPI). Cattle sera collected from the field showed 34.5% seroprevalence of fasciolosis by ELISA using MAg. Comparative immunoblot studies of metacercarial antigen with anti-*Gigantocotyle explanatum* and anti-*Paramphistomum epiclitum* sera revealed that 21 and 25 kDa polypeptides of metacercarial antigen did not cross-react with any of these sera and appear to be unique to *F. gigantica* and having the desirable qualities of early and specific immunodiagnosis.

**Keywords:** Bovine calves, ELISA, *Fasciola gigantica*, Metacercarial antigen

Tropical fasciolosis, caused by *Fasciola gigantica*, is a disease of grave economic importance in ruminants of India affecting animal health and production<sup>1</sup>. In adult cattle, the course of the disease is often referred to as chronic and sub clinical, whereas it is reported to be acute with a high rate of mortality in young ones<sup>2</sup>. Though effective novel vaccines are being developed and experimental trials are underway<sup>3</sup>, yet at present, in spite of a few reports of anthelmintic resistance, chemotherapy is the only available option to control this disease. For effective use of these drugs early and accurate diagnosis of the disease is of paramount importance. Confirmative coprological diagnosis is useful in patent infections, but it is not capable of detecting early infections. The pathology of immature fasciolosis is manifested as early as 2 weeks post infection (WPI)<sup>4</sup>, whereas coprological diagnosis is possible only after 13 – 14 WPI. The serodiagnostic methods are quite sensitive to detect the infection in the early stages and have been exploited for the diagnosis of fasciolosis and other helminth infections<sup>5-10</sup>. However, these serological assays lack the diagnostic specificity in the areas where animals are infected with different species of helminths. The possession of vast array of antigens associated with

the migrating flukes is one of the major factors responsible for the existence of common antigens between the flukes and other trematode parasites<sup>11</sup>. Lack of specificity in the serodiagnosis of fasciolosis may also be due to use of antigens derived from adult flukes. Of late, efforts are being focussed on the use of larval antigens for serodiagnosis of human<sup>12</sup> and ovine fasciolosis<sup>7</sup> with high level of specificity.

Therefore, the present study was carried out to evaluate the metacercarial antigen for serodiagnosis of fasciolosis in cattle using ELISA and Western blot.

### Materials and Methods

*Metacercariae*—Snails, *Lymnaea auricularia*, were collected (n=350) from different water bodies in and around Bareilly, India and screened for *F. gigantica* infection *in vitro*. The infected snails were fed on pipal leaves (*Ficus religiosa*) and metacercariae shed by them were harvested on transparent polythene sheets (2×2 cm) and were stored in sterile distilled water at 4°C, until use. Weekly changes of sterile distilled water were done.

*Preparation of metacercarial antigen (MAg)* — Metecercariae attached to polythene sheets were brushed into a Petri dish containing phosphate buffer saline (PBS), pH 7.2. The collected metacercariae were homogenized in the presence of cocktail of

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protease inhibitors (1mM each of ethylene diamine tetracetic acid, ethylene glycol bis-NNNN-tetracetic acid, N-ethylmaleimide and phenylmethyl sulphonyl fluoride) for 20 min under ice bath using a Teflon glass homogenizer<sup>7</sup>. The homogenate was, then, subjected to ultrasonication for 5 min to disrupt remaining intact metacercariae using an ultrasonicator (Misonix, USA). After sonication, the preparation was centrifuged at 14,000 g for 45 min at 4°C. The protein content of the supernatant containing metacercarial antigenic material was determined<sup>13</sup> and stored at -20°C until use.

*Preparation of somatic antigen (FSAg)*—Mature *F. gigantica* were collected in chilled phosphate buffer (PBS, pH 7.2) from the liver of slaughtered buffaloes, thoroughly washed, weighed and dried. The dried flukes were pulverized in pestle and mortar. The resultant fluke powder was suspended in chilled PBS containing a cocktail of protease inhibitors and stirred for further extraction. The extracted antigen was centrifuged at 4000 g for 30 min at 4°C. Supernatant was collected and centrifuged at 14000 g for 45 min. The collected supernatant was equilibrated against 20 mM, tris; 0.5 M, NaCl; pH 8.0, filtered by 0.45 µm syringe filter (Sartorius) and kept as FSAg<sup>14</sup>.

*Experimental design*—Eight male crossbred calves (*Bos taurus* × *B. indicus*), free from parasitic infection were procured from institutes dairy and were kept in the experimental large animal shed of the division. The animals were stall fed and were provided with milk, calf-starter ration, green fodder and clean water. The animals were randomly assigned to two groups (group 1 and 2) of 4 calves in each group. The *F. gigantica* naïve status of the calves was confirmed by ELISA using FSAg. The animals of group 1 were orally infected with 400 viable metacercariae of *F. gigantica*, whereas the animals of group 2 were maintained as uninfected control. Before giving infection to the animals, the viability of the metacercariae was tested microscopically based on the movements of the young flukes inside the cyst and presence of excretory granules in bunches. The infective doses of metacercariae were prepared in a bolus of wheat flour dough and administered orally to the experimental calves, which were kept off feed for 24 hr prior to infection.

*Collection of serum and faecal samples* — Sera from both infected (group 1) and uninfected control (group 2) group of animals were collected at weekly intervals over a period of 14 weeks and stored in

aliquots at -20°C until assayed for *F. gigantica* specific antibodies. Meanwhile, daily coprological examination of faecal samples was carried out from 7 WPI, until there was evidence of *F. gigantica* eggs in the faeces by sedimentation method<sup>15</sup>.

To evaluate the diagnostic potentiality of MAg, 84 cattle sera samples were randomly collected from Shahajahanpur, India, an area identified for *F. gigantica* infection. The sera samples were kept at -20°C until use.

*Raising of anti-Gigantocotyle explanatum serum*—Crude extracts of *G. explanatum* were prepared as per the method of Oldham<sup>16</sup>. Briefly, 300 µg of the extract in 100 µl of PBS, (pH 7.2) was mixed with an equal volume of Freund's complete adjuvant (Bangalore Genei, India) and two rabbits of 5 months old were immunized subcutaneously. Booster doses were administered twice at bi-weekly intervals with Freund's incomplete adjuvant (FIA). Sera samples were collected intracardially one-week after last immunization and stored at -20°C until use.

*Raising of anti-Paramphistomum epiclitum serum*—The sera were obtained from the other laboratory of the Division, working on paramphistomes. The procedure adopted for raising the sera was as follows. Two 4 month old buffalo calves, free from parasitic infection were orally infected with 10,000 (approx.) laboratory harvested metacercariae of *P. epiclitum* and bled at 5 days interval up to 15 WPI. The sera samples were stored at -20°C until use. All animal experimentations were carried out as per the guidelines of Institute Animal Ethics Committee.

*SDS-PAGE and immunoblotting* — SDS-PAGE was employed for characterization and analysis of MAg and FSAg on 12.5 % polyacrylamide gel at 150 V for 4 - 5 hr in a maxi gel apparatus (Bangalore Genei, India) as described earlier<sup>17</sup>. Metacercarial antigen was electrophoresed and transfer of resolved proteins from gel to nitrocellulose sheets (NCP) was performed at 100 mA for 2 hr in a max. trans blot cell (Bio-Rad, USA)<sup>18</sup>. The transfer efficiency was checked by staining the NCP with 0.1% Ponceau-S solution. NCP was cut into strips and incubated in 5% of skimmed milk in phosphate buffered saline containing 0.05% Tween 20 (PBS-T) at 4°C overnight to block the free sites. The optimum dilution of primary antibody was determined by incubating the NCP's in different dilutions and 1:100 dilution was found optimum. Then NCPs containing transferred

proteins was probed with primary antibodies for 2 hr at 37°C. This was followed by incubation in a 1:2000 dilution of horseradish peroxidase conjugated with rabbit anti-bovine/anti-rabbit IgG (Bangalore Genei, India) in PBS-T for 1 hr at 37°C. The strips were further incubated in substrate buffer containing DAB (3-3-diamino-benzidine tetra hydrochloride, Sigma) in dark room until the bands were visible. The colour reaction was stopped after 10 min by rinsing with distilled water. The reaction pattern was analysed by gel documentation and analysis system using Genesnap programme (Syngene, UK).

**Antibody detection in sera of experimentally infected calves using ELISA** — ELISA was performed as per technique described earlier<sup>21</sup>. A 100 µl of MAg (10 µg/ml) in carbonate buffer (pH 9.6) was coated in each well of a micro ELISA plate (NUNC) and incubated overnight at 4°C. After 3 washes with PBS-T, 200 µl of 5% skimmed milk in PBS-T was added and incubated for 1 hr at 37°C to block the free sites. A 100 µl sample of 1:100 diluted cattle sera (experimentally infected and field collected) was added to the wells and incubated for 2 hr at 37°C. Wells were washed again and 100 µl of 1:5000 dilution of rabbit anti-bovine IgG - HRPO conjugate (Bangalore Genei, India) was added to the wells and incubated for 1 hr at 37°C. After 3 washes with PBS-T, 100 µl of substrate (OPD-10 mg, 10 ml of citrate buffer pH 5.0, 10 µl of H<sub>2</sub>O<sub>2</sub>) was added to the plates and kept in dark room for 10 min and the reaction was stopped by adding 100 µl of 3N H<sub>2</sub>SO<sub>4</sub> to each well. The absorbance values were read at 492 nm in a microplate reader (Electronic Corporation of India). Cut-off value was determined by mean absorbance values of control sera + 3 SD.

## Results

**Animal infection** — The eggs of *F. gigantica* started appearing in the faecal samples of all infected calves at 13 WPI.

**SDS-PAGE and immunoblot analysis** — SDS-PAGE profile of MAg and FSAg is shown in Fig.1. In MAg, twenty four polypeptides of 96, 92, 78, 76, 66, 62, 60, 54, 49, 46, 43, 39, 37, 33, 32, 30, 28, 26, 25, 21, 18, 16, 14 and 12 kDa were resolved. Almost similar polypeptide profile was observed in FSAg, except the 21 kDa polypeptide which was a specific prominent band in MAg.

When pooled sera from experimentally *F. gigantica* infected calves were probed with

fractionated MAg, bands of 43, 32, 25 and 21 kDa were recognized. Among these polypeptides, 21 kDa was detected as early as 10 days post-infection (DPI; Fig. 2). On 2 WPI, an additional band of 25 kDa was found reacting and persisted till the end of the experiment (14 WPI). Besides, 21 and 25 kDa proteins, band of 43 kDa was detected on 8<sup>th</sup> WPI. It is interesting to note that the 21 kDa specific polypeptide of MAg, sharply reacted from 10 days to 14 WPI.

**Detection of antibodies in sera of experimentally infected calves using ELISA** — Analysis of the ELISA results is illustrated in Fig.3. The anti-*F. gigantica* antibody was detected as early as 2 WPI, thus confirming the high level of sensitivity of the antigen and test procedure. The gradual increasing trend of antibody level was observed from 2 to 7 WPI, followed by a plateau till the end of the experiment (14 WPI).

**Screening of field sera** — Eighty four cattle sera collected randomly from the field were screened for fasciolosis using MAg. Out of 84 sera samples screened by ELISA, 29 (34. 5%) gave OD values in the range of 0.208 to 0.249 which were well above the cut off value of 0.194. The rest of the sera gave a range of insignificant OD values of 0.018 to 0.07.

**Cross-reactivity studies** — A comparative immunoblotting study of MAg with anti-*G. explanatum* and anti-*P. epiclitum* sera revealed cross reactivity. Anti-*G. explanatum* serum recognized six

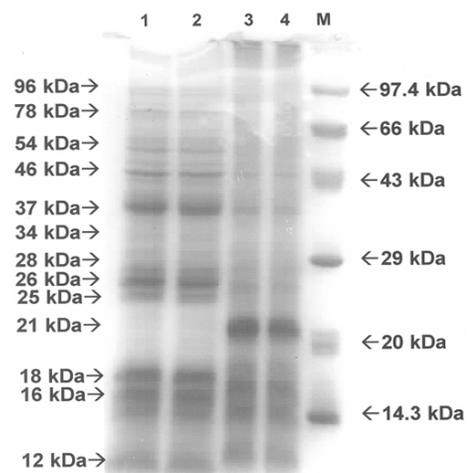


Fig. 1— Comparative SDS-PAGE (12.5 %) profile of the polypeptides of somatic and metacercarial antigens of *F. gigantica* [Lanes : 1 & 2 – FSAg; Lanes : 3 & 4 – MAg; and Lane : M-- Standard molecular weight marker]

protein bands of the molecular weight of 12, 18, 28, 33, 37 and 66 kDa, while anti-*P. epiclitum* serum recognized protein bands of 28, 33, 37, 49 and 66 kDa. However, the 21 polypeptide of MAG did not cross-react with the above sera raised against the parasites (data not shown).

**Discussion**

The pathological effects of bovine fasciolosis due to *F. gigantica* infections are paramount importance

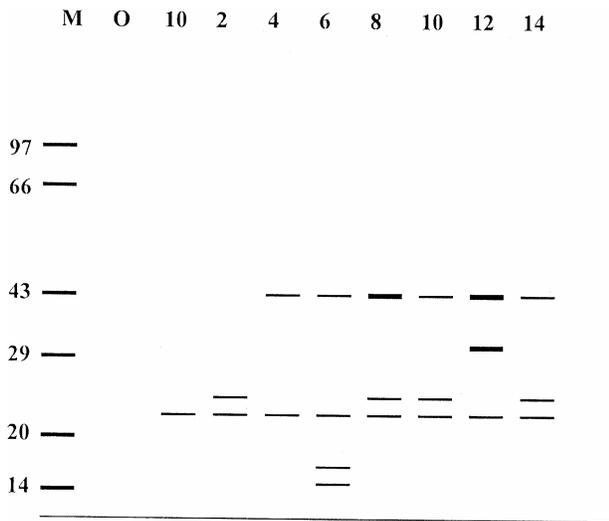


Fig.2— Schematic presentation of immunoreactive bands of *F. gigantica* metacercarial antigen using sera of bovine calves experimentally infected with *F. gigantica* by Western blot [Lanes: 0 day to 14 WPI; and Lane : M - Standard molecular weight marker]

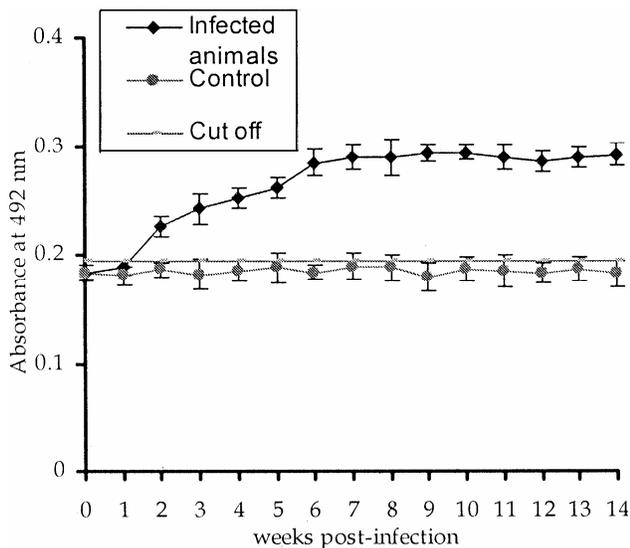


Fig. 3— Detection of antibodies to *F. gigantica* metacercarial antigen in experimentally infected bovine calves by indirect ELISA [The error bars represent SD]

because of traumatic hepatitis caused by immature flukes during first 6 – 8 WPI. At this stage of infection, serodiagnosis of the disease is the best option to coprological detection of the fluke eggs, as these are seen only after 13 -14 WPI. Several serodiagnostic techniques have been used to capture anti-*Fasciola* antibodies<sup>19- 23</sup>. However, these studies were confined to evaluate the efficacy of antigens derived from the adult stage of the parasite.

Comparative SDS-PAGE analysis of the polypeptides of MAG with FSAG revealed a 21 kDa protein which was unique to MAG. Moreover, the polypeptide was found immuno-reactive in Western blot as early as 10 DPI.

In the present study, MAG was used to detect anti-*Fasciola* antibodies in sera of experimentally infected bovine calves using ELISA and Western blot. We observed that the antibody titre was increased gradually from 2 to 7 WPI, followed by persistency of this titre up to 14 WPI. The kinetics of antibody response in the present study is in agreement with earlier workers who reported similar findings in sheep<sup>7, 22-25</sup> and in cattle<sup>19, 20</sup>.

In immunoblot studies, the immunoreactive proteins recognized against MAG were 43, 32, 25 and 21 kDa. It is interesting to note that the polypeptide, which is unique to MAG (21 kDa) reacted strongly as early as 10 DPI and continued to react till the end of the experiment, while reaction with 25 kDa polypeptide started on 2 WPI and persisted till the end of the experiment. The early recognition of these protein bands increases their usefulness for immunodiagnosis in the early prepatent stage of infection. Moreover, the rapid immune recognition of MAG by bovine calves may have significance in determining the course of infection and in defining the degree of immunity in this species. Recognition of other bands cannot be used for diagnosis of fasciolosis as they were cross-reacting and delaying the detection of infection. In contrast to our study, Mousa<sup>7</sup> has observed a band of 32.5 kDa in cercarial antigen as early as 2 WPI using sera from sheep infected with *F. gigantica*. The result of field screening of cattle sera using MAG was also encouraging and could detect infection in 34.5% of cattle in endemic area of Uttar Pradesh, India. However, further screening of more samples is required.

Comparative immunoblot studies of MAG with anti-*G. explanatum* and anti-*P. epiclitum* sera

identified cross reactive proteins. The major cross-reactive bands were 18, 28, 33, 37 and 66 kDa. Interestingly, the two early recognizable polypeptides of MAg i.e., 21 and 25 kDa were not reacting with anti-*G. explanatum* or anti-*P. epiclitum* sera. The present study suggested that the polypeptide bands of 21 and 25 kDa derived from MAg were sensitive and specific for detection of early *F. gigantica* infection in cattle. Isolation and purification of these polypeptides might further enhance the sensitivity and specificity of the test for early prepatent diagnosis of fasciolosis and that could be used as a promising alternative to the conventional method of faecal egg detection. This would also encourage early chemotherapy to save the animals prior to damage in the form of traumatic hepatitis and cirrhosis.

### Acknowledgement

Authors are thankful to ICAR and PIU-NATP for financial assistance through World Bank funded National Agricultural Technology Project (MM) to carry out the research work. The authors are grateful to Dr A Prasad of Paraamphistome laboratory of the division for providing *P. epiclitum* infected buffalo sera.

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