Comparative diagnostic potentiality of ELISA and Dot–ELISA in prepatent diagnosis of experimental Fasciola gigantica infection in cattle

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A glycoprotein (27 kDa) was isolated from crude somatic antigen of Fasciola gigantica by two steps affinity chromatography and was used in early detection of experimental fasciolosis in cattle by indirect ELISA and in dot-ELISA formats. Although, anti-27 kDa antibodies could be detected after 3 weeks post infection (WPI) by dot – ELISA which was one week later than indirect ELISA. The test, dot-ELISA, was more convenient in field application. By the test (dot-ELISA) the infection could be equally detected in animals infected with 100, 200 and 300 metacercariae of F. gigantica with high sensitivity. Further, the antigen (27 kDa) was not found to react with goat sera infected with Parasphistomum epithelium, which are giving strong reaction to homologous immature and mature fluke antigens of P. epithelium.

Keywords: Dot–ELISA, ELISA, Fasciola gigantica. Prepatent diagnosis

In fasciolosis detection of fluke eggs in faeces is considered as most reliable diagnostic method. However, coprological confirmation of the disease prior to application of strategic anti-fluke medication seems of a little or no significance to avoid heavy economic losses. While advantages of immunodiagnostic techniques lie in detecting early infection during the prepatent stage and in mild infection¹⁵. Sustained efforts have been made towards developing specific serodiagnostic tests for earliest possible detection of this infection in animals for controlling the negative impact of F. gigantica infection on the livestock productivity⁶⁻¹³. Fagbemi et al.¹⁴ have identified a protein (88 kDa) for detection of circulating antigen in the sera of cattle experimentally and naturally infected with F. gigantica. In the same year, Fagbemi and Guobadia² have purified a 28 kDa cysteine protease from the adult homogenates, which besides detecting anti-protease antibodies in the sera of cattle, sheep and goat experimentally infected with F. gigantica was also found to be cross-reactive with Parasphistomum microbothrium and Schistosoma bovis. Similarly, 28 kDa F. gigantica cysteine proteinase was isolated and tested for the diagnosis of ovine fasciolosis¹² and the antigen was found effective in detecting experimental infection at 4th week post-infection. Our earlier efforts to develop a suitable diagnostic assay for tropical fasciolosis using 27 kDa glycoprotein as an antigen have shown that this protein could detect Fasciola gigantica mono-infected infection in cattle with 100% sensitivity¹⁵. But due to the complexity involved in conducting indirect ELISA, the method cannot be recommended as a field diagnostic test.

Accordingly, comparative diagnostic potentiality of 27 kDa antigen was evaluated for the diagnosis of experimental F. gigantica infection in cattle using ELISA and dot-ELISA.

Materials and Methods

Metacercariae—Metacercariae (mc) were harvested on polythene sheets from F. gigantica infected Lymnaea auricularia maintained under laboratory conditions. These were processed and stored at 4°C in sterile distilled water until use. The viability of each batch was microscopically tested prior to oral administration of infection dose to the animals

Animal infection
Rabbits—Six New Zealand white rabbits weighing 1.5 to 2.0 kg each, were orally infected with 100 viable mc of F. gigantica and were bled by cardiac puncture on 10th week post-infection (WPI). The sera collected were used for isolation of infection induced immunoglobulins.
Cow calves—Cattle (Bos indicus X B. taurus) sera experimentally infected in our previous experiment by 100, 200 and 400 viable mc of F. gigantica per animal was used for evaluating the efficiency of the serological tests. Sera collected up to 13 weeks post infection (WPI) were tested. The previously collected post mortem data were compared with serological tests.

Goat—Goat sera experimentally infected with 3500 mc of Paramphistomum epiclitum were collected from other laboratory of the department working on paramphistomosis for cross reactivity study. All the experimental animals were maintained as per the approved guidelines of committee for the purpose of control and supervision of experimentation on animals (CPCSEA). Besides, the institute animal ethics committee (IAEC) constantly monitored the animal experimentation.

Preparation of somatic antigen of Fasciola gigantica (FSAg)—Mature F. gigantica were collected in chilled phosphate buffer (PBS), pH 7.2 from the liver of slaughtered buffaloes, thoroughly washed and dried off completely. The dried flukes were pulverized and the resultant fluke powder was suspended in chilled PBS containing cocktail protease inhibitors (1 mM each of ethylene glycol bis-NNNN-tetracetic acid, ethylene glycol bis-N,N,N,N-tetraacetic acid, N-ethylmaleimide and phenylmethyl sulphonyl fluoride; pH 7.2), and stirred for further extraction. The extracted antigen was centrifuged at 4000 g for 30 min at 4°C. Collected supernatant was further centrifuged at 13000 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. The protein concentration was determined.

Preparation of somatic antigen of P. epiclitum—Adult parasites from rumen and immature parasites from duodenum and jejunum of goats were collected in 0.1M PBS, (pH 7.2). After thorough washing, the somatic antigens were prepared as described above. The prepared antigens were designated as APRAg for adult and IPIAg for immature parasites.

Coupling of anti-F. gigantica IgG to CNBr activated sepharose 4B and affinity purification of FSAg—The sera of rabbits experimentally infected with mc of F. gigantica were treated with 40% ammonium sulphate for the precipitation of immunoglobulins. IgG was isolated from the precipitated immunoglobulins by anion exchange chromatography and coupled with CNBr activated sepharose 4B. The detail methodology for coupling and two steps affinity purification of FSAg has already been reported. The protein concentration of the antigen isolated after second steps of purification was determined by a spectrophotometric method and designated as H2.

SDS–PAGE—The proteins isolated after first (F2) and second (H2) step chromatography was electrophoretically resolved on gel (1.0 mm thick) using a discontinuous system. The stacking gels were constituted of 3% acrylamide in 0.5 M tris (pH 6.8) with 0.25% SDS and resolving gels were 12% acrylamide in 1.5 M tris (pH 8.8) with 0.25% SDS. Gels were stained with coomassie brilliant blue (CBB) R-250 to identify the marker proteins in the range of 14 to 100 kDa (Bangalore Geniei, India), F2 and H2. Gels were scanned by gel documentation and analysis system (Syngene, U.K.) using Genesnap and Genetool programs. Both F2 and H2 were stained by periodic acid silver stain as per the methods of Oakley et al. with some modifications.

Positive and negative reference sera—The sera of two cattle heavily infected with F. gigantica were chosen as positive reference sera. Negative sera were collected from 10 uninfected animals maintained in the experimental animal shed of the Division of Parasitology of the Institute.

Enzyme linked immunosorbent assay—Microtitration plates (Nunc) were coated with H2 antigen diluted (8 µg ml⁻¹) in carbonate bicarbonate buffer (pH 9.6) and incubated at 37°C for 1 hr before keeping overnight at 4°C. Coated plates were blocked with 5% non fat milk in PBS. After washing the plates, 100 µl of test sera diluted to 1:1000 with PBS, was added to each well and incubated at 37°C for 2 hr. The plates were again washed and 100 µl of anti-bovine IgG peroxidase conjugate (1:15000 dilution, Sigma Chemical Company, USA) was added and the plates were kept at 37°C for 2 hr. After washing, O-phenylene diamine (OPD) (Sigma chemical company, USA) 40 mg, OPD in 100 ml of phosphate citrate buffer (pH 5.0) and 40 µl H₂O₂ was added and the plates were kept in dark for 7 min. The reaction was stopped by adding 50 µl of 1N H₂SO₄ per well and the optical density (OD) was measured at 492 nm using ELISA reader (ECL, India). For cross reactivity study, microtitration plates were coated with APRAg, IPIAg and H2 at the coating level of 8 µg ml⁻¹.
Anti-P. epiclitum goat sera collected on different weeks were diluted (1:1000) and tested against the antigens. Peroxidase-conjugated anti-goat IgG was used as conjugate and OD values were measured. The cut off point for the OD was determined from the mean OD obtained from the sera of 10 uninfected animals. Three standard deviations of these measurements were added to the mean to give a cut off point for a positive test of 0.39 or greater for OD.

Dot-ELISA—Optimum concentration of the antigen required for dipstick ELISA was determined by checkerboard titration. Dipstick ELISA was done using experimentally infected cattle and goat sera. Each dipstick was coated with 360 ng of antigen and air-dried. The dried sticks were blocked with 5% skimmed milk in PBST for 1 hr at RT. The sticks were thoroughly washed in PBST for five times and then each stick was incubated separately for 45 min in respective sera diluted (1:50) in 1% skimmed milk in PBST. After, five washes the sticks were dipped in anti—bovine/anti-goat peroxidase conjugate at a dilution of 1:1000 in 1% skimmed milk in PBST for 45 min at RT. Final washing was carried out as described earlier. Chromogenic substrate, DAB was used for color development. The sticks were finally washed in distilled water and dried. Positive reactions were characterized by the formation of a distinct colored dot on the membrane.

Statistical analysis—Results were tested for statistical correlation using Student’s t test and P values of 0.05 or lower were considered statistically significant.

Results

Purification of FSAg—After second step purification a total of 5.5 mg bound protein (H2) was obtained from 520 mg FSAg. Electrophoretic separation of F2 in the presence of SDS and 2-mercaptoethanol, proteins were resolved in dimer forms (Fig. 1). Further purification of F2 eluted a 27 kDa glycoprotein (H2), stained intensely by CBB. The nature of the protein was confirmed by specific staining procedure (Fig. 2).

Experimental infection of animals

Cattle—In the faeces of experimentally infected calves, F. gigantica eggs were first detected on 14 WPI. The fluke recovery was varied from 39.5 to 76%, however, the mean percentage fluke establishment was lowest in group 2 followed by 3 and 1. The mean egg per gram (EPG) in animals of groups 1, 2 and 3 was 98.5, 112 and 168 eggs/gram of faeces. While on 25th WPI the mean EPG was declined to 36, 60 and 61, respectively for the animals of groups 1, 2 and 3.

Goat—Infected goats showed clinical signs such as anorexia, polydypsia and fluid foetid diarrhoea at 7th WPI. Immunodiffusion test using APRAg and IPIAg gave precipitin reaction in the sera collected on 7th and 8th WPI.

Detection of anti-H2 antibodies by ELISA using sera of cattle experimentally infected with F. gigantica—In an indirect ELISA, a detectable antibody level in all the groups of animals was
observed by 2nd WPI. Thereafter, a uniform slightly decreasing trend in all the groups of animals was recorded from 3rd WPI and a level of 2.37 x NV (normal value) in group 1. 2.4 x NV in group 2 and 2.4 x NV in group 3 was reached on 8th WPI. On 9th WPI, anti- H2 antibody showed an upward trend and a plateau was maintained until 13th WPI. Analysing the mean OD values obtained in the animals of group 1 and 3 it was observed that although the initial mc dose was four times higher in group 3, the anti-H2 antibody response was almost similar in groups 1, 2 and 3 (Fig. 3).

Detection of anti-H2 antibody by Dot-ELISA—Anti-H2 antibodies were detected in all the sera collected on 3rd WPI. There is no visible differences in the intensity of reaction in the dots developed using sera of cattle experimentally infected with different dosages of mc. No reaction was observed in the control sera (Fig. 4, dots 1-9). A strong signal was noted in the sera of goats experimentally infected with P. epiclitum when tested by homologous antigen (Fig. 4, dots 10-12) however, the same sera was found negative when tested with H2 coated dots (Fig. 4, dots 13-17).

Comparative sensitivity and specificity of antigen and test formats—All the experimentally infected cattle which were passing eggs in faeces were found positive in ELISA on 2 WPI, but positive signals in dot-ELISA was recorded on 3rd WPI irrespective to fluke establishment, thus establishing 100% sensitivity of both the test format (Fig. 3, Fig. 4, dots

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**Fig. 3**—Anti-27 kDa antibody response in experimentally infected cattle by indirect-ELISA on 0 to 13 WPI [OD values are mean ± SD of four animals for each group. Horizontal line represent cut off point].

**Fig. 4**—Detection of anti-27 kDa antibodies in F. gigantica infected cattle and in P. epiclitum infected goats by dot-ELISA. [1- positive control; 2 and 3 – H2 coated dots developed using sera of animals experimentally infected by 100 mc. 4 to 6 - H2 coated dots developed using sera of animals experimentally infected by 200 mc. 7and 8 - H2 coated dots developed using sera of animals experimentally infected with 400 mc. 9 - dot using control sera, 10 – APRAg coated dot reacted with P. epiclitum infected goat sera, 11 to 12- IPIAg coated dots reacted with P. epiclitum infected goat sera, 13 to 17- H2 coated dots probed with goat sera collected on 4th, 5th, 6th, 7th and 8th WPI, respectively]

**Fig. 5**—The mean OD values obtained in P. epiclitum infected goat sera collected on 4-8 WPI. [1-4th week, 2-5th week, 3-6th week, 4-7th week, 5-8th week. Horizontal line represent cut off point].
dot-ELISA format. It is interesting to note that although the presence of isolated protein in minute quantity in adult flukes. Previously, Cornelissen et al. have isolated 27 kDa cysteine protease, Cornelissen et al. have reported high specificity by ELISA with sera from experimentally infected cattle (98.5%) and sheep (96.5%), but the specificity was as low as 75.3% when sera were collected from naturally infected animals. Antigen (27 kDa) was not cross reactive in both the test formats with sera of goats experimentally infected with *P. oviporum*.

When efficiency and applicability of the two tests is compared, it has been observed that although indirect ELISA is detecting infection one week earlier, the field applicability of Dot–ELISA is higher than the indirect ELISA. It has also been observed that the antigen coated dots can be stored for at least 80 days in room temperature (Ghosh, personal communication). Generally speaking, the indirect ELISA was the most sensitive test, however, a drawback for this test is the need of an absorbance reader, which is expensive and therefore, difficult to have in laboratories with limited resources. On the other hand, a relevant point in favour of dot–ELISA is that it can easily be set up in a moderately equipped laboratory. The test proved to be useful in field diagnosis considering the advantage of eliminating the need of an absorbance reader. The test is also cheaper than indirect–ELISA. This also complies with one of the recommendation issued by the International Office of Epizootics (OIE), in the sense that it is necessary to develop diagnostic methods for parasitic diseases which should be simple, cheap and useful under field conditions. Further studies using more number of sera samples from known positive vis-à-vis known negative sera samples from slaughter house seem essential for evaluation of specificity of 27 kDa glycoprotein before the application of reported findings in field on large scale, a prerequisite for standardization of the test.

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**References**