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Serodiagnosis of bovine fasciolosis by enzyme linked immuno sorbent assay in Chennai, Tamil Nadu

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ABSTRACT

Excretory/ secretory (E/S) antigen of adult *Fasciola gigantica* worms was used for diagnosis of fasciolosis in cattle and buffaloes sera collected from different places in and around Chennai, Tamil Nadu using enzyme linked immuno sorbent assay (ELISA). Alcoholic fractionation (75%) and DEAE-sephadexA-25 anion exchange chromatography were used for the purification of E/S antigen. In this study, optimum dilution of E/S antigen, serum and conjugate were 1 in 100 (5 µg/ml), 1 in 200 and 1 in 5000, respectively, were used as standardized by checker board titration. A total of 321 sera samples consisting of 168 cattle and 153 buffaloes were tested using ELISA and also dung samples examined by sedimentation method for comparing the sensitivity of both tests. Out of 321 animals examined 19 (5.91%) were positive for egg of *Fasciola* sp., 41 (12.77%) for amphistome egg, 38 (11.83%) for strongyle egg, 6 (1.86%) for *Strongyloides* sp. egg and 2 (0.62%) for mixed (both amphistome and strongyle eggs) infection in coprological examination. A total of 29 sera samples (17.26%) of cattle and 38 sera samples (24.83%) of buffaloes were found to be positive in ELISA test. Cattle sera samples showed 100% sensitivity and 95.09% specificity in ELISA. Similarly, sera samples from buffaloes also showed 100% sensitivity and 92.10% specificity in comparison to faecal examination method. ELISA was highly sensitive and specific in detecting higher percentage of positive animals than faecal examination and can be recommended for routine use in seroepidemiological surveys of *Fasciola gigantica* infection.

Key words: Buffaloes, Cattle, *Fasciola gigantica*, ELISA, Excretory/ Secretory antigen

Fasciolosis, one of the major parasitic diseases caused by digenetic trematodes *Fasciola gigantica* (Linnaeus 1758) and *Fasciola hepatica* (Cobbold 1885) in cattle, buffaloes, sheep and goats, leads to a significant economic loss (FAO 1994). The economic loss by fasciolosis includes death of the infected animals, reduced milk yield, poor body weight gain, poor carcass quality and liver condemnation (Carnevale *et al*. 2001, Charlier *et al*. 2007). Prevalence of bovine fasciolosis was 18% in Switzerland (Rapsch *et al*. 2006), 18% in Turkey (Toparlak and Gull 1988), 31.7% in Zimbabwe (Pfukenyi and Mukaratirwa 2004), 44.7% in China (Liu *et al*. 2009) and 72% in England (McCann *et al*. 2010). Bovine fasciolosis was reported in Andaman and Nicobar Islands (Rai *et al*. 1996), Punjab (Maqbool *et al*. 2002), Uttar Pradesh (Bhatia *et al*.1989), Haryana (Gupta *et al*. 1986), Uttaranchal (Yadav *et al*.2007), Meghalaya (Roy and Tandon 1989), and Jammu and Kashmir (Sharma *et al*. 1989). There is no published report on bovine fasciolosis from Tamil Nadu except 19.7% mortality rate recorded in sheep from Nilgiris district (Soundararajan *et al.* 2000).

Detection of eggs in faeces is the routine diagnostic procedure for live animals, but the method is cumbersome and labour intensive (Happich and Boary 1969, Soulsby 1982). Faecal examination technique does not allow the detection of early-stage prepatent infection, which lasts approximately 13 weeks (Gupta and Yadav 1992). Failure to detect prepatent and acute fasciolosis by coprological examination is one of the contributing factors which hinder the control of this disease in India. The antibody-based serologic test was used successfully to detect *F. hepatica* as early as 2–4 weeks after infection (Itagaki *et al*. 1989, Fagbemi and Guobadia 1995). ELISA with E/S products as antigens proved to be sensitive, specific and an early method for detection of fasciolosis in cattle and sheep (Sinclair and Wassall 1998). Serodiagnosis of bovine fasciolosis has not been tried to elucidate the prevalence of acute fasciolosis and prepatency of the disease in Tamil Nadu. Hence, the study was undertaken to know the seroprevalence of *F. gigantica* using ELISA to detect the infection in field conditions in and around Chennai, Tamil Nadu.

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MATERIALS AND METHODS

Collection of worms: Adult *Fasciola gigantica* worms were collected in normal saline solution from bile duct of cattle and buffaloes slaughtered at Corporation slaughter house, Perambur, Chennai during the period from December 2009 to April 2010. Worms were washed in phosphate buffer saline solution. Excretory/secretory antigen was prepared from adult flukes by keeping the live flukes in RPMI-1640 media for 24 h and purifying E/S antigen by alcoholic fractionation method (Coles and Rubano *et al.* 1988) and DEAE-SephadexA-25 anion exchange chromatography (Sriveny *et al.* 2006).

Sera samples (321) consisting of 168 cattle (86 Jersey cross, 27 Hallikar and 55 non-descript) and 153 buffaloes (93 Murrah graded and 60 non-descript) were collected from animals during slaughter and also from suspected cattle and buffaloes reared in and around Chennai. Faecal samples were also collected from the same 321 animals separately. Coprological examination was done by sedimentation method to detect endoparasitic infection (Soulsby 1982).

Enzyme linked immuno sorbent assay: The test was carried out as per Knox *et al*. (2003). The optimum dilution of E/S antigen, serum and conjugate were determined using checker board titration. The 96 well flat bottomed ELISA plates were coated with 100 µl of total E/S antigen 1 in 100 dilution in carbonate-bicarbonate buffer (containing 5 µg/ml) and incubated at 4°C for overnight. The plates were washed 5 times with washing buffer and then blocked with 300 µl of blocking solution for 2 h at room temperature to prevent non-specific reaction. After blocking, plates were again washed 5 times with PBS-T and then the wells were added with 100 µl of 1 in 200 dilution of serum (in duplicates) obtained from test and control cattle/buffaloes incubated at 37°C for 2 h. After washing 5 times with PBS-T, 100 µl of 1 in 5000 dilution of anti-bovine IgG - HRP conjugate was added and incubated at 37°C for 2 h. Then, the plates were again washed 5 times with PBS-T. Wells were added with 100 µl of substrate solution and incubated at 37°C in dark room for 15 min to observe colour development. Then, the reaction was stopped by adding 100 µl of 0.1M sulphuric acid to each well and the colour reaction was read in ELISA at absorbance wavelength of 492 nm. Sera from cattle and buffaloes naturally infected with *Fasciola gigantica* showing optical density value of 0.322 and 0.378, respectively, were used as positive control sera in ELISA. Sera samples of a day-old healthy crossbred calf showing optical density value of around 0.092 was used as negative control. A total of 321 sera samples were tested for fasciolosis. The test sera samples showing the optical density value twice that of the known negative control sera were considered as positive for presence of *F. gigantica* antibodies in ELISA.

Statistical analysis of results was carried out using the Chi-square test and the significance of ELISA was assessed. The sensitivity and specificity of ELISA was calculated as description by Smith (1995) in comparison to the faecal examination method.

Excretory/ secretory antigen of Fasciola gigantica: Live adult *Fasciola gigantica* worms (60) were collected from slaughter houses for preparation of E/S antigen. E/S antigen was prepared by keeping the flukes in RPMI-1640 medium and incubated at 37°C for 24 h which is similar to Coles and Rubano *et al.* (1988). The protein concentration of total E/S antigen varied from 1.5 to 1.8 mg/ml. A total of 6.4 mg of E/S antigen was obtained from the 60 flukes. On the contrary, Vongpakorn *et al.* (2001) who has reported 3.57 mg/ml of protein concentration in E/S antigen while Dixit *et al.* (2003) reported 66.71 mg of total cysteine proteinase antigen from 580 adult flukes.

E/S antigen of *F. gigantica* purified by 75% alcoholic fractionation was having a protein concentration of 3.2 mg/ml and DEAE-Sephadex A-25 anion exchange chromatography purified fraction was having a protein concentration of 2.1 mg/ml, respectively. Purification of *F. gigantica* functional antigens by chromatography method is expected to remove host components, which if present are liable to cross react with the conjugate and elicit false results in ELISA. Chromatography methods could be used as very effective tools for isolation of candidate diagnostic molecule from the parasites (Willadsen *et al.*1989, Fagbemi *et al.*1997).

RESULT AND DISCUSSION

Faecal examination: Out of 321 animals, 19 (5.91%) were positive for eggs of *Fasciola* sp., 41 (12.77%) for amphistome sp., 38 (11.83%) for strongyle, 6 (1.86%) for *Strongyloides* sp. and 2 (0.62%) for mixed (both amphistome and strongyle eggs) infection. In cattle, 8 (4.76%), 16 (9.52%), 22 (13.09%), 3 (1.78%) and 1 (0.59%) for eggs of *Fasciola* sp., amphistome, strongyle, *Strongyloides* sp. and mixed (both amphistome and strongyle eggs) infection respectively. Similarly in buffaloes, 11 (7.18%), 25 (16.33%), 16 (10.45%), 3 (1.96%) and 1 (0.65%) were positive for *Fasciola* sp. egg, amphistome egg, strongyle egg, *Strongyloides* sp. egg and mixed (both amphistome and strongyle eggs) infection, respectively.

In the present study 5.91% showed the presence of *Fasciola* sp. eggs in faecal sample, which is much lower than the level of infection 11% by faecal examination and 19% by bile examination in buffaloes in Pantnagar, India as reported by Vinod Kumar *et al.* (2002).

Enzyme linked immuno sorbent assay: Out of 321 animals (168 cattle and 153 buffaloes) screened 67 (20.87%) sera showed positive result in ELISA, which includes 29 cattle (17.26%) and 38 buffaloes (24.83%) as compared to 8 (4.76%) and 11 (7.18%) were found positive in coprological examination of cattle and buffaloes, respectively. Statistical analysis showed significant differences in percent positivity between ELISA and faecal examination tests (Table 1).

Table 1. Comparison of ELISA and faecal examination for *Fasciola* infection

Animal species	No. of samples	Dung examination by sedimentation method		Sera positive in ELISA	χ^2 value
		Total +ve for helminth infection	Total +ve for *Fasciola* sp. egg		
Cattle	168	50 (29.76)	8 (4.76)	29 (17.26)	13.39**
Buffaloes	153	56 (36.60)	11(7.18)	38 (24.83)	17.71**
Total	321	106 (33.02)	19 (5.91)	67 (20.87)	

Figures in parentheses indicate percentage;Chi square value of 13.39** in cattle and 17.71** in buffaloes showed highly significant difference between ELISA and faecal examination.

Out of 118 cattle which showed negative result in faecal examination, 16 animals (55.17%) showed positive result in ELISA. Similarly in 97 buffaloes, 21 animals (55.26%) showed positive absorbance value in ELISA.

Cross reaction in ELISA test with other gastrointestinal parasites infected animal, viz. amphistomes (6.86% and 7.89%), strongyle (10.34%, and 5.26%) of cattle and buffaloes were detected. This may be due to previous exposure of animals or mixed infection with *Fasciola* infection. Ibarra *et al.* (1998) reported cross reactivity of *Paramphistomum* sp. infected cattle (1 serum out of 10) with *Fasciola* antigen in dot-ELISA.

Sensitivity and specificity of ELISA: All animals found positive for *Fasciola* sp. eggs by faecal examination were also positive in the ELISA. Cattle sera samples showed 100% sensitivity and 95.09% specificity in ELISA. Similarly, the sera samples from buffaloes also showed 100% sensitivity and 92.10% specificity in comparison to faecal examination technique.

The application of ELISA with E/S antigen allows the detection of specific antibodies in cattle and buffaloes. Molloy *et al.* (2005) reported 98.2% sensitivity on detecting antibodies to *F. hepatica* and *F. gigantica* in cattle and buffaloes in Australia using commercially available ELISA. On the contrary, Rapsch *et al.* (2006) reported diagnostic sensitivity of 91.7% for antibody ELISA to diagnose *F. hepatica* infection in cattle under field condition in Switzerland and Hillyer *et al.* (1996) reported 82% sensitivity in ELISA for detection of *F. hepatica* in cattle by using E/S antigen.

Specificity of ELISA was 95.09% in cattle which is similar to that of Sanchez-Andrade *et al.* (2000) who has reported 94.4% specificity for ELISA in cattle to diagnose *F. hepatica* infection and Molloy *et al.* (2005) also reported 98.3% specificity on detecting antibodies to *F. hepatica* and *F. gigantica* in cattle and buffaloes in Australia using commercially available ELISA.

Overall cattle and buffaloes showed 17.26% and 24.83% positive result in ELISA, respectively compared to 4.76% and 7.18% by coprological examination. This may be due to the fact that ELISA can detect circulating antibodies against the infection even in the pre-patent period, whereas the routinely used parasitological tests can detect the eggs only when they are passed out in the faeces of the animals. On the contrary, Hillyer *et al.* (1996) reported high prevalence of 57% in cattle by ELISA compared to faecal examination method for fluke eggs (26%).

It is concluded that ELISA was highly sensitive and specific in detecting higher percentage of positive animals than faecal examination and also high number of samples can be tested in short time. The findings together suggest that *Fasciola gigantica* total excretory/ secretory protein may be used as easily available, safe and inexpensive antigen for immunodiagnosis of fasciolosis. ELISA can be recommended for routine use in sero-epidemiological surveys of *Fasciola gigantica* infection. The ability to diagnose and treat infections early is great advantage of ELISA because it minimizes tissue damage caused by immature flukes in the liver. The only disadvantage in antibody tests is that they do not discriminate between active and past infections.

In our study, there was no attempt made to screen the cross reactivity between *Fasciola gigantica* and other helminths species affecting ruminants. In future, a study required to explore the possibility of cross reaction between the *Fasciola gigantica* and other helminths affecting cattle and buffaloes most appropriately using purified excretory/ secretory protein antigen based serological tests.

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