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ORIGINAL ARTICLE

Comparison between in-house indirect ELISA and Dot-ELISA for the diagnosis of *Fasciola gigantica* in cattle

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Abstract This survey was done to investigate the efficacy of the in-house indirect ELISA (iELISA) and Dot-ELISA methods Prepared from excretion–secretory (ES Ag) and Crude (Cr Ag) antigens of *Fasciola* for sero-diagnosis of *Fasciola gigantica* in cattle. The liver specimens of slaughtered cattle were collected and their liver examined macroscopically and microscopically for infestation to fasciolosis. Sera from two groups of cattle, one infected with fasciolosis (n = 60) and the other non-infected with fasciolosis (n = 60), were used in the iELISA and Dot-ELISA test; grouping based on histopathology results. Except specificity, other parameters such as, sensitivity, accuracy, positive and negative predictive values of both Dot-ELISA and iELISA done with ES Ag were better than those of tests performed with Cr Ag. Interestingly, the reliability of two methods was very good similar to one another.

Keywords Comparison · In-house Dot-ELISA · In-house i-ELISA · *Fasciola gigantica* · Cattle

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Introduction

Fasciolosis (liver flukes) is a parasitic diseases caused significant economic losses in domestic livestock, particularly cattle and sheep. On the other hand, it is one the important zoonotic disease in some area of the world (Moghadami and Mardani 2008). *Fasciola hepatica* and *Fasciola gigantica* are the most commonly parasites caused Fasciolosis (Dolton 1998). The pathogenicity of fasciolosis is based on expansion of lesions in the liver of host by migrating and feeding immature flukes as well as host immune responses to parasite secreted molecules and tissue damage alarm signals (Molina-Hernández et al. 2015). In general clinical signs appear three weeks post-infection. Unfortunately, conventional detection methods can only reveal the infestation between 10 and 14 weeks after infection when eggs can found in faeces of hosts (Conboy and Stromberg 1991). Therefore, designing a more accurate diagnostic assay may be valuable.

It seems that serological methods can been evaluated as an alternative assay for the diagnosis of Fasciolosis because of the relative simplicity and early sero-conversion (usually 1–2 weeks) (Sanchez-Andrade et al. 2000). Accordingly, designing the enzyme-linked immunosorbent assay (ELISA) and Dot-ELISA could be mentioned as a rapid and reliable immunoassay for screening of *Fasciola* in cattle herds. Former studies had been mostly focused on the serodiagnosis of *F. hepatica* infestation in ruminants (Hillyer and De Galanes 1988; Hillyer and de Galanes 1991; Zimmerman et al. 1985) while few documents had been found on serodiagnosis of *F. gigantica* infection in domestic livestock (Swarup et al. 1987; Fagbemi and Obarisiagbon 1991). Accordingly, Current survey was set out to investigate the efficacy of the in-house indirect ELISA (iELISA) and Dot-ELISA methods Prepared from

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excretion–secretory (ES Ag) and Crude (Cr Ag) antigens of *Fasciola* for sero-diagnosis of fasciolosis in cattle.

Materials and methods

Cattle slaughtered at Ahvaz slaughterhouses were bled and examined macroscopically for the presence of mature and immature *Fasciola* flukes in their livers, bile ducts, and gall bladder according to the method of Anderson et al. (1999). Each liver was placed in a large basin; all the flukes in the gall bladder and the major bile ducts were collected into a small glass. The liver was then sliced into strips of about 1 cm in thickness and soaked in normal saline for about 1 h. Flukes emerging from the cut bile ducts were put into the small glass and each sliced strip was thoroughly squeezed from end-to-end, washed in saline, and discarded. The contents of the basin were sieved; placed in a Petri dish; and the adult, immature, and cut pieces of flukes were added to the glass. Identification Of the live flukes was performed according to Andrews (1999). The number of *Fasciola* worms was counted as Anderson et al. (1999). Examined cattle were classified into *Fasciola*-infected and *Fasciola*-free groups. Blood samples were allowed to clot on the bench at room temperature at an inclined position for 2 h, centrifuged at 3000 rpm for 30 min, and serum samples were obtained and stored at −20 °C until tested. A total of 50 serum samples from *Fasciola*-infected and 60 serum samples from *Fasciola*-free cattle were collected.

Preparation of crude worm antigen

Fasciola crude worm antigen (Cr Ag) was prepared as described by Oldham and Willams (1985) with some modification. Briefly, adult *Fasciola* flukes were washed three times in phosphate-buffered saline (PBS; 0.01 mM, pH 7.4), drained and freeze- dried for 24 h at −70 °C. The dried flukes were ground into a fine powder and suspended in PBS, then homogenized in a high-speed mixer for 15 min and were stored overnight at 4 °C. After centrifugation at 3000 rpm for 15 min, the supernatant was filtered and sterilized by passing through 0.45 and 0.22 μm filters, aliquoted, and stored at −20 °C until assayed.

Preparation of *Fasciola* excretory/secretory antigens

Fasciola excretory/secretory antigen (ES Ag) was prepared according to Simsek et al. (2006). Briefly, adult *Fasciola* helminthes were washed several times in 0.01 mM PBS (pH 7.4). The specimens were incubated in PBS (5 flukes per 10 mL) at 37 °C and 5 % CO_2 for 6 h. PBS containing E/S products were centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant was filtered through filter with 0.22 μm in size. The products were dialyzed against distilled water for 24 h, aliquoted, and stored at −20 °C until analysed.

Measurement of protein concentration in prepared antigens

Protein concentration of each antigen (Cr and ES) was measured according to the method described by Lowry et al. (1951).

Dot-ELISA method

Dot-ELISA was conducted as described earlier, with some modification. Optimal serum, antigen, and bovine anti-IgG peroxidase conjugate (Abcam) concentrations were determined after preliminary checkerboard titration. Briefly, 1 μg of *Fasciola* ES and Cr antigen was dotted on nitrocellulose membrane discs and allowed to be dried thoroughly. The discs were placed into flat bottom micro plate wells. Nonspecific binding sites were blocked by addition of skimme milk (Merk, Darmstadt, Germany). Blocking solution was then aspirated off and antigen disks were washed by shaking (three times, 10 min each) with 0.05 % Tween 20 (Riedel-de-Haen AG, Seelze, Germany). Amount of 100 μl of 1:5 dilution of serum was added to each disk before incubation for 1 h at room temperature. After washing (see above) 100 μl of a 1:5000 dilution of anti-bovine IgG peroxidase conjugate was added to each disk and the plate was incubated for 1 h at room temperature. After washing, 100 μl substrate including tetramethyl benzidine and H_2O_2 (Serotech, Seoul, South Korea) was added into each well and incubated for 25 min at room temperature. The development of a deep brown color dot on disks when compared with negative serum control was considered to be evidence of positivity. In addition to *Fasciola*, two recent parasites are the main causes of liver parasite infestation in ruminant. Therefore, serum samples of positive infected cattle with dicrocoeliasis and hydatidosis were used for evaluation of cross reactivity.

Indirect ELISA method

The method used for the ELISA was similar to that described by Wijffels et al. (1994) and Zimmerman et al. (1982) with some modification. Optimal serum, antigen, and bovine anti- IgG peroxidase conjugate (Abcam) concentrations were determined after preliminary checkerboard titration Catty and Raykundalia (1989). Briefly,

ELISA plates (Nunc, Denmark) were coated with 100 µl of crude and ES antigens solution per well and incubated overnight at 4 °C. The wells were washed three times with PBS containing 0.05 % Tween and incubated with 200 µl of 5 % skimmed milk (Merk, Germany) in PBS for 1 h at room temperature, another three washes were undertaken as before and 100 µl of 1:10 dilution of serum was added to each well before incubation for 1 h at room temperature. After washing, as before, 100 µl of a 1:8000 dilution of antibovine IgG peroxidase conjugate was added to each well and the plate was incubated for 1 h at room temperature. Plate were washed three times and 100 µl substrate (tetra methyl benzidine + H_2O_2; Serotech, South Korea) was added into each well. After incubation for 15 min at room temperature, 100 µl of 2 N sulphuric acid (Merk, Germany) was added to stop the reaction and the optical density (OD) of the wells was measured at 405 nm in a spectrophotometer (Dynatech, Netherland). The cutoff point for the OD from the ELISA method was determined from the mean OD obtained from the negative sera of 60 uninfected bovine samples. All negative control sera were obtained from cattle, less than 2 months of age that had no eggs of *Fasciola* spp. detected in their faeces with two successive negative faecal examinations. Three standard deviations of this measurement were added to the mean to give a cutoff point for a positive test. In each ELISA plate, two negative control sera and two positive control sera were included. Negative control sera were obtained from 1 month old with two successive negative faecal examinations for *Fasciola* eggs. The positive control serum was a pool of sera obtained from slaughtered cattle with adult liver fluke in their livers. Three positive bovine blood samples of dicrocoeliasis and three positive bovine blood samples of hydatidosis for cross-reaction evaluation were tested on iELISA by Cr and ES antigens.

Results

Antigens (Cr and ES) were prepared from adult *F. gigantica* collected from the bile ducts of cattle. Total protein of Cr and ES antigens were determined as 900 and 85 g/dl, respectively. The results of both examination of livers for the presence or absence of liver fluke and the corresponding results from the i-ELISA and Dot-ELISA by Cr and ES antigens were obtained and presented in Tables 1 and 2.

The diagnostic sensitivity, specificity, precision positive predictive value and negative predictive value percentages of Dot-ELISA using Cr and ES antigens for diagnosis of *Fasciola* infection in cattle were calculated and recorded in Tables 3 and 4.

Table 1 Results of examination of cattle livers for liver flukes and i-ELISA on their sera using Cr and ES antigens

Antigen type	Result	Infected	Non-infected
Cr Ag	Positive	54	15
Negative	6	45	
ES Ag positive	53	12	
Negative	7	48	

Table 2 The diagnostic sensitivity, specificity, precision, positive predictive value and negative predictive value percentages of i-ELISA using Cr and ES antigens for diagnosis of *F. gigantica* infection in cattle

Antigen type (%)	Cr Ag (%)	Es Ag (%)
Sensitivity	90	88.33
Specificity	75	80
Precision	82.5	84.16
Positive predictive value	78.26	81.53
Negative predictive value	88.23	87.27

Table 3 Results of examination of cattle livers for liver flukes and Dot-ELISA on their sera using Cr and ES antigens

Antigen type	Result	Infected	Non-infected
Cr Ag	Positive	54	9
Negative	6	51	
ES Ag positive	56	7	
Negative	4	53	

Table 4 The diagnostic sensitivity, specificity, precision, positive predictive value and negative predictive value percentages of Dot-ELISA using Cr and ES antigens for diagnosis of *F. gigantica* infection in cattle

Antigen type (%)	Cr Ag (%)	Es Ag (%)
Sensitivity	90	93.33
Specificity	85	88.33
Precision	87.5	90.83
Positive predictive value	85.71	88.88
Negative predictive value	89.47	92.98

Based on our data, Cross-reactions were not detected in iELISA by ES antigen but in 1:3600 dilution of Cr antigen detected with dicrocoeliasis and hydatidosis. Moreover, cross-reactions were not detected in Dot-ELISA by ES antigen but in 2, 3, 4 and 5 µg of dilution Cr antigen detected with dicrocoeliasis and hydatidosis.

Discussion

Serological techniques have been evaluated for the diagnosis of fasciolosis (Dalimi et al. 2004). ELISA-based techniques or variant like Dot-ELISA are considered as rapid and reliable immunoassay with easy procedure for detection of antibody or antigen (Hillyer and De Galanes 1988). For these reasons, the vast majority of researchers nowadays have applied an ELISA-based assay or variant in diagnosis of different parasitological diseases such as fasciolosis, toxoplasmosis, schistosomiasis, hydatidosis and cysticercosis (Hassan et al. 2002). The level of antibody can be detected by FAST-ELISA during 1–2 weeks after infestation of cattle with *Fasciola* (Dolton 1998). Serodiagnosis of *F. gigantica* infection in cattle as described by (Awad et al. 2009; Charlier et al. 2008; Hillyer et al. 1996; Ibarra et al. 1998; Molloy et al. 2005; Mousa 1994; Reichel 2002; Santiago and Hillyer 1988; Zimmerman et al. 1985). In present survey, two *F. gigantica* antigens (Cr and ES) were applied to evaluate the diagnostic sensitivity, specificity, precision, positive predictive value, and negative predictive value of iELISA and Dot-ELISA for the diagnosis of *F. gigantica* infection in cattle. Results of iELISA showed that Higher sensitivity for the diagnosis of bovine fasciolosis was detected by Cr Ag (90 %) compared with ES Ag (88.33 %). High specificity (80 %) was recorded when ES Ag was used in iELISA for diagnosis of *F. gigantica* infection in cattle compared with 75 % using Cr Ag. The high sensitivity (90 %) and high specificity (80 %) of iELISA using ES Ag for the diagnosis of *F. gigantica* infection in cattle supports the use of this antigen in the Higher precision (84.16 %) was obtained when using iELISA with ES Ag for diagnosis of bovine fasciolosis compared with using Cr Ag for diagnosis (82.5 %). Using ES Ag in iELISA gives high-accuracy rates. Dot-ELISA isone of the valuable and simple methods in diagnosis of different infectious illness (Hassan et al. 2002). This technique can be simply employed in the clinic or fields and gives useful data in a short time. However, ELISA is a very sensitive test, but this assay is a time consuming method and needs some expensive instruments and reagents. Based on our results, Higher sensitivity for the diagnosis of *F. gigantica* was recorded using ES Ag (93.33 %) compared with Cr Ag. (90 %) However, high specificity (88.33 %) was determined when ES Ag was used in Dot-ELISA for diagnosis of *Fasciola* infection compared with 85.00 % using Cr antigens. Higher precision (90.83 %) was obtained when using Dot-ELISA with ES Ag for diagnosis of bovine fasciolosis compared with using Cr Ag (87.50 %). Using ES Ag in Dot-ELISA gives high-accuracy rates. Recently, native cathepsin-L cysteine proteinase was purified from the excretory secretory products of *Fasciola* and used for sero-diagnosis of *Fasciola* infection in buffaloes by Dot -ELISA. The results demonstrated that cathepsin-L cysteine proteinase based Dot-ELISA achieved 90.00 % sensitivity and 100 % specificity (Varghese et al. 2012). Our data indicated when ES antigens of *F. gigantica* used for i-ELISA or Dot-ELISA designing, cross-reaction was not detected. Nevertheless, three positive bovine blood samples of dicrocoeliasis and three positive bovine blood samples of hydatidosis may be detected when i-ELISA or Dot-ELISA was performed by crude antigens of *F. gigantica.* We propose that because Cr antigens possess more numerous antigenic components compared to ES antigen, using the Cr antigens for designing of ELISA produce more sensitive and less specific assay compared with using ES antigen. The false negative results reported in this study may be due to modulation of the host immune response by liver flukes as reported by Anderson et al. (1999). In conclusion, these data suggest that ES antigen based Dot-ELISA or iELISA can be used as a reliable and with the same potential for sero-diagnosis of *Fasciola* infection in cattle. Of note, in-house Dot-ELISA is a very rapid and simple test with remarkable accuracy compared to in-house iELISA.

Conclusion

ES antigen based Dot-ELISA or iELISA can be used as a reliable and with the same potential for sero-diagnosis of *Fasciola* infection in cattle. Of note, in-house Dot-ELISA is a very rapid and simple test compared to in-house iELISA.

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