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Araştırma Makalesi ***Research Article***

Comparison of Dot-ELISA and ELISA Techniques for Detection of *Fasciola hepatica* in Sheep Using Excretory-Secretory Antigens

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Abstract

Fascioliasis is one of the most important zoonosis with worldwide distribution, which causes great losses in livestock production. Serological methods are used for early detection of disease. The current investigation undertook to challenge dot-ELISA and ELISA techniques in diagnosing of *Fasciola hepatica* in sheep using excretory-secretory (E/S) antigen to select the most appropriate method. The serum samples (n=281) were collected from 50 cases of fascioliasis, 81 animals with parasitic diseases other than fascioliasis, and 150 healthy control cases. The E/S antigen used was obtained from infected livers (*F. hepatica*) of sheep slaughtered in Hamedan industrial abattoir (Iran). The result of dot-ELISA showed 100% sensitivity and 92.2% specificity. Also, positive and negative predictive values, and accuracy of assay were 73.52%, 100% and 93.59%, respectively. In the case of ELISA, the results were as follows: 98% sensitivity, 100% specificity, 100% positive predictive values, 99.56% negative predictive values, and 99.64% accuracy of assay. In both techniques, cross reaction with fascioliasis was not observed. In conclusion, although these two tests had very similar results, dot-ELISA was more acceptable with respect to its higher sensitivity, simplicity in practice and cheaper than ELISA. Therefore, dot-ELISA can be recommended as a routine test in paraclinical laboratories and epidemiological studies.

Özet

Koyunlarda Fasciola hepatica Enfeksiyonunun Tespitinde Ekskresyon / Sekresyon Antijenleri Kullanılarak Dot-ELISA ve ELISA Tekniklerinin Karşılaştırılması

Fasiolazis, dünya çapında hayvancılıkta büyük kayıplara neden olan önemli zoonozlardan biridir. Hastalığın erken tanısı için serolojik metotlar kullanılmaktadır. Bu araştırmada, koyunlarda *Fasciola hepatica* enfeksiyonlarının ekskresyon/sekresyon antijenleri kullanılarak tanısında Dot-ELISA ve ELISA tekniklerinden hangisinin en uygun olduğu karşılaştırılmıştır. Serum numuneleri (n=281) 50 adet fascioliasis olgusundan, 81 adet fasiolasis dışındaki paraziter hastalık olgusundan ve 150 adet sağlıklı kontrol olgularından toplanmıştır. Kullanılan E/S antijeni, Hamedan mezbahasında (İran) kesilen enfekte koyun karaciğerinden (*F. hepatica*) temin edilmiştir. Dot ELISA sonuçları %100 duyarlılık ve %92,2 belirleyicilik göstermiştir. Ayrıca, pozitif, negatif tahmini değerler ve testin doğruluğu sırasıyla %73,52, %100, %93,59 bulunmuştur. ELISA metodunda sonuçlar, %98 duyarlılık, %100 belirleyicilik, %100 pozitif tahmini sonuç, %99,56 negatif tahmini sonuç ve %99,64 testin doğruluğu olarak belirlenmiştir. Her iki teknikte de fasiyolazis ile çapraz reaksiyon gözlenmemiştir. Sonuç olarak bu iki testin benzer sonuçları olmasına rağmen, Dot ELISA metodu, ELISA metoduna göre daha yüksek duyarlılığa sahip olması, uygulamadaki basitliği ve ucuz olması açısından daha kabul edilebilirdir. Bu nedenle Dot-ELISA, paraklinik laboratuvarlarında ve epidemiyolojik çalışmalarda rutin bir test olarak tavsiye edilebilir.

Introduction

Fascioliasis is an economically important disease of domestic livestock, in particular sheep and cattle, and occasionally man. The disease is caused by digenean trematodes of the genus *Fasciola*, commonly referred to as liver flukes (Soulsby, 1982).

The two species most commonly implicated as the aetiological agents of fascioliasis are *F. hepatica* and *F. gigantica* (Fasciolidae). *F. hepatica* has a worldwide distribution but predominates in temperate zones while *F. gigantica* is found on most continents, primarily in tropical regions (Kooshan et al., 2010). The disease is transmitted to animals and human by eating contaminated plants with infective metacercaria, which are derived from an intermediate molluscan host (Rokni et al., 2006).

The infections in animals are detected by microscopic observation of *F. hepatica* eggs in the faeces. However, early diagnosis by coprological examination is not possible because eggs are not found in the faeces until 10-12 after infection (Kooshan et al., 2010; Noureldin et al., 2004).

Early diagnosis and management of fascioliasis is important before irreparable damage of the liver occurs (Rokni et al., 2004). For these reasons, serology assay is the most dependable diagnostic method. Many serological techniques, therefor, have been challenged to diagnose human and animals fascioliasis, most of them vary in specificity and sensitivity due to differences in materials and methods, and of course, may owe to differences in the nature of the parasite, being utilized to prepare antigen. Enzyme linked immune sorbent assay (ELISA), dot-ELISA, indirect fluorescence antibody test (IFAT), haemaglutination (HA), immuneperoxydase (IP) and counter-electrophoresis (CEP) can be mentioned for detection (Hillyer et al., 1985; Pfister, 1990).

The current investigation undertook to challenge dot-ELISA and ELISA techniques in diagnosing of *F. hepatica* in sheep using excretory-secretory (E/S) antigens to select the most appropriate method.

Materials and Methods

Serum samples: Three groups including sheep infected with *F. hepatica* (n=50), sheep with some other parasitic diseases (n=81) and healthy control sheep (n=150), were implicated in the test. Non-fascioliasis serum cases were infected with hydatidosis (n=61) and dicroceliosis (n=20) which were acquired from Hamedan industrial abattoir using stool examination and inspection of organs. Fascioliasis cases were diagnosed based on gross inspection of livers after slaughter in abattoir and stool examination. All sera were removed after centrifugation at 1500×g for 10 min and stored at -20°C until assayed.

Preparation of E/S antigen: The E/S antigen was prepared from a spent culture medium (RPMI 1640, pH7.3) containing 0.1 mM tosylamide 2-phenylethyl-chloromethyl ketone, 1 mM of L-trans-3-carboxyoxiran-2-carbonyl-L- leucylagmatine, 30 mM hepes, 0.53 g/l N-acetyl-L-glutamina alanine, 7 ml/l sodium bicarbonate 7.5% and 25 mg/ml gentamaycine.

The worms had been maintained in RPMI solution for 24 hours at 37°C (2.5 ml for each mature worm). For checking aliveness of flukes, their motion was observed by stereomicroscope every 12 hours. After incubation, the worms were removed and the collected spent medium was clarified by centrifugation at 10,000×g for 30 min at 4°C (Kooshan et al., 2010; Rokni et al., 2006).

The concentration of each antigen was measured using Bradford method (Bradford, 1976). All antigens were preserved at -20 °C until used (Rokni et al., 2006).

Dot-ELISA: Dot-ELISA was conducted as described earlier (Diab et al., 2011; Rokni et al., 2006). Briefly, 1.5 µg of *Fasciola* E/S antigen was dotted on nitrocellulose membrane discs and allowed to be dried thoroughly. The discs were placed into flat bottom micro plate wells. Non-specific binding sites were blocked by addition 100 µl of tris buffer solution containing 0.5% Tween 20 (TBS/T) to each well. Blocking solution was then aspirated off and antigen disks were washed by shaking (three times, 10 min each) with 0.05% Tween 20 in TBS (vol/ vol).

100 µl of serum samples diluted 1:320 in TBS/T (after checkboard for serum dilution) was added to each disk and incubated for 45 min at room temperature (RT). The serum samples were removed and washings were conducted as described above. The washing solution was removed and 100 µl of anti-sheep IgG conjugate diluted in TBS/T (1:5000, Institute Razi of Iran) was added to each well and incubated for 45 min at RT. The optimum dilution of the conjugate was found to be 1:500 by block titration of two-fold dilutions of the conjugate. The conjugate was removed and other washings were conducted as mentioned before. 100 µl of the substrate chromogen diamino 3, 3’ benzidin tetrahydrocholoride (Sigma) was added to each well and incubated for 30 min at RT. The development of a deep brown colour dot on disks when compared with negative serum control was considered to be evidence of positivity. Colour development in controls was negligible or completely absent.

ELISA: This method was performed in 96-well immunolon essentially as described earlier (Kooshan et al., 2010). In brief, the E/S antigen was diluted 0.125, 0.25, 0.5 and 1 µl/ml with PBS (pH 7.5). Polystyrene micro titer plates were coated with 100 µl of diluted antigens per well. After incubation for 1 hour at RT, plate was sensitized overnight at 4°C. Then, the wells were washes 3 times for 5 min, with 300 µl PBS- 0.05% Tween 20. 200 µl of BSA 1% in PBS-T was added to each well as blocking and incubated for 1 hour at RT.

Serum samples were diluted at 1:10, 1:20, 1:50, 1:100 and 1:500 with PBS-T. 100 µl of diluted serum was added to each well an incubated at RT for 30 min. After a further washing, 100 µl anti-sheep IgG peroxidase conjugate diluted at 1:1000 in PBS-T was added to each well. The plates were incubated at RT for 30 min and then washed as previously described. Then 100 µl of substrate solution containing TMB/H_2O_2 was added to each well and the plate was incubated for 15 min in darkness at RT. The reaction was stopped with 100 µl of 1M sulfuric acid.

The absorbance was measured at 450 nm using a micro plate ELISA reader. In the plate, 2 wells were considered for each of the blank, negative and positive controls, separately.

Statistical analysis: Standard diagnostic indices including sensitivity, specificity, positive and negative predictive values and accuracy were calculated as previously described (Galen, 1980).

Results

Table 1 shows all fascioliasis cases (1st group) are positive by dot-ELISA (P<0.0001). However, 98% (49/50) of cases were positive by ELISA (P<0.0001).

The result of dot-ELISA showed 100% sensitivity, 92.2% specificity, 73.52% positive predictive values (PPV), 100% negative predictive values (NPV) and 93.59% accuracy.

In the case of ELISA, the results were as follows: 98% sensitivity, 100% specificity, 100% PPV, 99.56% NPV, and 99.64% accuracy. In the case of the 2nd group (non-fascioliasis animals, which were expected negative for fascioliasis), we did not detect false positive for both techniques. In healthy group, 18 sample of 132 was reported false positive (Table 1).

Table 1. Comparison of sensitivity, specificity, accuracy, positive and negative predictive values of dot-ELISA and ELISA in sero-diagnosis of *F. hepatica* in sheep.

Tablo 1. Koyunlarda *F. hepatica* Enfeksiyonunun serolojik tanısında dot-ELISA ve ELISA metotlarının duyarlılık, belirleyicilik, doğruluk, pozitif ve negatif tanımlama oranlarının karşılaştırılması.

Type of Technique	Fascioliasis Animals (n=50)		Non-Fascioliasis Animals (n=81)		Healthy Animals (n=150)		Sen (%)	Spe (%)	PPV (%)	NPV (%)	ACC (%)
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.					
Dot-ELISA	50	0	0	81	18	132	100	92.2	73.52	100	93.59
ELISA	49	1	0	81	0	150	98	100	100	99.56	99.64

Sen: sensitivity; Spe: specificity; PPV: positive predictive value; NPV: negative predictive value; ACC: Accuracy of test

Discussion

A serological detection of fascioliasis was developed in 1976 and then, ELISA method was used to detect experimental ovine fascioliasis in 1982 (Van-Tiggele and Over, 1976; Zimmerman et al., 1982).

Arriaga et al. (1989) and Zimmerman et al. (1982) have separately introduced a dot-ELISA against E/S antigens to detection of *F. hepatica* infections. Santiago and Hillyer (1988) were able to detect ELISA antibodies to E/S and somatic antigens. Yamasaki et al. (1989) used ELISA to detection of fascioliasis using E/S antigens as sensitive and specific assay in 1989. They used E/S antigen to diagnosis of *F. hepatica* similar to other studies (Rabia et al., 2010; Rokni et al., 2004, 2006; Yamasaki et al., 1989).

Rodriguez and Hillyer (1995) described that E/S antigens were more specific than somatic and surface antigens for sero-diagnosis of fascioliasis in sheep. A recent study Rokni et al. (2004) compared the diagnostic potential of somatic and E/S antigens using enzyme linked immunotransfer blot (EITB) technique. The sensitivity and specificity values for somatic antigens were 91.0% and 96.2%, and for E/S antigen were 95.2% and 98.0%, respectively. On the other report, sensitivity and specificity of somatic antigen was reported 100% and 93%, respectively (Shaker et al., 1994).

In this work, sensitivity and specificity of dot-ELISA (100%, 92.2%) and ELISA (98%, 100%) is calculated in naturally infected sheep, separately (Table 1).

Hassan et al. (2002) performed dot-ELISA using anti-*Fasciola* IgG isotypes. They reported that IgG2 and IgG4 demonstrated the highest specificity (>99%), followed by IgG1 (90%) and the least specific test was obtained with detection of IgG (85%).

Shaheen et al. (1989) compared dot-ELISA with micro-ELISA to detect fascioliasis in human. They found that dot-ELISA results completely agreed with those of micro-ELISA.

Diab et al. (2011) reported the sensitivity and specificity of dot-ELISA 98.9% and 98.3%, respectively. In fact, all previous studies used dot-ELISA for screening anti-*Fasciola* antibodies in both ruminant and human. In this manner, Intapan et al. (2003) used *F. gigantica* 27 kDa (FG 27) as a target antigen in dot-ELISA and found that the sensitivity, specificity and accuracy were 100%, 97.4% and 98.2%, respectively.

Dalimi et al. (2004) used *F. gigantica* partially purified antigen in dot-ELISA and reported 94.2% and 99.4% sensitivity and specificity, respectively. Also, Rokni et al. (2006) was found 96.8% sensitivity and 96.1% specificity in dot-ELISA using E/S antigen.

The difference in sensitivities and specificities of reports may be due to various target antigens and protocols of antigen purification (Diab et al., 2011).

In current investigation, there was no cross-reaction between non-fascioliasis group and other animals (Table 1). In similar study, Yamasaki et al. (1989) reported only a single case of cross-reactivity with sera from *Schistosoma japonicum* infected. In research from Thailand, 25.9% of sera with cholangiocarcinoma indicated cross-reactivity with *Fasciola* antigen (Maleewong et al., 1999). Rabia et al. (2010) reported, cross reactivity with sera of two sheep with Schistosomiasis, a hydatid cyst, a Trichostrongyloidiasis and a hook worm infection.

In sero-epidemiological study of sheep from Ethiopia, the superiority and practicability of ELISA were indicated successfully (Njau et al., 1989). Diab et al. (2011) described the dot-ELISA has advantage over ELISA due to nitrocellulose papers spotted with antigen which are stable for at least three months at -20°C, also all incubation steps are performed at RT, applicable to diagnosis in the field and simpler than other tests.

In conclusion, dot-ELISA is a cheaper, easier, more rapid, sensitive and specific assay than ELISA to detect fascioliasis. Its ability to implement in field trials, little use of antigen and antibody, require no special equipment, validity in screening tryouts and a lot of other benefits make it as an authentic and applicable test in early diagnosis of ovine fascioliasis. Therefore, dot-ELISA can be recommended as a routine test in paraclinical laboratories and epidemiological studies.

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