Journal of Microbiology, Immunology and Infection (2015) xx, 1–5

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

ORIGINAL ARTICLE

Serologic detection of antibodies against *Fasciola hepatica* in sheep in the middle Black Sea region of Turkey

Mustafa Acici [a,*], Ozlem Buyuktanir [b], Cenk Soner Bolukbas [a], Gokmen Zafer Pekmezci [c], Ali Tumay Gurler [a], Sinasi Umur [a]

[a] *Department of Parasitology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey*
[b] *Department of Microbiology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey*
[c] *Department of Preclinical Sciences, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey*

Received 23 January 2015; received in revised form 21 May 2015; accepted 30 June 2015

Available online ■ ■ ■

KEYWORDS
ELISA;
fasciolosis;
sheep;
Turkey

Abstract *Background/Purpose:* The aim of the present study was to estimate the prevalence of *Fasciola hepatica* infection in sheep in the Black Sea region of Turkey.
Methods: Samples from 213 sheep were collected randomly in Samsun, Tokat, and Sinop from September 2005 to January 2007 and tested by indirect enzyme-linked immunosorbent assay (ELISA) and Western blot analysis using *F. hepatica* excretory-secretory (E/S) antigens.
Results: The distribution of ELISA-positive samples for *F. hepatica* infections out of a total of 213 sheep serum samples was 23/71 (32.4%), 15/59 (25.4%), and 29/83 (34.9%) in Samsun, Sinop, and Tokat, respectively. The immunodominant proteins were determined by Western blot analysis using molecular weight markers of 14 kDa, 20 kDa, 24 kDa, 27 kDa, 33 kDa, 45 kDa, and 66 kDa and extracted from sera of sheep that were positive for *Fasciola* spp. eggs and also hyperimmune sera from rabbits immunized with E/S antigens.
Conclusion: The ELISA-positive results were confirmed by Western blot analysis. As a result, seroprevalence of *F. hepatica* infection was found in 31.4% of sheep from the Karayaka breed in the Middle Black sea region of Turkey.

* Corresponding author. Department of Parasitology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, 55139 Samsun, Turkey.
E-mail address: acicim@omu.edu.tr (M. Acici).

http://dx.doi.org/10.1016/j.jmii.2015.06.005

Introduction

Fasciolosis is an economically important disease in domestic livestock, particularly cattle and sheep, and occasionally in humans. The disease is caused by digenean trematodes of the genus *Fasciola*, commonly referred to as liver flukes.[1] *Fasciola hepatica* has a worldwide distribution, but predominates in temperate zones, while *Fasciola gigantica* is found primarily in tropical regions.[1,2] Additionally, *F. hepatica* infection in sheep is prevalent in many parts of Turkey.[3–5] In a previous study, prevalence of *F. hepatica* was 3.99% in sheep and ranged from 0.48% to 2.65% in cattle in the Trakya region of Turkey.[6] In another study based on fecal examination in Samsun, *Fasciola* spp. was found in 20.99% of sheep.[7] The assays based on antibody detection are overwhelmingly the preferred method for immune diagnosis of fasciolosis. The reasons include the relative simplicity of the assays and early seroconversion during primary infections. Consequently, most investigators today use enzyme-linked immunosorbent assay (ELISA) and immunoblots for the immune diagnosis of fasciolosis. Antigenic preparations used have been primarily derived from adult worm extracts, excretion–secretion products of adult worms, or the partially purified fractions.[8–11] Although *F. hepatica* is endemic in many rural areas of Turkey, there have been few serodiagnostic studies by ELISA and Western blotting undertaken in sheep far.[3–6,12,13]

The aim of the present study was to estimate the prevalence of fasciolosis in sheep by indirect ELISA and Western blot tests using *F. hepatica* excretory–secretory (E/S) antigens in the Middle Black sea region, Turkey.

Methods

Sera and feces

Serum samples from 213 Karayaka sheep randomly collected in Samsun, Sinop, and Tokat from September 2005 to January 2007 were tested for antibodies against *F. hepatica*.

Initially, fecal, serum samples, and liver flukes were collected from sheep ($n = 40$) at the local slaughterhouse in Samsun. All of the animals were not given anthelmintic, were $\geq$1 year old, and had grazed in the pasture for at least spring, summer and autumn. The sera were stored at −20°C until use and *Fasciola* spp. eggs per gram (EPG $\geq$ 50) of feces were counted by a standard sedimentation method using McMaster chambers.[14]

Preparation of E/S antigens

F. hepatica E/S antigens were obtained from adult *F. hepatica* as described by Zimmerman et al[15] Briefly, viable *F. hepatica* adult flukes collected from the bile duct of sheep were washed several times in 0.15M NaCl (pH 7.2), then incubated at 37°C (1 fluke/mL) for 17 hours in phosphate-buffered saline [PBS (pH 7.2)] with penicillin (100 IU/mL) and streptomycin (100 mg/mL). After the incubation, the supernatant was collected and centrifuged at 5000*g* for 30 minutes at 4°C. The supernatant fluids were filtered and then dialysed against distilled water. The total protein concentration of the antigen was measured as described by Bradford.[16]

Production of hyperimmune serum

To obtain anti-*F. hepatica* E/S antibodies, two New Zealand rabbits (2 kg) were immunized with *F. hepatica* E/S antigens according to Almazan et al.[17] On day zero, 1 mL (0.8 mg/mL) *F. hepatica* E/S antigen emulsified with an equal quantity of Freund's complete adjuvant (Sigma-Aldrich, USA) was administered subcutaneously. Five additional immunizations were given in equal doses of the antigen in Freund's incomplete adjuvant (Sigma-Aldrich, USA) at 15-day intervals subcutaneously. In the 10th week, the rabbits had an antibody titer >100,000 as determined by Dot blot assay. The hyperimmune rabbit sera were used for confirmation of the immunogenicity of *F. hepatica* E/S antigens and also for optimization of the ELISA and Western blot tests along with the infected and negative sheep sera. Preimmune sera were used as a negative control.

Indirect ELISA

ELISA was performed on microtiter plates as described by Guobadia and Fagbemi[18] and Ferre et al[19] with some modifications. Briefly, polystyrene microtiter plates (Nunc Maxisorp, Thermo Fisher, Denmark) were coated with 100 μL of the E/S antigen (5 μg/mL) diluted in coating buffer (pH 9.6) per well, and incubated overnight at 4°C. The plates washed with PBS + 0.1% Tween-20 (PBST) were blocked with 200 μL 1% non-fat dry milk in PBST and incubated for 1 hour at 37°C. Subsequently, 100 μL rabbit and sheep sera (1:1000 and 1:100, respectively) diluted in 1% non-fat dry milk were added and incubated for 1 hour at 37°C.

After 100 μL of anti-sheep and anti-rabbit immunoglobulin IgG alkaline-phosphatase conjugate (1:10,000 and 1:15,000, respectively) were added, the plates were incubated for 1 hour at 37°C.

The plates were washed with PBST between each step. The paranitrophenyl phosphate substrate (1 mg/mL) was added and the optical density measured at 405 nm using an ELISA reader.[20]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were carried out according to Laemmli[21] and Towbin et al[22] Briefly, *F. hepatica* E/S antigen was separated by 12% SDS-PAGE and then transferred to nitrocellulose (NC) membrane (0.2 μm pore size, Sigma-Aldrich) using a semi-dry system with a 25mM Tris and 192mM glycine buffer. The NC membranes were cut into strips and washed with PBST (0.05%) between each step.

The NC strips were blocked with 1% non-fat dry milk in PBST (also used as dilution buffer) incubated overnight at 4°C, followed by incubation with 1:1000 rabbit and 1:100 sheep serum sample dilutions for 1 hour at 37°C. The strips

Please cite this article in press as: Acici M, et al., Serologic detection of antibodies against *Fasciola hepatica* in sheep in the middle Black Sea region of Turkey, Journal of Microbiology, Immunology and Infection (2015), http://dx.doi.org/10.1016/j.jmii.2015.06.005

were incubated for 1 hour at 37°C with anti-sheep and anti-rabbit IgG alkaline-phosphatase conjugates diluted 1:10,000 and 1:15,000, respectively. Finally, reaction development was evaluated by adding substrate buffer containing nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich, USA).

Statistical analysis

Statistical analysis was performed by Chi-square test and receiver operating characteristic (ROC) analysis using SPSS version 20.0 (IBM SPSS Statistics, Armonk, NY, USA, commuter license).[23–25]

Results

Initially, serodiagnosis of *F. hepatica* infection based on IgG antibody detection by ELISA was optimized using 40 fecal samples from grazing flocks in three different Samsun provinces and then confirmed by Western blot analysis. Immunized and unimmunized rabbit sera were used as the positive and negative controls, respectively. Eight of 40 (20%) fecal samples from the grazing flocks in three different Samsun provinces in Samsun were observed as positive for *Fasciola* spp. eggs by sedimentation and McMaster methods (EPG $\geq$ 50). These *Fasciola* spp. infections confirmed the significant increase in total IgG levels observed by ELISA and supported by Western blot analysis.

Protein bands with molecular weights of 14 kDa, 20 kDa, 24 kDa, 27 kDa, 33 kDa, 45 kDa, and 66 kDa were revealed from *F. hepatica* E/S antigens in the sera from both coprologically and serologically positive sheep by Western blot analysis. Seven specific bands at 20 kDa, 24 kDa, 27 kDa, 33 kDa, 39 kDa, 42 kDa, and 66 kDa were also observed from the sera from rabbits immunized with *F. hepatica* E/S antigens. No bands were detected in any of the coprologically and serologically negative sheep using the pre-immunized rabbit sera (Figure 1).

In this study, a total of 213 sheep serum samples were analyzed by ELISA for anti-E/S antigen of *F. hepatica* and 67/213 (31.4%) sera were found positive for anti-*F. hepatica* antibodies in the central Black Sea region of Turkey. The anti-*F. hepatica* antibody prevalence in sheep was higher in Samsun and Tokat relative to Sinop Province (Table 1). However, there was no significant difference between any of the provinces for ovine fasciolosis ($p > 0.05$). The cut-off value used for indirect ELISA was determined to be 0.400 using ROC analysis at OD405. Sensitivity and specificity were determined at 94% and 76%, respectively, with a 95% confidence interval using ROC analysis for the ELISA.

Discussion

F. hepatica has a widespread distribution in South America, Africa, Eastern Europe, and the Middle East, as well as South and Eastern Asia.[26–28] Surveys based on fecal and slaughterhouse examinations in some Turkish counties demonstrated that among domestic animals, sheep were found to suffer more frequently from fasciolosis.[4,5,29,30] In a previous study,[6] 19/476 (3.99%) $\geq$ 1 year old sheep were infected with

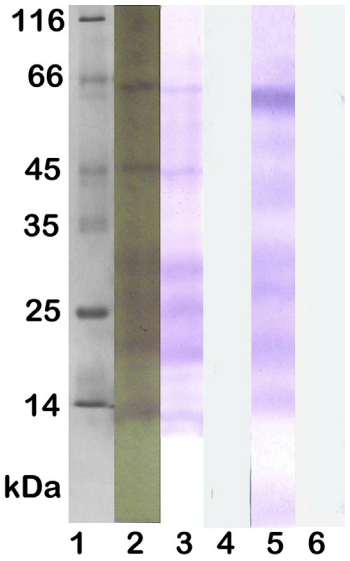

Figure 1. Western blot of *Fasciola hepatica* E/S antigen to detect antibodies against *F. hepatica* in sera collected from rabbit with immunized *F. hepatica* E/S antigens and sheep. Molecular weight marker (lane 1); pool of sera from naturally infected seropositive sheep (lanes 2 and 3); negative control for sheep (lane 4); immunized rabbit (lane 5); negative control for rabbit (lane 6).

F. hepatica in the slaughterhouses in Trakya. In the Black Sea region, fasciolosis was found in 20.99% of sheep by coprologic examination.[7] In this study, serodiagnosis of fasciolosis by ELISA demonstrated levels as high as 31.4% in the middle Black Sea region, with all seropositive and seronegative animals confirmed using Western blot analysis.

Immunodiagnosis of *F. hepatica* infections has been studied in cattle rather than sheep from 2007 to 2015 in Turkey. The following studies have been carried out during this period. For adult cattle, the prevalence of *F. hepatica* infections was found at 3.03% in Nevşehir using copro-ELISA, 60.5% using indirect ELISA in Elazığ, and 21% by slaughter examination in Erzurum.[31–33] In one study, *F. hepatica* infection was observed in 5.97% of sheep in Sivas using copro-ELISA.[13] Additionally, an abattoir survey showed that the main causes of organ condemnation were fasciolosis in sheep and cattle in Bursa, Turkey.[34]

The prevalence of *F. hepatica* infections in sheep was found at 23/71 (32.4%), 15/59 (25.4%), and 29/83 (34.9%) in Samsun, Sinop, and Tokat, respectively. Our findings are comparable with others describing high prevalence of infection observed

Table 1 Detection of *Fasciola hepatica* antibodies of infected sheep by ELISA in the middle Black Sea region of Turkey.

Counties	Examined No.	Positive No.	Prevalence (%)	$\chi^2_{\upsilon = 2}$ (p)
Samsun	71	23	32.4	1.492 (0.474)
Sinop	59	15	25.4	
Tokat	83	29	34.9	
Total	213	67	31.4	

Please cite this article in press as: Acici M, et al., Serologic detection of antibodies against *Fasciola hepatica* in sheep in the middle Black Sea region of Turkey, Journal of Microbiology, Immunology and Infection (2015), http://dx.doi.org/10.1016/j.jmii.2015.06.005

in different regions of Turkey, indicating epidemiological importance of the disease in ruminants in Turkey.

The high frequency of infection may be due to the serodiagnostic technique used, the size of the sample, and the location of the animals, given the greater annual rainfall associated with high soil moisture and wetlands in the middle Black Sea region. A similar study was performed by Gonenc et al[12] showing that *Fasciola* spp. eggs were observed by coprological examination in all seropositive sheep by Western blot analysis in Central Anatolia. In the present study, anti-*F. hepatica* antibody prevalence revealed by ELISA in sheep was higher in the cities of Samsun and Tokat relative to Sinop, however, with no statistically significant difference. Both this and a previous study[7] may indicate that ovine fasciolosis has become endemic in sheep populations in the Black Sea region of Turkey. These findings suggest that variations in agro-climatic conditions in the Black sea region in Northern Turkey and in the grazing practices adopted by livestock owners have direct effects on the epidemiological pattern of *F. hepatica* infection in sheep.

In a previous study, the SDS-PAGE profile of *F. hepatica* E/S antigens in sheep sera contained six major protein bands with molecular weights of 15 kDa, 16 kDa, 20 kDa, 24 kDa, 33 kDa, and 42 kDa.[35] Similar results were observed in this study for *F. hepatica* E/S proteins, which included seven major protein bands with molecular weights ranging from 14 kDa to 66 kDa, predominately from infected sheep. Santiago et al[36] reported that seven polypeptides with molecular weights between 23 kDa and 28 kDa were the major protein bands that reacted with sera from rabbits with experimental fasciolosis. In the present study, seven major protein bands ranging from 20 kDa to 66 kDa recognizing the hyperimmune rabbit sera were detected.

In conclusion, the overall prevalence percentage of ovine fasciolosis was found to be 31.4% in the middle Black Sea region using ELISA based on *F. hepatica* E/S antigens. Diagnosis of ovine fasciolosis can be accomplished using serodiagnostic assays based on E/S antigens of liver flukes and using ELISA and Western blot assays as screening and confirmation tests, respectively. Both assays are sufficient for use in seroepidemiological studies.

Conflicts of interest

There are no conflicts of interest.

Acknowledgments

This study was supported by The Scientific Research Committee of Ondokuz Mayis University, Samsun, Turkey (Project number, Vet-023). Part of the study was submitted to the XV National Parasitology Congress (November 18–23, 2007) Urgup, Kayseri, Turkey, as an oral presentation.

References

1. Andrews SJ. The Life cycle of *F.hepatica*. In: Dalton JP, editor. *Fasciolosis*. Wallingford, Oxon, UK: CABI Publishing; 1998. p. 1–20.
2. Mas-Coma S, Bargues MD. Human liver flukes: a review. *Res Rev Parasitol* 1997;**57**:145–218.
3. Akyol CV. Tarihce ve epidemiyoloji. In: Tınar R, Korkmaz M, editors. *Fasciolosis, Türkiye Parazitoloji Derneği Yayın no: 18. Bornova İzmir*. Turkey: Meta Basım; 2003. p. 85–106 [in Turkish].
4. Toparlak M, Gul Y. Van ili Belediye Mezbahası'nda kesilen koyunlarda karaciğer trematod enfeksiyonları üzerine araştırmalar. *Ankara U Vet Fak Derg* 1988;**35**:269–74 [in Turkish].
5. Vural A, Onar E, Ozkoc U, Everett G. İstanbul iline bağlı köylerde koyunlarda gastrointestinal nematod, moniezia ve karaciğer trematodlarının yayılış durumu. *Pendik Vet Kont Arst Derg* 1968;**1**:146–59 [in Turkish].
6. Gargili A, Tuzer E, Gulanber A, Toparlak A, Efil I, Keles V, et al. Prevalence of liver fluke infections in slaughtered animals in Trakya (Thrace), Turkey. *Tr J Vet Anim Sci* 1999;**23**:115–6.
7. Celep A, Acici M, Cetindag M, Gurbuz I. Samsun yöresi koyunlarında paraziter epidemiyolojik çalısmalar. *T Parazitol Derg* 1995;**19**:290–6 [in Turkish].
8. Guobadia EE, Fagbemi BO. Time-course analysis of antibody response in EITB and ELISA before and after chemotherapy in sheep infected with *F. gigantica*. *Vet Parasitol* 1995;**58**:247–53.
9. Hillyer GV. Immunodiagnosis of human and animal fasciolosis. In: Dalton JP, editor. *Fasciolosis*. Wallingford, Oxon, UK: CABI Publishing; 1998. p. 435–43.
10. Hillyer GV, Soler De Galanes M. Identification of a 17-kilodalton *F. hepatica* immunodiagnostic antigen by enzyme- linked immuno-electrotransfer blot technique. *J Clin Microbiol* 1988;**26**:2048–53.
11. Pfister K, Daveau CH, Ambroise-Thomas P. Partial purification of somatic and excretory-secretory products of adult *F. hepatica* and their application for the serodiagnosis of experimental and natural fascioliasis using an ELISA. *Res Vet Sci* 1984;**37**:39–43.
12. Gonenc B, Sarimehmetoglu HO, Kara M, Kircali F. Comparison of crude and excretory/secretory antigens for the diagnosis of *F. hepatica* in sheep by Western blotting. *Tr J Vet Anim Sci* 2004;**28**:943–9.
13. Karapınar A, Yıldırım A, Bişkin Z, Düzlü Ö İnci A. Zara yöresindeki koyunlarda fasciolosis'in koproantijen ELISA ve sedimentasyon-çinko sülfat flotasyon yöntemi ile araştırılması. *Kafkas Univ Vet Fak Derg* 2012;**18**(Suppl-A):A7–12 [in Turkish].
14. Thienpont D, Rochette F, Vanparijs OFJ. *Diagnose von Helminthosen Durch Koproskopische Untersuchung*. Belgium: Beerse: Janssen Research Foundation; 1979 [in German].
15. Zimmerman GL, Jen LW, Cerro JE, Franswoth KL, Westcott RB. Diagnosis of *F. hepatica* in sheep by enzyme-linked immunosorbent assay. *Am J Vet Res* 1982;**43**:2097–100.
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;**72**:248–54.
17. Almazan C, Avila G, Quiroz H, Ibarra F, Ochoa P. Effect of parasite burden on the detection of *F. hepatica* antigens in sera and feces of experimentally infected sheep. *Vet Parasitol* 2001;**97**:101–12.
18. Guobadia EE, Fagbemi BO. The isolation of F. gigantica-specific antigens and their use in the serodiagnosis of fasciolosis in sheep by the detection of circulating antigens. *Vet Parasitol* 1997;**68**:269–82.
19. Ferre I, Ortega-Mora LM, Rojo-Vazquez FA. Serum and bile antibody response (IgG and IgA) during subclinical *F. hepatica* infection in sheep. *Vet Parasitol* 1997;**68**:261–7.
20. Mousa WM. Evaluation of cercarial antigen for the serodiagnosis of fasciolosis in experimentally and naturally infected sheep. *Vet Parasitol* 2001;**97**:47–54.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–5.

Please cite this article in press as: Acici M, et al., Serologic detection of antibodies against *Fasciola hepatica* in sheep in the middle Black Sea region of Turkey, Journal of Microbiology, Immunology and Infection (2015), http://dx.doi.org/10.1016/j.jmii.2015.06.005

22. Towbin H, Stachelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci* 1979;**76**: 4350.
23. Greiner M, Sohr D, Gobel P. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. *J Immunol Methods* 1995;**185**: 123—32.
24. Reichel MP. Performance characteristics of an enzyme-linked immunosorbent assay for the detection of liver fluke (*F. hepatica*) infection in sheep and cattle. *Vet Parasitol* 2002; **107**:65—72.
25. Thrusfield M. Diagnostic testing. In: Thrusfield M, editor. *Veterinary Epidemiology*. 2nd ed. Oxford, UK: Blackwell; 1995. p. 266—85.
26. Hillyer GV, Soler De Galanes M, Buchon P, Bjorland J. Herd evaluation by enzyme- linked immunosorbent assay for the determination of *F. hepatica* infection in sheep and cattle from the Altiplano of Bolivia. *Vet Parasitol* 1996;**61**:211—20.
27. Torgerson P, Claxton J. Epidemiology and control. In: Dalton JP, editor. *Fasciolosis*. Wallingford, Oxon, UK: CABI Publishing; 1998. p. 113—39.
28. Matanovic K, Severin K, Martinkovic F, Simpraga M, Janicki Z, Barisic J. Hematological and biochemical changes in organically farmed sheep naturally infected with *F. hepatica*. *Parasitol Res* 2007;**101**:1657—61.
29. Leloglu S. A comparative study of livers with or without liver flukes (*F. hepatica*) for clostridial infections by means of culture and by fluoroscent antibody technique. *Turk Vet Hek Der Derg* 1972;**42**:19—22.
30. Oguz T, Kalkan A. Kurşunlu ilçesi Devrez yöresinde *F.hepatica*'nın epidemiyolojisi ve ekolojisi üzerine araştırmalar. *Ankara U Vet Fak Derg* 1978;**25**:568—83 [in Turkish].
31. Sen M, Yıldırım A, Bişkin Z. Düzlü Ö İnci A. The investigation of sasciolosis in cattle by copro-ELISA and stool examination techniques around the Derinkuyu region. *Turkiye Parazitol Derg* 2011;**35**:81—5.
32. Simsek S, Risvanlı A, Utuk AE, Yuksel M, Saat N, Koroglu E. Evaluation of relationship between repeat breeding and *Fasciola hepatica* and hydatid cyst infections in cows in Elazig district of eastern Turkey. *Res Vet Sci* 2007;**83**:102—4.
33. Balkaya I, Simsek S. Erzurum'da kesilen sığırlarda Hidatidosis ve Fasciolosis'in yaygınlığı ve ekonomik önemi. *Kafkas Univ Vet Fak Derg* 2010;**16**:793—7 [in Turkish].
34. Yibar A, Selcuk O, Senlik B. Major causes of organ/carcass condemnation and financial loss estimation in animals slaughtered at two abattoirs in Bursa Province, Turkey. *Prev Vet Med* 2015;**118**:28—35.
35. Meshgi B, Eslami A, Hemmatzadeh F. Determination of somatic and excretory- secretory antigens of *F. hepatica* and *F. gigantica* using SDS-PAGE. *Iran J Vet Res* 2008;**9**:77—80.
36. Santiago N, Hillyer GV, Garcia-Rosa M, Morales MH. Identification of functional *F. hepatica* antigens in experimental infections in rabbits. *Am J Trop Med Hyg* 1986;**35**:135—40.

Please cite this article in press as: Acici M, et al., Serologic detection of antibodies against *Fasciola hepatica* in sheep in the middle Black Sea region of Turkey, Journal of Microbiology, Immunology and Infection (2015), http://dx.doi.org/10.1016/j.jmii.2015.06.005