Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Prevalence and Molecular Diagnosis of *Fasciola hepatica* in Sheep and Goats in Different Districts of Punjab, Pakistan

Waseem Shahzad*, Khalid Mehmood[1], Rashid Munir[2], Waqar Aslam, Muhammad Ijaz[1], Rashid Ahmad, Mohammad Sarwar Khan[1] and Ahmad Jawad Sabir[1]

Livestock Production Research Institute Bahadurnagar, Okara; [1]University of Veterinary and Animal Sciences, Lahore-54000; [2]Veterinary Research Institute, Lahore, Pakistan
*Corresponding author: waseem1971@hotmail.com

ARTICLE HISTORY

Received: January 16, 2012
Revised: February 20, 2012
Accepted: March 11, 2012

Key words:
Bile
Fecal
Fasciolosis
Microscopy
PCR

ABSTRACT

A study was conducted to determine the prevalence and molecular diagnosis of fasciolosis in Lohi sheep and Beetal goats in Okara, Sahiwal and Lahore districts of Punjab province. A total of 800 samples including 200 fecal and 200 bile, each from Lohi sheep and Beetal goats were collected and processed for microscopic examination of *Fasciola* eggs as well as its confirmation through polymerase chain reaction (PCR). Of the fecal samples, 3.5 and 2% samples whereas from bile samples, 6.5 and 4% bile samples from sheep and goats were found microscopically positive for *Fasciola hepatica*, respectively. By PCR, 4.5 and 3.5% fecal samples and 8.5 and 5% bile samples from sheep and goats, respectively were confirmed for *F. hepatica*. Statistically sheep and goats species were found equally likely acquiring the *F. hepatica* diagnosed by either source of material (fecal and bile samples) similarly fecal and bile samples were equally likely showing fasciolosis in either of sheep and goats.

To Cite This Article: Shahzad W, K Mehmood, R Munir, W Aslam, M Ijaz, R Ahmad, MS Khan and AJ Sabir, 2012. Prevalence and molecular diagnosis of *Fasciola hepatica* in sheep and goats in different districts of Punjab, Pakistan. Pak Vet J, 32(4): 535-538.

INTRODUCTION

Among diseases which are not often apparent to farmers, the liver fluke infections are of considerable economic and public health importance. Fasciolosis, largely caused by *Fasciola hepatica* in temperate climates and by *Fasciola gigantica* in tropical regions, is characterized by sudden death with blood stained froth at the natural orifices in acute cases (McGarry *et al*., 2007; Mas-Coma *et al.*, 2009; Raza *et al*., 2010), while diarrhea, jaundice, ascites and bottle jaw are predominant features in chronic cases. Annual economic loss caused by the disease is mainly due to mortality (mild to heavy), cost of diagnosis and treatment, condemned livers (Ahmadi and Meshkehkar, 2010), reduced milk yield, fertility disorders and reduced meat production (Rokni *et al*., 2010; Hossain *et al*., 2011). *Fasciola* spp. are also capable of causing disease in humans (Ai *et al.*, 2011) and is a significant public health threat due to emerging food-borne zoonosis in developing countries like Pakistan (Qureshi *et al*., 2005). These parasites inhabit the hepato-biliary system of the affected hosts and rarely can they be found in ectopic sites within the host body (Nguyen *et al*., 2009).

The detection of infections in ruminants relies on the microscopic observation of *Fasciola* eggs in the feces of infected animals. However, early diagnosis by coprological examination is not possible because eggs are not found in the feces until 10–12 weeks after infection, when flukes reach maturity, and when hepatic injury has been produced. A few Lohi sheep and Beetal goats maintained at Livestock Production Research Institute, Bahadurnagar, district Okara showed sub-mandibular swelling, progressive weakness, emaciation, stunted growth, rough body coat and occasional deaths. Fecal sedimentation method revealed the presence of *Fasciola* eggs, which was later confirmed as *F. hepatica* through polymerase chain reaction (PCR). This study was initiated to investigate the prevalence of fasciolosis in Lohi sheep and Beetal goats and also to confirm the *Fasciola* spp. by applying molecular diagnostic tool such as PCR in Okara, Lahore and Sahiwal districts of Punjab province.

MATERIALS AND METHODS

Using random sampling technique 200 each of fecal and bile samples were collected each from Lohi sheep and

Beetal goats from Livestock Production Research Institute (LPRI) Bahadurnagar, Okara and slaughter houses of districts Okara, Sahiwal and Lahore during September to December 2010, thus a total of 800 samples were collected for this study. Animals of all age groups and either sex were included in this study and all samples were obtained prospectively for the purpose of study from different sheep and goats flock (from 7 to 200 sheep/goats in each flock) in each district mainly kept on traditional grazing of weeds, trees and seasonal green fodder. The affected animals were identified through clinical signs and symptoms of the disease, post-mortem lesions of affected died sheep/goats and also by taking the history of the affected animals from the owners at the time of sampling. For qualitative and quantitative microscopic examination of *Fasciola* eggs, all fecal samples were processed by fecal centrifugal sedimentation method as described by Zajac and Conboy (2006) and bile samples were processed according to the protocol described by Suhardono *et al*. (2006). For the isolation and DNA extraction, the *Fasciola* eggs from fecal and bile samples were recovered by using a standard washing–sieving procedure as described by Suhardono *et al*. (2006) with a little modification by using 3 g of each fecal sample, 1% solution of commercial washing powder (Surf Excel) as suspending fluid and sieves of 1 mm, 450 µm and 266 µm aperture in tandem to retain fibrous debris. The recovered eggs from both types of samples were then re-suspended in physiological saline to give a pool of eggs in a final volume of 100 ml which were then vortexed with glass beads for over night to disturb the egg shells before the DNA was extracted (Ahmed and Khosa, 2010). After vortexing the resultant fluids were centrifuged (Sorvall® Ultraspeed Centrifuge, Thermo, USA) at 12,000 rpm for 15 min, supernatant was discarded and 300 µl sediment were used for the extraction of total genomic DNA by using a commercially available DNA extraction kit (Cat.# K0512, Fermentas) according to the manufacturer's instruction. Thirty healthy goats with no history of fasciolosis or free of *Fasciola* eggs were maintained at LPRI, Bahadurnagar, Okara and were used as known control negative group. DNA concentration was determined spectrophotometrically at 260 and 280 ηm and the samples were stored at - 20°C till further use. The primer pair DSJF consisted of a forward primer (5' -ATA TTG CGG CCA TGG GTT AG-3') and a species specific reverse primer known as DSJ3 (5'- CCA ATG ACA AAG TGA CAG CG-3') specific for *F. hepatica* were used. Another species specific reverse primer DSJ4 (5'- CCA ATG ACA AAG TAA CAG CA-3') specific for *F. gigantica* was also used with DSJF forward primer. These *Fasciola* specific primers (Ai *et al*., 2010) were used at a concentration of 10 ρmol for the amplification of approximately 300 bp fragment for *Fasciola* species. All PCR amplification reactions, including control negative samples, were carried out in a final volume of 25 µl containing DNA template, 1.5 µl $MgCl_2$ (Cat # EP0402, Fermentas) , 0.5 µl *Taq* DNA polymerase (5u/ µl) (Cat # EP0402, Fermentas) and 12.5 µl commercially available PCR master mix (PyroStart™ Fast PCR Master Mix-2X, Fermentas). Ten micro liters of the PCR product were sized by electrophoresis on a 1% agarose gel (1 h at 90 V) with a 1-Kb ladder as size marker. The gels were stained with ethidium bromide (2 µl 50 ml^{-1} gel) and analyzed in a UV transilluminator (Dolphin-Doc, Wealtec, USA).

RESULTS

Fecal and bile samples examined under the microscope revealed large brown eggs with an operculum identified as *F. hepatica* eggs based on their morphology. Out of 800 samples, thirty two (8%) positive samples were positive microscopically (based on eggs per gram (EPG) and subsequent copro-cultures) and out of these 32 samples, 21 (10.5%) were positive from bile samples while 11 (5.5%) were positive from fecal samples (Table 1). Data recorded for extracted DNA concentration by spectrophotometric analysis showed optical density values of 1.67 and 0.63 at 260 and 280 ηm wavelengths, respectively. The approximate 300-bp fragment was generated in all *F. hepatica* positive samples tested with DSJF/DSJ3 primers (Fig.1). No amplification was obtained when *F. gigantica* specific primers (DSJF/DSJ4) were applied on the template DNA. Similarly no such amplicon was detected in control negative samples. Forty-three (10.75%) bile and fecal samples were found positive for *F. hepatica* through PCR and out of these 43 samples, 27 (13.5%) were positive from bile samples where as 16 (8%) were positive from fecal samples (Table 1). All samples positive by microscopic method were also positive by PCR, whereas, out of the 43 samples positive by PCR test, 11 were negative by microscopy.

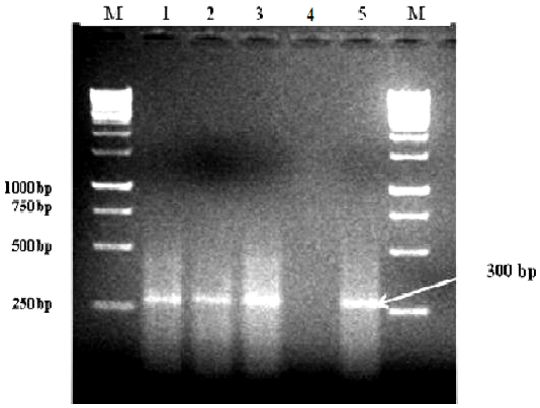

Fig. 1: PCR amplification of DNAs from sheep and goats isolates of *Fasciola hepatica* with specific primers. Lanes 1-2 show the PCR products of *Fasciola hepatica* from sheep bile samples with sized 300bp, Lane 3= sheep fecal sample with sized 300bp, Lane 4= control negative, Lane 5= PCR products of *Fasciola hepatica* from goat bile sample, M= Molecular ladder

Statistical significance of prevalence of *F. hepatica* in sheep and goats by either source of material (fecal or bile samples) has been measured by Chi-square test-statistic at level of significance $\alpha = 0.05$; under the characteristics attributed to animal species and sample source (fecal & bile samples) as mentioned in Table 1. The sheep and goats species were equally likely acquiring the *F. hepatica* diagnosed by either source of material (fecal and bile samples), showing (χ^2) test-statistic with P-value (0.223). Similarly fecal and bile samples were equally likely showing fasciolosis in either of sheep and goats, showing (χ^2) test- statistic with P-value (0.056).

Table 1: Prevalence of *F. hepatica* in sheep and goats by using microscopic and PCR methods from fecal and bile samples (n= 200)

Animal spp.	No. Positive Microscopically		No. Positive by PCR	
	Fecal Sample	Bile Sample	Fecal Sample	Bile Sample
Sheep	07(3.5)	13(6.5)	09(4.5)	17(8.5)
Goat	04(2.0)	08(4.0)	07(3.5)	10(5.0)
Total	11(2.75)	21(5.25)	16(4.0)	27(6.75)

Figures in parenthesis indicate percentage.

DISCUSSION

Several studies in Pakistan have revealed that *F. hepatica* is endemic in cattle, buffaloes, sheep, goats and humans (Qureshi *et al*., 2005; Iqbal *et al*., 2007; Ijaz *et al*., 2009; Khan *et al*., 2010) but throughout the country, there has been no molecular diagnostic reported case of sheep and goats being infested with this parasite. This is the first molecular diagnostic report about the prevalence of *F. hepatica* in Lohi sheep and Beetal goats in populated districts of Punjab province of Pakistan. However, Ijaz *et al*. (2009) has reported 14.67% infection rate of *F. hepatica* by coprological exam in diarrheic sheep in and around Lahore, about the same area where present study was conducted. In that study, the *Fasciola* ova were identified by using key as described by Soulsby (1982) whereas in the present study molecular diagnostic technique such as PCR has been applied for the species identification and confirmation which is a more reliable, sensitive and more accurate diagnostic method. Furthermore, the higher prevalence (14.67%) of fasciolosis in fecal samples in the previous study can be compared with the low prevalence (2.75%) reported in the present study by coprological exam due to random selection of samples and diarrheic animals.

In earlier works, fasciolosis was diagnosed through symptoms and microscopy in fecal samples but in this study, a molecular technique (PCR) was applied for the first time to diagnose this fasciolosis infection in sheep and goats, not only in the fecal samples but also bile samples were used to confirm the *Fasciola* spp. in Pakistan. So molecular approaches such as PCR and similar other molecular techniques like DNA sequence analysis of the nuclear ribosomal internal transcribed spacers, 28 S rRNA genes etc (Li *et al*., 2009; Ai *et al*., 2010; Alasaad *et al*., 2011; Amor *et al*., 2011) have greatly enhanced the ability to differentiate members of the genus *Fasciola* thus indicate the importance of molecular techniques for the identification and differentiation of *Fasciola* spp.

In present study, the percentage of positive samples by PCR for *F. hepatica* was higher (4 and 6.75%) in sheep and goats fecal and bile samples than by microscopic method (2.75 and 5.25%) in sheep and goats fecal and bile samples, indicating PCR as more sensitive technique. Although microscopy of fecal samples is an easy and economic diagnostic technique, but this technique proved less sensitive as compared to PCR. Another limitation of microscopy is that it cannot clearly differentiate different species of *Fasciola* while the molecular technique does so.

The results of the present study are in agreement with the findings of Ai *et al*. (2010) who used DSJF/DSJ3 primers for the identification and differentiation of *F. hepatica* from different samples collected from naturally infected buffaloes, cattle, sheep or goats and found PCR to be the most sensitive method for detection and identification of *Fasciola* infection. The identification of *Fasciola* to species level is traditionally based on differences in size and shape of ova but recent studies have demonstrated this method to be unreliable. Species of *Fasciola* can be distinguished by staining and comparing the morpho-anatomy of the gut and ovaries or by iso-enzyme analysis but such approaches are time consuming and require specialist skills. Our study is also in agreement with the findings of McGarry *et al*. (2007), Ichikawa and Itagaki (2010) and Saki *et al*. (2011) who also found PCR and other molecular techniques more reliable and specific method for identification and differentiation of *Fasciola* species in and of cattle and sheep.

Since Fasciolosis is now recognized as an emerging food-borne zoonosis in many parts of the world (Freites *et al*., 2009; Meray y Sierra *et al*., 2011; Karahocagil *et al*., 2011) and World Health Organization has also included human fascioliasis on its list of priorities among neglected tropical diseases (WHO, 2008) and on the other hand the incidence of human infection in Lahore, Pakistan has been reported (0.31 %) by Qureshi *et al*. (2005), it is very important to apply such molecular diagnostic tool for epidemiological study for human and animal fasciolosis in a developing country like Pakistan.

During the present study it was observed that amplification of approximate 300bp was obtained as described by Ai *et al*. (2010) but with little modification by the addition of 1.5 µl of $MgCl_2$ and 0.5 µl *Taq* DNA polymerase in the reaction mixture.

Conclusion: PCR for the detection of *F. hepatica* is specific and sensitive. The test is suitable for tracing infected animals and provides a quantitative validated measure that is useful in epidemiological surveys and follow up for drug treatment in cattle, buffalo, sheep and goats. In addition, it would be useful for designing fasciolosis control programs in endemic areas.

Acknowledgement: The author is thankful to Dr. Rashid Ahmad, Director Livestock Production Research Institute, Bahadurnagar for providing necessary research facilities. Partially support for this study was provided by the project "Up-gradation of research facilities at LPRI, Bahadurnagar, Okara." The authors gratefully acknowledge Mr. Sohail Ahmad (Research Officer, Statistics) for his valuable contribution to statistical inferences.

REFERENCES

Ahmadi NA and M Meshkehkar, 2010. Prevalence and long term trend of Liver fluke infections in sheep, goats and cattle slaughtered in Khuzestan, southwestern Iran. J Paramed Sci, 2: 26-31.

Ahmed S and AN Khosa, 2010. An introduction to DNA technologies and their role in livestock production: a review. J Anim Plant Sci, 20: 305-314.

Ai L, SJ Dong, WY Zhang, HM Elsheikha, YS Mahmmod, RQ Lin, ZG Yuan, YL Shi, WY Huang and XQ Zhu, 2010. Specific PCR-based assays for the identification of *Fasciola* species: their development, evaluation and potential usefulness in prevalence surveys. Annals Trop Med Parasitol, 104: 65-72.

Ai L, MX Chen, S Alasaad, HM Elsheikha, J Li, HL Li, RQ Lin, FC Zou, XQ Zhu and JX Chen, 2011. Genetic characterization, species differentiation and detection of *Fasciola* spp. by molecular approaches. Parasit Vectors, 4:101-106.

Alasaad S, RC Soriguer, M Abu-Madi, A El Behairy, PD Baños, A Píriz, J Fickel and XQ Zhu, 2011. A fluorescence-based polymerase chain reaction-linked single-strand conformation polymorphism (F-PCR-SSCP) assay for the identification of *Fasciola* spp. Parasitol Res, 108: 1513-1517.

Amor N, S Farjallah, K Said and BB Slimane, 2011. First report of *Fasciola hepatica* in *Equus caballus* host species from Tunisia based on the ribosomal internal transcribed spacer regions. Turk J Vet Anim Sci, 35: 319-324.

Freites A, C Colmenares, B Alarcon-Noya, ME Garcia and O Diaz-Suarez, 2009. Human fasciolosis in Mara municipality, Zulia state. Venezuela: prevalence and associated factors. Invest Clin, 50: 497-506.

Hossain MM, S Paul, MM Rahman, FMA Hossain, MT Hossain and MR Islam, 2011. Prevalence and economic significance of caprine fascioliasis at Sylhet district of Bangladesh. Pak Vet J, 31: 113–116.

Ichikawa M and T Itagaki, 2010. Discrimination of the ITS1 types of *Fasciola* spp. based on a PCR-RFLP method. Parasitol Res, 106: 757–761.

Ijaz M, MS Khan, M Avais, K Ashraf, MM Ali and MZU Khan, 2007. Infection rate and chemotherapy of various helminthes in diarrheic sheep in and around Lahore. J Anim Plant Sci, 19: 13-16.

Iqbal MU, MS Sajid, A Hussain and MK Khan, 2007. Prevalence of helminth infections in dairy animals of Nestle milk collection areas of Punjab (Pakistan). Italian J Anim Sci, 6: 935-938.

Karahocagil MK, H Akdeniz, M Sunnetioglu, M Cicek, R Mete, N Akman, E Ceylan, H Karsen and K Yapici, 2011. A familial outbreak of fascioliasis in Eastern Anatolia: a report with review of literature. Acta Trop, 118: 177-89.

Khan MN, MS Sajid, MK Khan, Z Iqbal and A Hussain, 2010. Gastrointestinal helminthiasis: prevalence and associated determinants in domestic ruminants of district Toba Tek Singh, Punjab, Pakistan. Parasitol Res, 107: 787-94.

Li QY, SJ Dong, WY Zhang, RQ Lin, CR Wang, DX Qian, ZR Lun, HQ Song and XQ Zhu, 2009. Sequence-related amplified polymorphism, an effective molecular approach for studying genetic variation in *Fasciola* spp. of human and animal health significance. Electrophoresis, 30: 403–409.

Mas-Coma S, MA Valero and MD Bargues, 2009. Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv Parasitol, 69: 41–146.

McGarry JW, PL Ortiz, JE Hodgkinson, I Goreish and DJL Williams, 2007. PCR-based differentiation of *Fasciola* species (Trematoda: Fasciolidae), using primers based on RAPD-derived sequences. Annals Trop Med Parasitol, 101: 415-421.

Mera y Sierra R, VH Agramunt, P Cuervo and S Mas-Coma, 2011. Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. Parasit Vectors, 4: 104-121.

Nguyen TG, N Van De, J Vercruysse, P Dorny and TH Le, 2009. Genotypic characterization and species identification of *Fasciola* spp. with implications regarding the isolates infecting goats in Vietnam. Exp Parasitol, 123: 354-361.

Qureshi AW, A Tanveer, SW Qureshi, A Maqbool, TJ Gill and SA Ali, 2005. Epidemiology of human Fasciolosis in rural areas of Lahore, Pakistan. Punjab Univ J Zool, 20: 159-168.

Raza MA, S Murtaza, HA Bachaya, A Qayyum and MA Zaman, 2010. Point prevalence of *Toxocara vitulorum* in large ruminants slaughtered at Multan abattoir. Pak Vet J, 30: 242-244.

Rokni MB, M Mirhendi, M Behnia, MF Harandi and N Jalalizand, 2010. Molecular characterization of *Fasciola hepatica* isolates by RAPD-PCR and ribosomal ITSI sequencing. Iran Red Cres Med J, 12: 27-32.

Saki J, S Khademvatan and E Yousefi, 2011. Molecular identification of animal *Fasciola* isolates in Southwest of Iran. J Appl Sci Res, 7: 1878-1883.

Suhardono, JA Roberts and DB Copeman, 2006. Optimization of the procedure for counting the eggs of *Fasciola gigantica* in bovine feces. Trop Anim Health Prod, 38: 383–387.

Soulsby EJL, 1982. Helminths, Arthropods and Protozoa of Domesticated animals. 7th Ed. Bailliere Tindall, London, UK, pp: 579-624, 765-766.

World Health Organization, 2008. Fact sheet on fascioliasis. Action against Worms, Geneva, Switzerland: World Health Organization Headquarters.10: 1-8.

Zajac AM and GA Conboy, 2006. Veterinary Clinical Parasitology, 7th Ed. Blackwell Publishing, Iowa, USA, pp: 11-12.