

Differentiation of cattle and sheep originated *Fasciola hepatica* by RAPD-PCR

O. S. ALDEMIR

Department of Parasitology, Faculty of Veterinary Medicine, Adnan Menderes University, Aydın-TURKEY
E-mail : oselcuk9@yahoo.com, oselcuk9@hotmail.com, osaldemir@adu.edu.tr

SUMMARY

In this study, a RAPD-PCR (Random Amplified Polymorphic DNA-PCR) technique was used to differentiate between cattle and sheep originated *Fasciola hepatica* DNA. In order to isolate the parasitic DNA, phenol extraction/ethanol precipitation methods were employed. After optimization of the PCR conditions, three primers were selected to amplify the DNA of each *F. Hepatica*. The results show that the amplification fragments were between 150 and 700 bp and, the use of random genetic markers allowed to identify genetic variations of *F. hepatica* between species. Thus the RAPD-PCR method can be useful for the differentiation of cattle and sheep originated *F. hepatica*.

Keywords : Differential identification - *Fasciola hepatica* - RAPD-PCR - sheep - cattle.

RÉSUMÉ

Distinction entre *Fasciola hepatica* d'origine bovine et ovine par RAPD-PCR. Par O. S. ALDEMIR.

Au cours de cette étude, une technique de RAPD-PCR (amplification aléatoire d'ADN polymorphe par PCR) a été utilisée afin de différencier les ADN de *F. hepatica* issue de mouton ou de veau. L'ADN parasitaire a été isolé après extraction au phénol et précipitation à l'éthanol puis amplifié à partir de 3 amorces différentes après optimisation des conditions de PCR. Les fragments d'ADN ainsi amplifiés ont présenté des tailles comprises entre 150 et 700 bp et l'utilisation de ces amorces aléatoires a permis de montrer que *F. hepatica* présentait des variations génétiques en fonction de l'espèce parasitée. Par conséquent, la méthode de RAPD-PCR peut être utile pour différencier les *F. hepatica* d'origine bovine et ovine.

Mots-clés : Identification - *Fasciola hepatica* - RAPD-PCR - mouton - veau.

Introduction

Fasciola hepatica, a liver fluke, is a causative agent of fascioliasis in mammals (sheep, cattle, goat, ox and other ruminants, pig, hare rabbit, beaver, elephant, dog, cat and kangaroo). In the unusual hosts, such as man and the horse, the fluke may be found in the lungs, under skin or other situations. The parasite *Fasciola hepatica* is a hermaphroditic trematode, which is particularly common in sheep and cattle. Transmission of *F. hepatica* is dependent on the presence of its lymnae snail intermediate hosts. Following ingestion of metacercariae by the hosts, the juvenile worms burrow through the host gut walls and migrate to the liver, where they cause extensive damage before moving into the bile ducts. Finally, the parasites pass through the bile duct walls and develop into mature forms that live in the microenvironment of the bile ducts. This worm causes important economic losses due mainly to liver damage and reduced production of meat and milk [11, 13, 14].

Molecular techniques such as PCR and its variants are used for the diagnosis of parasitic diseases and the identification of parasites, for the development of specific antigens for serological tests and for studying the immune response in the patients. Molecular techniques have become widely accepted through the world. They provide a more specific method than methods conventionally employed in epidemiological studies [1, 2, 4, 6, 8, 10, 11].

The aims of this study were to characterize molecularly and to differentiate between cattle and sheep originated *Fasciola hepatica* using a RAPD-PCR assay. This study is not particularly aimed to phylogenically analyze *Fasciola*

spp. However, it can help to better understand the evolution of these worm species.

Material and Methods

ISOLATION OF PARASITES

Adult *Fasciola hepatica* were obtained from the livers of cattle and sheep. The collected worms were washed several times with phosphate-buffered saline (PBS [pH 7.4]) and then incubated in the same buffer at 37°C for 3 h to eliminate any residual host matter. Afterwards the parasites were washed with PBS several times.

EXTRACTION OF DNA

The extraction of DNA was performed according to the protocol described previously by KAPLAN *et al.* [7], MC MANUS and BOWLES [9] ; VARGAS *et al.* [15]. The method was slightly modified as follows :

The parasite samples were homogenized in a lysis solution (8 % Triton 100X, 0,25 M Sucrose, 50 mM EDTA, pH 7,4). The homogenates were centrifuged at 10.000 g for 10 min. at 4°C. Genomic DNA was extracted by SDS-proteinase K digestion, followed by phenol chloroform extraction. The recovered DNA was dissolved in 10 mM Tris-HCl, 1 mM EDTA pH 7,6 (Tris-EDTA buffer) and contaminating RNA was removed by incubation with RNase for 1 h at 37°C, followed by a second phenol chloroform extraction and ethanol precipitation.

The suspensions were stored at -20°C until required.

CHOICE OF PRIMERS

In order to optimize the PCR conditions, three primers (5'-TCG TCG CATT -3' (OSA 09), 5'- AGC AGC AGGC -3' (OSA 10) and 5'- GGG TAA CGCC -3' (OSA 11)) were randomly selected to amplify the DNA of cattle and sheep originated *Fasciola hepatica*, as reported by VARGAS *et al.* [15].

OPTIMIZATION

It was necessary to optimize the conditions for the amplification to obtain complete and reproducible band patterns for the genomic DNA (5 ng) of each parasite used as a template DNA. The DNA concentrations were determined spectrophotometrically at 260 nm (A_{260}). The reaction was carried out in a final volume of 50 μ l containing 10X buffer (500 mM KCl, 200 mM Tris- HCl pH 8.4), 2.0 mM $MgCl_2$, 0.4 μ M dNTPs (Promega, USA). Each reaction tube contained 2 units of Taq polymerase. PCR was performed in a Hybaid Omni-Gene thermocycler (Hybaid, UK). DNA was amplified in 75 cycles with a denaturing step at 94°C for 5 seconds, primer annealing at 36°C for 30 seconds, and an extension step at 72°C for 10 seconds. Incubation at 72°C for 5 seconds for primer extension has been suggested by previous studies [15]. However, a longer extension period produced shadow formation. On the basis of several attempts over different time periods, it is concluded that 10 seconds at 72°C is optimum for primer extension.

AGAROSE GEL ELECTROPHORESIS

1.4 % agarose gel was stained with ethidium bromide prior to visualization and photography. It was used as indicating of molecular weight a 100 pb ladder.

Results

The results of this study show that different primers gave different fragments (Table I). These fragments are species-specific for cattle and sheep originated *F. hepatica*. Different DNA fragments were obtained from parasites of 2 species (cattle and sheep) according to the primers used (Table I). The sizes of DNA fragments from cattle and sheep originated *F. hepatica* respectively amplified by the OSA 09 primer were approximately 700 bp and 150 bp (Figure 1). Using the OSA 10 primer, only DNA fragments (150 bp) from sheep parasite have been evidenced, whereas no amplification has been achieved with cattle worm DNA (Figure 2). Finally,

after amplification by the OSA 11 primer, 2 DNA fragments of approximately 200 and 400 bp were obtained only from cattle originated *F. hepatica* and no band from sheep worm was visualized (Figure 3).

Discussion

The identification of closely similar species based on morphological characteristics can be difficult. This is particularly the case for soft-bodied animals such as digenean trematodes. However, recent advances in molecular biology, in particular the amplification of specific DNA regions via the polymerase chain reaction (PCR) and the improvement of direct deoxy sequencing techniques, may allow to distinguish closely related species by comparing their DNA [1-3, 9, 10].

DNA probes as well as PCR primers based on variable regions of the ribosomal DNA sequences have been developed for the detection and differentiation of *Fasciola species*. However, since variable regions are rare in the ribosomal DNA genes of *Fasciola species*, and since other gene sequences are not yet widely available for species of *Fasciola species*, a method which does not require prior sequence information, such as the isolation of RAPD-PCR-derived polymorphic markers, is a convenient alternative to the methods previously used [7-9].

The RAPD-PCR technique allows to amplification of short regions of an organism's genome without prior sequence information. This technique has a great potential in identifying genetic markers, tagging genes and chromosomes, and performing population studies. Moreover, this method evidences species-specific DNA fragments which may be used as diagnostic probes among organisms and consequently would allow determination of their genetic relatedness [5, 16].

In the present study, 3 random primers gave different DNA fragments in size according to the host species origin of the worm, demonstrating that these fragments were specific for cattle and sheep originated *F. hepatica*. With the OSA 09 primer, a 150 bp length DNA fragment was obtained from the sheep worm whereas a 700 bp length DNA fragment was identified from the cattle worm. These results were in agreement with previous reports [12, 15]. The present study concluded that RAPD-PCR technique is able to evidence of the genetic relation between cattle and sheep originated *Fasciola hepatica* and also to identify species specific parasite worms.

Primers	Sheep originated worm		Cattle originated worm	
	Number	Size (bp)	Number	Size (bp)
OSA-09	1	150	1	700
OSA-10	1	150	0	--
OSA-11	0	--	2	200 - 400

TABLE I. — Number and size of amplified genomic DNA fragments from cattle and sheep originated *F. hepatica* using different primers.

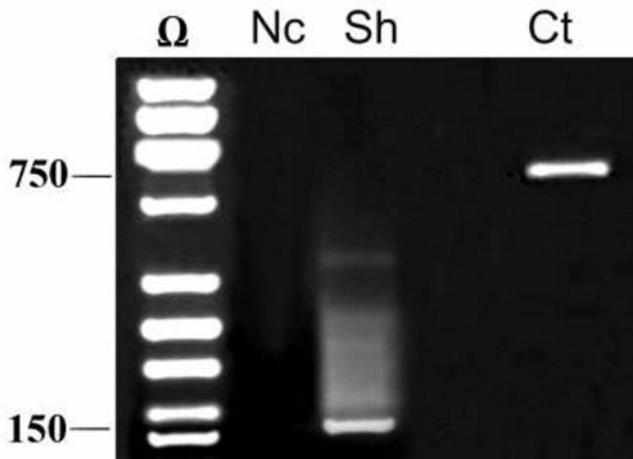


FIGURE 1. — Amplification of genomic DNA from sheep and cattle originated *F. hepatica* with OSA-09 primer. ϕ : Marker, Nc: Negative control, Sh: Sheep origin, Ct: Cattle origin.

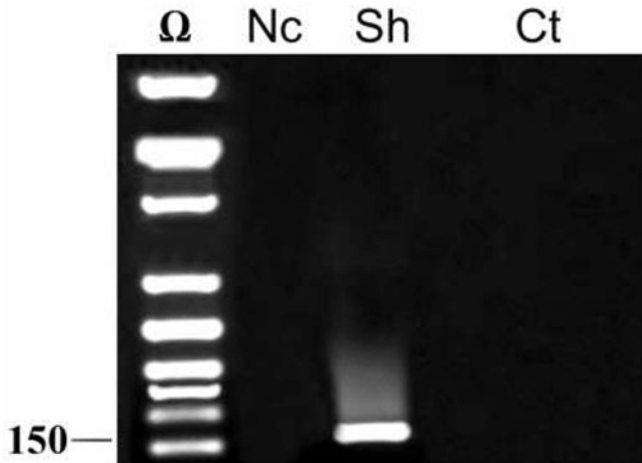


FIGURE 2. — Amplification of genomic DNA from sheep and cattle originated *F. hepatica* with OSA-10 primer. ϕ : Marker, Nc: Negative control, Sh: Sheep origin, Ct: Cattle origin.

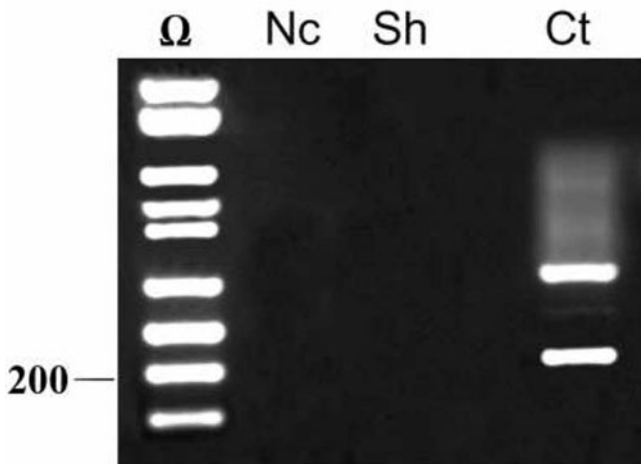


FIGURE 3. — Amplification of genomic DNA from sheep and cattle originated *F. hepatica* with OSA-11 primer. ϕ : Marker, Nc: Negative control, Sh: Sheep origin, Ct: Cattle origin.

In conclusion, RAPD-PCR method used for the differentiation of cattle and sheep originated *Fasciola hepatica* produced amplified products in different numbers and sizes. Therefore this technique has the potential to differentiate these two worm species. These findings may also be useful for determining genetically the phylogenetic evolution of worm species in future studies.

References

1. — BARKER RH. : Use of PCR in field. *Parasitol. Today.*, 1994, **10**, 117-119.
2. — COOTE UG. : Amplification of Nucleic Acids by the Polymerase Chain Reaction. *Article.*, 1990, **4**, 57-59.
3. — ERLICH HA., ARNHEIM N. : Genetic analysis using the Polymerase Chain Reaction. *Annu. Rev. Genet.*, 1992, **26**, 479-506.
4. — ERLICH HA., GELFAND D., SNINSKY JJ. : Recent advances in the Polymerase Chain Reaction. *Science.*, 1991, **252**, 1643-1651.
5. — ESPINOSA L., BOROWSKY R. : Evolutionary Divergence of AP-PCR (RAPD) Patterns. *Mol. Biol. Evol.*, 1998, **15**, 408-414.
6. — HECKEROTH AR., TENTER AM. : Development and validation of species-specific nested PCR for diagnosis of acute Sarcocystosis in sheep. *Int. J. Parasitol.*, 1999, **29**, 1331-1349.
7. — KAPLAN RM., DAME JB., REDDY GR., COURTNEY CH. : A repetitive DNA probe for the sensitive detection of *Fasciola hepatica* infected Snails. *Int. J. Parasitol.*, 1995, **25**, 601-610.
8. — KRAMER F., SCHNIEDER T. : Sequence heterogeneity in a repetitive DNA element of *Fasciola*. *Int. J. Parasitol.*, 1998, **28**, 1923-1929.
9. — MC MANUS D., BOWLES J. : Molecular genetic approaches to parasite identification : their value in diagnostic parasitology and systematics. *Int. J. Parasitol.*, 1996, **26**, 687-704.
10. — MOSTAFA OM., TAHA HA., RAMADAN G. : Diagnosis of *Fasciola gigantica* in snail using the polymerase chain reaction (PCR) assay. *J. Egypt. Soc. Parasitol.*, 2003, **33**, 733-742.
11. — ROGNLIE M., DIMKE K., KNAPP S. : Detection of *Fasciola hepatica* in infected intermediate hosts using RT-PCR. *J. Parasitol.*, 1994, **80**, 748-755.
12. — SHUBKIN C., WHITE M., ABRAHAMSEM M. : A nucleic acid-based test for detection of *Fasciola hepatica*. *J. Parasitol.*, 1992, **78**, 817-821.
13. — SINEO L., MARTINI R., BORGHI G., FAILLI M. : Analysis of genetic markers by Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR). *Bull. Chim. Farm.*, 1993, **132**, 201-202.
14. — SOLSBY E.J.L. : 7. Ed. The English Language Book Society and Bailliere Tindal, p 40. 1986, Printed in Great Britain by Williams Clauses Limited, London.
15. — VARGAS D., VEGA M., GLORIA C. : Aproximacion a una caracterizacion molecular *Fasciola hepatica* por la tecnica RAPDs-PCR. *Parasitol. Latinoam.*, 2003, **58**, 11-16.
16. — WILLIAMS JGK., KUBELIK AR., JIVAK KS., RAFALKSI JA., TINGEY SV. : DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic acids Res.*, 1990, **18**, 6531-6535.