Parasitol Res
DOI 10.1007/s00436-015-4310-9

ORIGINAL PAPER

Approach to molecular characterization of different strains of *Fasciola hepatica* using random amplified polymorphic DNA polymerase chain reaction

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Received: 12 November 2014 / Accepted: 7 January 2015

Abstract The aim of the present study was to genetically characterize *Fasciola hepatica* strains from diverse ecogeographical regions (America and Europe), susceptible and resistant to Triclabendazole, using the random amplified polymorphic DNA fragments (RAPDs-PCR) technique to elucidate genetic variability between the different isolates. Ten different oligonucleotide primers of 10 bases with GC content varying from 50–70 % were used. A polymerase chain reaction (PCR) was carried out in 25 μl of total volume. Duplicate PCR reactions on each individual template DNA were performed to test the reproducibility of the individual DNA bands. The size of the RAPD-PCR fragments was determined by the reciprocal plot between the delay factors (Rf) versus the logarithm of molecular weight ladder. The phenogram obtained showed three main clusters, the major of which contained European Strains (Cullompton and Sligo) showing a genetic distance of 27.2 between them. The American strains (Cedive and Cajamarca) on the other hand formed each their distinctive group but clearly maintaining a closer genetic relationship among them than that to their European counterparts, with which showed a distance of 33.8 and 37.8, respectively. This polymorphism would give this species enhanced adaptability against the host, as well as the environment. The existence of genetically different populations of *F. hepatica* could allow, against any selection pressure, natural or artificial (for use fasciolicides products and/or control measures), one or more populations of *F. hepatica* to be able to survive and create resistance or adaptability to such selective pressure.

Keywords *Fasciola hepatica* · Molecular characterization · RAPDs-PCR

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Introduction

The common liver fluke (*Fasciola hepatica*) is a major cause of economic losses to agriculture in temperate regions, with cost estimated at US$ 2000 million per annum (Boray 1994). The absence of an effective vaccine against the fluke control is achieved by chemotherapy. Triclabendazole (TCBZ) has been the drug of choice for treating liver fluke infections in livestock for over 20 years. More recently, it has been used successfully to treat human cases of fascioliasis. Anthelmintic resistance in parasitic of livestock is a chronic problem in the world. The intensive use of TCBZ has resulted in the development of resistant liver flukes; the resistance to TCBZ first appeared in farm animals in Australia in the mid-1990s (Overend and Bowen 1995), and since then has been reported in Europe and South America (Fairweather 2011; Olaechea et al. 2011; Ortiz et al. 2013). In 1990, a method was presented that proposes the use of initiators ("primer") for arbitrary molecular markers in any genome using the technique of polymerase chain reaction (PCR). The method described as "random amplified polymorphic DNA" (RAPD) (Williams et al. 1990). It came out as a simple process based on the amplification of genomic DNA with primers (one for each PCR reaction). Nucleotide sequence arbitrary function detects polymorphisms as genetic markers. The initiators have only 9 or 10 nucleotides in length and G+ C composition between 50

Published online: 18 January 2015

and 80 %. Each primer PCR amplified several segments of, and many of them are polymorphisms between the species analyzed (from both prokaryotic and eukaryotic organisms). Another important feature to consider is the dominant feature: You can tell when a segment of DNA has been amplified from a locus heterozygous (one copy) or homozygous (two copies) (Staub et al. 1996). In the rare cases where markers are co-dominant, RAPD seen as segments of different sizes amplified from the same locus. RAPD method was adopted by its simplicity and low cost, does not require radioactive tracers, and uses minimal amounts of DNA. Bands generated can be classified according to their intensity staining (strong, medium, weak) versus dye; this may be a reflection of the specificity of the amplification. Because of its "dominant inheritance," RAPD markers are expressed as presence or absence of a product amplified, which results in a loss of information when compared with markers inherited as "codominant" as is the case of the isoenzymes. However, RAPDs provide a huge source of data and thus may be more informative about the population structure and genetic diversity and that the isoenzymes (Campbell et al. 1999) is a useful genetic marker for the identification and characterization of parasite populations (Gasser 2006). Genetic polymorphisms establish the possible existence of genomic variants between individuals of the same species (Gomes et al. 2000).

A previous study with fluke samples from different countries (China, Niger, France, the USA, and Spain) shows that comparative analysis of different isolates of the same species from the same country exhibited small genetic variations for pcox1, pnad4, and pand5 (Ai et al. 2011). In Northern Europe, the information suggests that the colonization of the liver fluke involved at least two populations with distinct origins (Walker et al. 2011). This could occur either as a result of selection for variants best adapted to that environment or by genetic drift. In species which reproduce panmictically and exclusively by sexual means, there is a tendency for any nuclear mutations that occur in a population to be lost as a result of subsequent sexual reproduction. In contrast, the flukes forming the infrapopulation in the mammalian definitive host have the potential for self-fertilization or asexual reproduction, reviewed by Fletcher et al. (2004), which is followed by asexual polyembryony in the intermediate host. These processes may favor the relatively rapid establishment in the population of neutral or favorable mutations (Walker et al. 2011). In another study that shows genetic diversity of liver fluke populations in three different countries from Eastern Europe (Greece, Bulgaria, and Poland), it was revealed that there is a distinction of polymorphisms in populations in Eastern Europe on the South/North direction. The results verified the theory that such distinction does exist. It was hypothesized that this distinction is due to the territorial and population division of the chosen geographic regions in the past eras (Teofanova et al. 2011).

The present study shows the comparative molecular characterization of different strains of *F. hepatica* susceptible and resistant to TCBZ using the random amplified polymorphic ADN fragments (RAPDs-PCR) technique.

Materials and methods

Isolate used, experimental infection, and collecting of flukes

Four different isolates (Table 1) were used. For details of the history of this fluke isolate, please see (Fairweather 2011; Ortiz et al. 2013; Sanabria et al. 2013). Two parasite-free Corriedale weaned lambs were orally inoculated with 200 metacercariae of *F. hepatica* of each strain, contained in a gelatin capsule. The liver fluke infection was confirmed 16 weeks later by the presence of eggs in the feces and indirect estimation of liver damage by measurement of high levels of serum glutamate dehydrogenase and gamma glutamyl transferase activities in the host, assayed as described previously (Solana et al. 2001).

The animals were stunned and exsanguinated immediately. Animal procedures and management protocols were approved by the Ethics Committee according to Animal Welfare Policy (Act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar), and to the internationally accepted animal welfare guidelines (AVMA 2001). Flukes were recovered from the common bile ducts of each lamb and rinsed thoroughly with warm (37 °C) sterile saline solution (0.9 %, *w*/*v*, sodium chloride) to remove bile and/or adhering materials. Samples were preserved in ethanol 99 % at −20 °C until their use.

DNA extraction and PCR conditions

The genomic DNA of each sample was isolated following the standard phenol–chloroform procedure (Sambrook et al. 1989). Ten commercial decamer oligonucleotides (Serie A Biodynamics®) were evaluated for the amplification of random DNA markers with the requirement that the percentage of guanine and cytosine (G&C) content fluctuated between 50 and 70 % (Williams et al. 1990) (Table 2).

Table 1 Isolates of *Fasciola hepatica* used in this study

Strains	ABZ	TCBZ	Geographic origin
Cullompton	Susceptible	Susceptible	UK
Cedive	Resistant	Susceptible	Argentina
Sligo	Susceptible	Resistant	UK
Cajamarca	Susceptible	susceptible	Peru

Table 2 Primers tested for random amplified polymorphic DNA analysis of *Fasciola*

Code	Sequence (5′–3′)	Size	G+C (%)
A01	CCC AAG GTC C	10	70
A02	GGT GCG GGA A	10	70
A03	AAG ACC CCT C	10	60
A04	CTT CAC CCG A	10	60
A05	CAC CAG GTG A	10	60
A06	GAG TCT CAG G	10	60
A07	CCC GAT TCG G	10	70
A08	ACG CAC AAC C	10	60
A09	CTA ATG CCG T	10	50
A10	ACG GCG TAT G	10	60

A polymerase chain reaction (PCR) was carried out in 25 μl of total volume containing Dntps 20 mM, 2 μl; Cl_2Mg 25 mM, 2 μl; buffer 10X 2.5 μl; Taq 500 U Invitrogen® 25 μl; primer 1 μl; DNA 20 ng/μl, 1 μl; H_2O 16.25 μl. The PCR amplifications were carried out as follows: 2 min of denaturation at 94 °C followed by 45 cycles at 94 °C for 15 s, 40 °C for 30 s, 72 °C for 90 s, and final extension step of 72 °C for 5 min. Duplicate PCR reactions on each individual template DNA were performed to test the reproducibility of the individual DNA bands. In all PCR reactions, a negative control was included containing the reaction components except DNA template.

Visualization of fragments

DNA was resolved by agarose gel electrophoresis stained with 1 % SYBR® Safe. Electrophoresis was performed at 90 V. An indicator of molecular weight of 100 bp ladder (Highway®) was used. The amplified fragments were observed on a UV transilluminator. The size of the RAPD-PCR fragments was determined by the reciprocal plot between the delay factors (Rf) versus the logarithm of molecular weight ladder.

Analysis of the data and construction of the phenograms

Digital processing of amplicon bands on agarose gels was done using PyElph gel image analysis software (Pavel and Vasile 2012). Data were transformed into 1 (present) or 0 (absent) matrix over all genotypes and fragments scored. Genetic distances between genotypes were calculated on the basis of Pearson's coefficient algorithm with 100 bootstrap replicates using Dendro UPGMA on line (http://genomes.urv.cat).

Results

The purification and concentration of genomic DNA for analytical studies from *F. hepatica* from different strains was achieved by a sequential cell lysis, deproteinization, phenol extraction, and ethanol precipitation of DNA. The Å260 to Å280 ratio was found to vary from 1.66 to 1.77 among various samples indicating the purity of DNA. The purity of the DNA was further checked on 1 % agarose electrophoresis. The isolated DNA was found to be relatively intact and free from smearing.

The results of RAPD profiles are shown in Figs. 1 and 2.

The phylogenic analysis was carried out based on Pierce similarity coefficients generated from 18 RAPD bands which ranged from 250 to 5500 bp. The cophenetic correlation values for the phenogram a degree of 0.867, $p<0.01$, and phenograms suggest that the cluster analysis strongly represents the similarity matrix. Bootstrapping analysis resulted in at least on an average of 72.5 % of confidence limits for the major clusters.

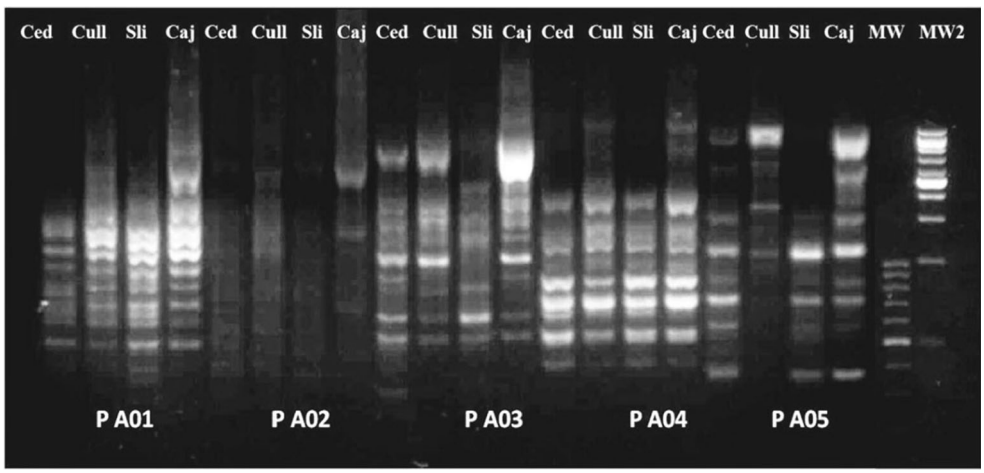

Fig. 1 Agarose gel electrophoresis of RAPD-PCR products of *Fasciola hepatica*. Patterns produced by primers 1–5.*Ced* Cedive strain, *Cull* Cullompton strain, *Sli* Sligo strain, *Caj* Cajamarca strain, *MW* 100 bp DNA marker, *MW2* 1 kp DNA marker

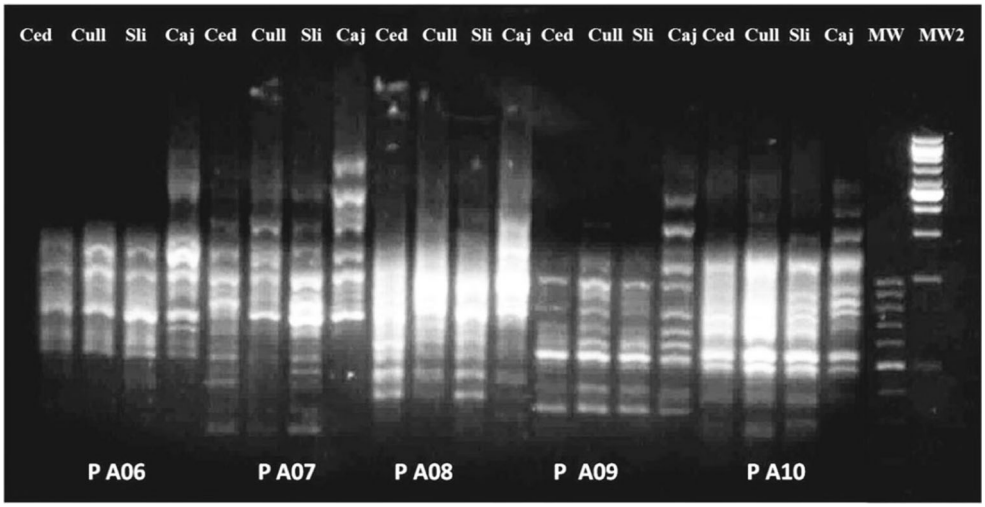

Fig. 2 Agarose gel electrophoresis of RAPD-PCR products of *Fasciola hepatica*. Patterns produced by primers 6–10. *Ced* Cedive strain, *Cull* Cullompton strain, *Sli* Sligo strain, *Caj* Cajamarca strain, *MW* 100 bp DNA marker, *MW2*: 1 kp DNA marker

The phenogram calculated by using Pierce coefficient (Fig. 3) algorithm showed three main clusters, the major of which contained European strain Cullompton and Sligo (UK and Ireland), sensitive and resistant to TCBZ, respectively, were grouped together showing a genetic distance of 27.2 between them. American strains Cedive (Argentina) and Cajamarca (Peru), sensitive and resistant to TCBZ, respectively, on the other hand formed each their distinctive group but clearly maintaining a closer genetic relationship among them than that to their European counterparts.

Discussion

The use of the RAPD marker has the advantage of being quick and simple and allows searching for polymorphisms in complete genomes (Gasser 1999). *F. hepatica* genomes analyzed by RAPDs appear to be grouped to their respective geographical origin. The phylogenetic tree for different trematodes as illustrated by the consensus phenogram generated by Pearce algorithm was clear to show a geographical grouping, beyond their degree of resistance to TCBZ; the topological phenogram shows (Fig. 3) that European parasites are grouped together regardless of their anthelmintic resistance; consensus phenogram reveals the facts that *F. hepatica* are grouped according to their genetic characteristics other than anthelmintic resistance.

The present investigation categorizes American flukes from diverse ecogeographical regions and morphological characteristics into different clusters than the European flukes.

The size of these fragments fluctuated between 250 and 55, 000 bp approximately. The results that show genetic polymorphism in *F. hepatica* of amplified DNA fragments were present in some samples and absent in others. This polymorphism would give this species enhanced adaptability against the host, as well as the environment. This has been suggested in previous studies to compare parasites from cattle, where the results suggest that populations from sheep show lower levels of gene

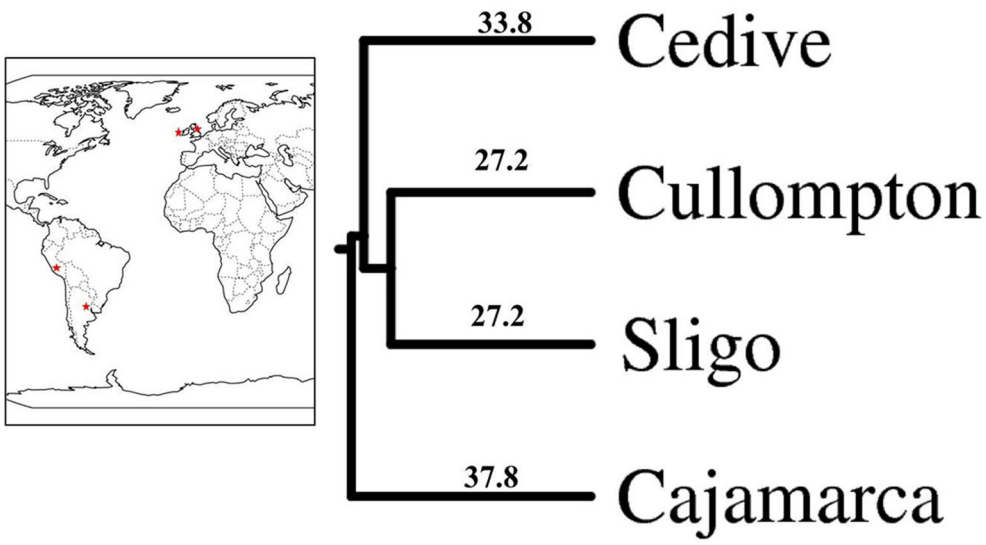

Fig. 3 Phenogram consensus algorithm coefficient Pearce able bootstrap replicates with 100 for primer (phenogram was constructed from 10 phenograms and the averages of the genetic distances between samples). *Numbers* indicate the genetic distances between strains. The map shows the localization of the different strains in the world

flow, higher degree of aggregate transmission, higher probability of mating within clones, and lower parasitic load. These differences have implications for the evolution of anthelmintic resistance because they affect the effective population size and the degree of inbreeding. The development and rapid spread of resistance seems likely in the parasites of cattle because populations from the study area are characterized by high gene flow. However, results also suggest that the efficient selection of a new recessive advantageous mutation would be favored in parasites of sheep due to a greater potential for inbreeding (Vilas et al. 2012). Finally, the analysis of fluke populations in South-Eastern Australia has revealed a high level of diversity in field and drug-resistant flukes; in contrast, TCBZ-susceptible Sunny Corner laboratory isolate exhibited very little diversity. This lack of diversity may have been caused from years of inbreeding of this isolate in the laboratory since being isolated in 1989 (Fairweather 2011). Similarly, they also observed little diversity in the Norvic field isolate, demonstrating that low diversity may be anormal property of certain fluke populations (Elliott et al. 2014).

The existence of genetically different populations of *F. hepatica* could allow, against any selection pressure, natural or artificial (for use fasciolicides products and/or control measures), one or more populations of *F. hepatica* to be able to survive and create resistance or adaptability to such selective pressure.

Conflict of interest The authors declare that they have no conflict of interest.

Declaration of animal welfare All the authors of the present manuscript declare that animal procedures and management protocols were approved by the Ethics Committee according to Animal Welfare Policy (Act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar), and to the internationally accepted animal welfare guidelines (AVMA 2001). The experiments comply with the current laws of the country in which they were performed.

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