"Development and validation of a mtDNA multiplex PCR for identification and discrimination of *Calicophoron daubneyi* and *Fasciola hepatica* in the *Galba truncatula* snail"

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Running title: *C. daubneyi* and *F. hepatica* mtDNA multiplex PCR diagnosis in snails.

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ABSTRACT

Paramphistomosis and Fasciolosis caused by *Calicophoron daubneyi* and *Fasciola hepatica*, respectively, are frequent and important trematodoses in ruminant livestock worldwide. Both parasites use the same snail, *Galba truncatula*, as intermediate host. The aim of this study was to develop and validate an analytical method based on a mitochondrial DNA (mtDNA) multiplex PCR technique which would allow the early and specific identification, in one step, of *C. daubneyi* and *F. hepatica* infection in *G. truncatula*. First of all, a 1035 bp fragment of mtDNA from adult *C. daubneyi* worms was obtained. Then specific mtDNA primers, which amplified a DNA fragment of 885 pb in the case of *C. daubneyi*, and of 425 pb in that of *F. hepatica,* were designed. By means of the multiplex PCR technique developed, there was always a specific amplification in samples from adult *F. hepatica* and *C. daubneyi*, but not from *Calicophoron calicophorum*, *Cotylophoron cotylophorum*, *Cotylophoron batycotyle* or *Dicrocoelium dendriticum*. Likewise, specific amplifications of the expected DNA fragments happened in all samples from snails harbouring larval stages of *C. daubneyi* or *F. hepatica*, previously detected by microscopy. However, amplifications were not seen when DNA from snails harbouring other Digenea (Plagiorchiidae, Notocotylidae and furcocercous cercariae) was analysed. Moreover, DNA from *G. truncatula* molluscs free from infection was not amplified. The multiplex PCR assay permitted infection in the snails experimentally infected with 4 miracidia to be detected as early as day 1 p.i. in the case of *F. hepatica* and with only 2 miracidia from day 2 p.i. in both, *C. daubneyi* and *F. hepatica*. Nevertheless it was necessary to wait until days 29 and 33 p.i. to see *C. daubneyi* and *F. hepatica* immature redia, respectively, using microscope techniques. The detection limit of the PCR technique was very low: 0.1ng pg of DNA from *C. daubneyi* and 0.001ng from *F. hepatica*. This allowed infection by either *F. hepatica* or *C. daubneyi* to be detected even when pools made up with only 1µl (60 ng) from infected snail plus 99 µl from non-infected ones were analyzed. Moreover, simultaneous detection of both parasites was experimentally possible in pools made up with uninfected (98 µl), *C. daubneyi* infected (1µl) and *F. hepatica* infected (1µl*)* snails*.* The most precise and early diagnosis of the infections using the multiplex PCR technique designed will allow more realistic epidemiological models of both infections to be established and consequently a better strategic control.

KEYWORDS: Digenea, *Calicophoron daubneyi*, *Fasciola hepatica*, *Galba truncatula* snail, Multiplex PCR identification, Mitochondrial DNA (mtDNA).

1. INTRODUCTION

The digestive Paramphistomosis caused by the *Calicophoron daubneyi* (Dinnik, 1962) Eduardo, 1983 and the hepatic Fasciolosis produced by *Fasciola hepatica* Linaeus, 1758 are frequent parasitoses in ruminant livestock all around the world. Fasciolosis, which is also a severe zoonosis, has been thoroughly studied (Hope Cawdery *et al.*, 1977; López-Díaz *et al.*, 1998; Spithill and Dalton, 1998; Mas-Coma *et al.*, 2005; Schweizer *et al.*, 2005; Elitok *et al.*, 2006; Mezo *et al.*, 2011, amongst others) and its negative impact on ruminant health and productivity is well known. On the contrary, there are very few studies on Paramphistomosis caused by *C. daubneyi*, a parasite that has been considered practically inoffensive for many years. Nevertheless, studies carried out in Europe (Mage *et al*., 2002; Cringoli *et al.*, 2004; Rinaldi *et al.*, 2005; Díaz *et al.*, 2007; Rieu *et al*., 2007; Foster *et al.*, 2008; Murphy *et al.*, 2008; González-Warleta *et al.*, 2012, amongst others) have shown that the prevalence of this parasitosis has significantly increased in the last few years. Moreover, several cases with clinical symptoms including death have been described (Dorchies *et al*., 2002), so this parasitosis must be considered in the differential diagnosis of enteric processes in ruminants.

Both *C. daubneyi* and *F. hepatica* have an indirect life cycle and use the same freshwater mollusc, *Galba truncatula* (Müller, 1774) (Lymnaeidae) as intermediate host. Natural infections by *F. hepatica* larvae have been frequently found in *G. truncatula* (Manga-González *et al.*, 1991; Augot *et al.*, 1996; Abrous *et al.*, 1999; Szmidt-Adjide *et al.*, 2000; Mas-Coma *et al.*, 2001; Relf *et al.*, 2011). When Dinnik (1962) attempted to infect water snails of different genera (*Bulinus, Physopsis, Physa, Anisus* and *Lymnaea*) with *C. daubneyi (= Paramphistomum daubneyi)* miracidia, larval development was only observed in *G. truncatula* (=*Lymnaea truncatula*). This snail species can also harbour double infections by both species of trematode (Abrous *et al.*, 1999; 2000). Moreover, *G. truncatula* can harbour larval stages of other Digenea, such as Notocotylidae, Plagiorchiidae and Echinostomatidae (Simón-Vicente *et al*., 1985; Skovronskii, 1985; Hourdin *et al*., 1991; Manga-González *et al*., 1994). In all these studies snail infections were diagnosed by dissection of the molluscs and subsequent examination by microscopy. These techniques are tedious, do not detect the early stages of the infection and require great experience to distinguish the larval stages from different parasites, so new diagnostic tools that allow early detection with high specificity are required.

Molecular techniques seem to be a good choice. By applying these methods for the first time Kaplan *et al.* (1995, 1997) were able to detect modest quantities of *F. hepatica* DNA in different host

snails. Later studies have confirmed the usefulness of Polymerase chain reaction (PCR) techniques for detecting *F. hepatica* infection in *G. truncatula* (Caron *et al.*, 2008, 2011; Kozak and Wedrychowicz, 2010), as well as in other *Lymnaea* species (Magalhaes *et al.*, 2004; Cucher *et al.*, 2006).

Molecular techniques have hardly been used for the study of Paramphistomidae (Sripalwit *et al*., 2007; Bazsalovicsova *et al.*, 2010; Sanabria *et al.*, 2011; Lofty *et al.*, 2010), and specifically in the case of *C. daubneyi*, only one technique has been described for the identification of adult worms, based on the use of the second internal transcribed spacer (ITS-2) of ribosomal DNA (Rinaldi *et al.*, 2005).

Bearing all the above in mind, the aim of this study was to develop and validate a sensitive and specific analytical method based on a mitochondrial (mtDNA) multiplex PCR technique for one-step early detection of infections by *C. daubneyi* and *F. hepatica* in the intermediate host snail. This multiplex PCR technique will allow reliable epidemiological models of both infections to be obtained and rational control strategies to then be established.

2. MATERIALS AND METHODS

2.1 Adult parasite and snail samples for DNA extraction

2.1.1 Adult parasites

DNA was extracted from the following parasites obtained at the slaughterhouse from naturally infected sheep and cattle:

A/ *Calicophoron daubney*i worms collected from rumens of cows slaughtered in Corunna (Spain).

B/ *Fasciola hepatica* and *Dicrocoelium dendriticum* worms collected from livers of sheep slaughtered in León (Spain).

C/ *Calicophoron calicophorum, Cotylophoron cotylophorum* and *Cotylophoron batycotyle* worms collected from cattle from Veracruz, Morelos and Tabasco (Mexico). These parasites were kindly provided by Dr. L. Trejo-Castro from the “Centro Nacional de Parasitología” (Cenapa-Sagarpa), Jiutepec (Morelos, Mexico).

The parasites from Spain were washed three times in phosphate-buffered saline, pH 7.4 (PBS), and gentamycin (40 mg/l) at 37ºC and stored at -85ºC until DNA extraction. Worms from Mexico were kept in 70º alcohol.

2.1.2. Snails

2.1.2.1. Experimentally infected

Experimental infections were carried out in order to determine the sensitivity of the new technique. Two hundred *G. truncatula* snails (4-6 mm in size) collected in the field from areas not grazed by domestic ruminants were kept in the laboratory for 2 months in order to allow the development of potential infection by trematode parasites of the wild fauna. At the end of this period, the 10% (n=16) surviving snails (n=160) were examined under stereomicroscope and no trematode larvae were found so the existence of natural infection was rejected. The rest of the snails (n=144) were divided into three groups, which were experimentally infected with a dose of 4 miracidia/snail of *F. hepatica* (n=48), 2 miracidia/snail of *F. hepatica* (n=48) and 2 miracidia/snail of *C. daubneyi* (n=48). Miracidia for infections came from eggs obtained from either gall bladders of *F. hepatica* infected cows or cultures of *C. daubneyi* worms collected from cattle rumens. The *C. daubneyi* worms were cultivated at 37ºC and 5% CO_2 in RPMI 1640 medium (Sigma-Aldrich, Madrid, Spain), supplemented with streptomycin (100 mg/l) and penicillin (100,000 IU/l). After 24 h, the medium was collected and centrifuged (1000 X g, 10 min) and the eggs were resuspended in distilled water. Both *F. hepatica* and *C. daubneyi* eggs were incubated in the dark at 25ºC for 12 days for their development and then exposed to the light for egg eclosion and to obtain miracidia.

One mollusc from each group was selected daily for helminthological examination from the first day post infection (p.i.). Each mollusc was removed from its shell, dissected and examined under the stereomicroscope. When larval stages of trematodes were observed, they were counted (if possible) and a representative sample was extracted for specific determination under the microscope, to check the degree of parasite development and take the corresponding microphotographs and do the scale drawings. The data obtained thus allowed us to check that the larval stages found in the experimental infections with *C. daubneyi* coincided with those originally described by Dinnik (1962) for that species. Those obtained in the experimental infection with *F. hepatica* were also the same as those described by various authors for that parasite (Manga-González, 1999, amongst others).

Uninfected and experimentally infected snails were stored at -85ºC until DNA extraction.

2.1.2.2. Naturally infected

Sensitivity and specificity of the new technique was also tested by analysing DNA extracted from 230 *Galba truncatula* specimens collected from pastures in NW Spain (Table 1) grazed by *C.*

daubneyi and *F. hepatica* infected cattle. All the molluscs were microscopically examined to detect trematode larvae following the same protocol described in the previous section. Snails were classified according to the infection status as follows: 1/ infected with *F. hepatica* (n=88); 2/ infected with *C. daubneyi* (n=44); 3/ infected with Plagiorchiidae (n=10); 4/ infected with Notocotylidae (n=10); 5/ infected with furcocercous cercariae (unknown family) (n=1) and 6/ non-infected specimens (n=77). Infected and non-infected molluscs were frozen and stored at -85ºC until DNA extraction.

2.1.3 DNA extraction

Genomic DNA was extracted from all the samples mentioned above using the commercial extraction kit "Speedtools tissue DNA kit" (Biotools, Spain) following the manufacturer's instructions. The purity (260/280 wave length ratio) and concentration of DNA recovered from the samples were determined by a spectrophotometer (NanoDrop ND-1000).

2.2. Development of the mitochondrial DNA based multiplex PCR

2.2.1. Obtaining and analysing a C. daubneyi mitochondrial DNA fragment

The mitochondrial DNA (mtDNA) sequence of *C. daubneyi* is unknown, so to obtain a fragment of mtDNA of this parasite we first designed a pair of general oligonucleotides for Platelminthes. Using the CLUSTAL W program, the mitochondrial sequences (from GenBank) of twenty species of Plathelminthes (2 Monogenea, 7 Digenea and 11 Cestoda) were aligned for this and the best conserved zones of the mitochondrial sequences were determined, following Martínez-Ibeas *et al.* (2011). The pair of oligonucleotides which functioned best for the amplification of the *C. daubneyi* mtDNA was that formed by:

Cox1F: 5'-T**N**TGTTTTTT**K**CC**K**ATGCA**Y**TA-3'

LrRNAR: 5'-TC**YYR**GGGTCTTTCCGTC-3'

IUB code for mixed base positions:
N=G,A,T,C; **K**=G,T; **Y**=C,T.

The amplification of *C. daubneyi* adult worm DNA samples with these nucleotides produced a fragment of 1035 bp. After electrophoretic separation the band was cut out of the agarose gel and purified using the Speedtools PCR clean-up (Biotools) commercial kit, in accordance with the manufacturer's instructions. These PCR products were cloned in the pGemT Easy plasmid and sequenced in the Instrumental Techniques Laboratory, University of León (Spain) from the plasmid

primers. The sequence was then analysed using MegAlign (DNASnastar Inc., Madison, WI, USA) software following the ClustalW (DNA Star) method. The sequence was sent to the databank GenBank (Access number JQ815200).

2.2.2. Multiplex PCR assay design

The mtDNA fragment of C*. daubneyi* obtained in the present study (JQ815200) and a known mitochondrial fragment of *F. hepatica* (AF216697) were aligned with the sequences of *F. gigantica* (AB553784.1), *Fascioloides magna* (EF534997.1) and *Fasciolopsis buski* (EF027094.1) to design two pairs of primers based on the variable regions; these produced amplicons of different size which allowed the different species to be distinguished. The following primers were designed: for *C. daubneyi* Cd_Cox1F (forward) 5'-TGGAGAGTTTGGCGTCTTTT-3', and Cd_Cox1R (reverse) 5'-CCATCTTCCACCTCATCTGG-3' which amplified an 885 pb fragment; and for *F. hepatica* Fh_Cox1F (forward) 5'- GCCGGGTCCTCAACATAATA-3' and Fh_Cox1R (reverse) 5'-AGCACAAAATCCTGATCTTACCA-3', which amplified a 425 pb fragment.

The PCR multiplex was developed in a single reaction, using the two pairs of primers simultaneously. Amplification was carried out in a 20 µl reaction volume containing 8 µl of HotMasterMix (2.5x) (5 PRIME) (2.5 mM Mg_2+), 5 µl of nuclease-free water and 2 µl of specific primer of *F. hepatica* 10 µM and 4 µl of specific primer of *C. daubneyi* 10 µM. Different quantities of template DNA of between 6 and 300 ngs were tested, finally establishing that of 60 ng (1 µl) as the most appropriate; it was then routinely added to each reaction. The reaction was done in an Applied Biosystems 2700 thermocycler. The amplification parameters consisted of initial denaturation at 92ºC for 2 min, followed by 38 denaturation cycles (95ºC, 30 sec), annealing (65ºC, 30 sec) and extension (72ºC, 1.5 min), with a final extension phase at 72ºC for 10 min. As a control, 60 ng of DNA template from a *C. daubneyi* and *F. hepatica* adult, respectively, were used in all the analyses. Water was added to all the reactions instead of DNA as a negative control. The PCR products were analysed after electrophoretic separation at 120 V for 30 min on 1.5% agarose gels stained with GelRed and photographed using the Gel Doc XR (Bio-Rad) image capturer.

The effectiveness of the designed primers was assessed by analysing the DNA samples from adults of *C. daubneyi*, *C. calicophorum*, *C. cotylophorum, C. batycotyle*, *F. hepatica* and *D. dendriticum* in multiplex PCR (Section 2.1.1).

215 ***2.2.3. Validation of the multiplex PCR assay for F. hepatica and C. daubneyi detection in snails***

216 The specificity of the assay was tested using: 1/ molluscs naturally infected with other Digenea
217 (Plagiorchiidae, Notocotylidae, furcocercous cercariae) (Section 2.1.2.2) and 2/ molluscs free from
218 infection (Section 2.1.2.1).

219 The sensitivity of the multiplex PCR technique was tested by analysing: 1/ snails experimentally
220 infected by *F. hepatica* and by *C. daubneyi*, respectively (Section 2.1.2.1.); 2/ snails with natural
221 infections by *F. hepatica* or by *C. daubneyi* confirmed by microscopic techniques (Section 2.1.2.2)

222 Two experiments were carried out to discover the detection limit of the technique. In the first one
223 we determined the minimum amount of parasite DNA detected by our technique. For this, we
224 analysed samples containing decreasing quantities of parasite DNA (from 10 ng until 0.001 ng) in
225 uninfected snail DNA.

226 In the second experiment, we determined the maximum number of snails that is possible to mix
227 and analyze as a pool in order to detect the presence of a single infected snail. For this, we analysed
228 samples obtained by mixing 1 µl (=60 ng) of template DNA from a mollusc infected with *F. hepatica* or
229 *C. daubneyi* with 9, 19, 49 and 99 µl, respectively, from a pool of uninfected molluscs. Moreover, in
230 order to discover the simultaneous detection limit of both parasites (experimentally), we analysed
231 samples obtained by mixing 1 µl of template DNA from a mollusc infected with *F. hepatica*, 1 µl from
232 a mollusc infected with *C. daubneyi* and 8, 18, 48 and 98 µl, respectively, from a pool of uninfected
233 molluscs.

234 All snails testing negative in multiplex PCR were again analysed after adding 60 ng of DNA from
235 adult parasites in order to rule out the presence of PCR inhibitors.

236

237 **3. RESULTS**

238

239 **3.1. Analysis of the mitochondrial DNA fragment sequence of *C. daubneyi***

240 The pair of general primers for mtDNA of Plathelminthes amplified a 1035 bp fragment of the
241 DNA from adult *C. daubneyi* worms. Sequentiation showed that this fragment includes the partial
242 sequence of the COI (285 pb) gene in the 5' end, the complete sequence of the tRNA-Thr (threonine)
243 (72 pb) and part of the LrRNA sequence in the 3' end (667 pb).

244

3.2. Effectiveness of the multiplex PCR for specific amplification of *C. daubneyi* and *F. hepatica* DNA

The designed primers (Cd_Cox1F/R for *C. daubneyi* and Fh_Cox1F/R for *F. hepatica*) always amplified two clear DNA fragments of the size expected for *C. daubneyi* (885 pb) and for *F. hepatica* (425 pb). A specific amplification always happened in samples from *F. hepatica* and *C. daubneyi* adult worms (Fig.1). However, no amplification was seen in samples from adult specimens of *C. calicophorum*, *C. cotylophorum*, *C. batycotyle* or *D. dendriticum* (Fig 1).

3.3. Validation of the multiplex PCR assay for detection of *C. daubneyi* and *F. hepatica* in infected snails

Using the multiplex PCR assay, specific amplifications of the expected DNA fragments happened in all samples from snails harbouring larval stages of *C. daubneyi* or *F. hepatica* (Fig 2) visible under the stereomicroscope in their hepatopancreas. However, amplifications were not seen when DNA from snails harbouring other Digenea (Plagiorchiidae, Notocotylidae and furcocercous cercariae) larvae was analysed (Fig 2). Moreover, DNA from *G. truncatula* molluscs free from infection was not amplified.

The multiplex PCR assay allowed us to detect infection in the snails experimentally infected with 4 miracidia as early as day 1 p.i., in the case of *F. hepatica* (Fig. 3), and with only 2 miracidia from day 2 p.i. in both, *C. daubneyi* and *F. hepatica* (Fig. 4). Nevertheless it was necessary to wait until days 29 and 33 p.i. to see *C. daubneyi* and *F. hepatica* immature redia, respectively, using microscope techniques.

Of the 77 snails which were negative to infection in the microscope studies, 5 were positive to *F. hepatica* and none to *C. daubneyi* when analyzed using our multiplex PCR assay. In order to rule out the presence of PCR inhibitors, all the negative samples were again analysed by PCR after adding parasite DNA and specific amplifications were always obtained.

The detection limit of our PCR assay was very low since it was able to detect 0.1 ng of DNA from *C. daubneyi* and up to 0.001 ng of DNA from *F. hepatica* (Fig. 5). This low detection limit allowed the infection by either *F. hepatica* (Fig. 6) or *C. daubneyi* (Fig. 7) to be detected even when we analysed pools made up with only 1 µl from infected snail plus 99 µl from non-infected ones. Moreover, simultaneous detection of both parasites, under experimental conditions, was possible in a mixture of

100 µl from snails, one infected with *F. hepatica*, another infected with *C. daubneyi* and 98 free from both infections (Fig. 8).

4. DISCUSSION

The infection by Digenea in molluscs has traditionally been diagnosed by identifying the larval stages of the parasites under the microscope. However, this procedure has low sensitivity and requires wide experience, especially for the specific identification of the early larval stages. Due to this other more sensitive techniques, such as those based on molecular biology, are required, principally when various species of parasites share the same mollusc species as intermediate host. This is the case of *G. truncatula* that can harbour *C. daubneyi*, *F. hepatica* and other species of Digenea, mainly Plagiorchiidae, Notocotylidae and Echinostomatidae families.

The PCR technique has already been used for detecting *F. hepatica* in *G. truncatula* (Caron *et al.* 2008, 2011; Kozak and Wedrychowicz, 2010), *L. columella* and *L. viatrix* (Magalhaes *et al.*, 2004; Cucher *et al.*, 2006) with satisfactory results. The latter two authors used PCR techniques based on mitochondrial DNA, which allow a considerable increase in sensitivity due to the high number of copies of this DNA present in most cells (Le *et al.*, 2002). PCR techniques have hardly been used, however, for the detection of *C. daubneyi* larvae in snails, probably due to the fact that the health and economic importance of this parasite has been underestimated for many years.

A mitochondrial DNA-based multiplex PCR technique, which amplifies specifically *F. hepatica* and/or *C. daubneyi* DNA in infected *G. truncatula* snails, has been developed in this study. This technique showed a high sensitivity since the parasite DNA was detected in the experimentally infected snails with 2 miracidia of *F. hepatica* or *C. daubneyi* from the 2nd day p.i., and even earlier (1st day p.i.) in those infected with 4 *F. hepatica* miracidia. However, larval stages could not be microscopically observed until day 29 p.i. in the case of *F. hepatica*, or day 33 p.i. in the case of *C. daubneyi*. The sensitivity of our PCR technique was similar to that reported by Magalhaes *et al.* (2004) and Kozak and Wedrychowicz (2010) in snails experimentally infected with one *F. hepatica* miracidium. Unfortunately, snails infected with only one miracidium were not available for our study. Nevertheless, the high detection limit of our technique (0.1 ng of *F. hepatica* DNA and 0.001 ng of *C. daubneyi* DNA) suggests that a single miracidium DNA (0.5-1 ng) could be detected according to Kaplan *et al*. (1997).

Our multiplex PCR technique was also tested on 230 *Galba truncatula* snails collected from pastures grazed by cattle naturally infected with *C. daubneyi* and *F. hepatica*, and its good performance in field conditions was confirmed. Indeed, it correctly identified the infection in all the snails with larval stages of *C. daubneyi* and *F. hepatica* detected by microscopy, while it gave negative results for the snails infected with other species of Digenea. The analysis by multiplex PCR of the 77 specimens of *G. truncatula*, which was negative by microscopy, provided *F. hepatica* positive results for 5 snails, probably due to the higher sensitivity of the molecular techniques for the detection of the early larval stages (Cucher *et* al, 2006). Nevertheless, these results should be interpreted with caution because the existence of unknown non-specific reactions cannot be completely excluded. All PCR-negative snails were retested after adding DNA from both parasites and positive results were always obtained, so the presence of inhibitors in the snail tissues could be ruled out. This agrees with what was reported by Caron et al (2011), who detected the presence of PCR inhibitors in samples containing high quantities (1 µg) of DNA, but not in samples with quantities similar to that used in our technique (60 ng).

An observation to note is that we did not find double natural infections by *C. daubneyi* and *F. hepatica* in snails either by microscopy or PCR, probably due to antagonism phenomena previously observed by Samnaliev *et al*. (1978) in *G. truncatula* between *F. hepatica* and *Paramphistomum microbothrium* and between the latter and *Echinostoma lindoense*. Under the microscope Manga-González *et al.* (1994) only observed double infections in 4 specimens of *G. truncatula* (1 with *F. hepatica* and Notocotylidae; 3 with *F. hepatica* and Plagiorchiidae), that is, 0.4% of the 973 specimens found infected with several Digenea species on examining 6291 specimens collected in León (Spain). Manga-González *et al.* (2009) only found one mollusc with a double infection (*F. hepatica* and possible Plagiorchiidae), when they studied 6208 *G. truncatula* collected in Galicia (Spain), 4.42% of them infected with *F. hepatica* and 1.70% with *C. daubneyi*. These results contrast with the 2.3% of double infections in *G. truncatula* specimens found infected at the same time with *F. hepatica* and *C. daubneyi* in France (Abrous *et al.,* 2000).

An interesting aspect in relation to the sensitivity of the technique is its capability to detect a single snail infected by *C. daubneyi* or *F. hepatica* in a batch of 100 snails, reducing analysis time and costs, thereby improving performance in field conditions. This is particularly important for carrying out

epidemiological studies, in which a large number of snails have to be analysed to discover the source of infection.

In conclusion, the mitochondrial-based multiplex PCR technique developed in this study proved to be a very sensitive and specific tool for the early detection of infections by *F. hepatica* and *C. daubneyi* in their intermediate hosts. Its high sensitivity, which allows analysis of pools of up to 100 snails, enables its use in epidemiological studies involving the analysis of a great number of molluscs. The most precise and early diagnosis of the mollusc infections by *F. hepatica* and *C. daubneyi* using the multiplex PCR technique designed will allow more realistic epidemiological models of both infections to be obtained and consequently better strategic control to be established.

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TABLE AND FIGURE LEGENDS

Table 1. Trematode infections detected by microscopy in the *Galba truncatula* snails used for determining the specificity of the mt-PCR multiplex technique. Places in Spain from which these snails were collected.

Fig. 1. Products of PCR amplification of adult specimens of *Fasciola hepatica* and *Calicophoron daubneyi* in agarose gel with Gel Red, using specific mtDNA primers. Marker; **1/** *F. hepatica*; **2/** *C. daubneyi*; **3/** *Cotylophoron cotylophorum* (Tabasco, Mexico), **4/** *Cotylophoron batycotyle* (Acayucan, Veracruz, Mexico); **5/** *Calicophoron Calicophorum* (Tuxpan, Veracruz, Mexico); **6/** *Dicrocoelium dendriticum*; **7/** Negative control.

Fig. 2. Products of PCR amplification of *Fasciola hepatica* and *Calicophoron daubneyi* in agarose gel with Gel Red, using specific mtDNA primers. Marker; **1/** *F. hepatica* adult; **2/** *C. daubneyi* adult; **3/** *Galba truncatula* naturally infected with *C. daubneyi* rediae containing germinal mass and immature cercariae, visible under the stereomicroscope. **4/** *G. truncatula* naturally infected with *F. hepatica* rediae containing germinal mass and mature cercariae, visible under the stereomicroscope. **5/** *G. truncatula* naturally infected with Notocotylidae. **6/** *G. truncatula* naturally infected with Plagiorchiidae. **7/** *G. truncatula* naturally infected with furcocercous cercariae; **8/** Non-infected; *G. truncatula*; **9/** Negative control.

Fig. 3. Products of PCR amplification of *Fasciola hepatica* and *Calicophoron daubneyi* in agarose gel with Gel Red, using specific mtDNA primers. Marker; **1/** *F. hepatica* adult; **2/** *C. daubneyi* adult; **3/** *Galba truncatula* experimentally infected with 4 miracidia of *F. hepatica* slaughtered on day 1 p.i.; **4-7/** *G. truncatula* experimentally infected with 2 miracidia of *F. hepatica* slaughtered 2, 5, 6, 7 days p.i., respectively; **8/** Negative control.

Fig. 4. Products of PCR amplification of *Fasciola hepatica* and *Calicophoron daubneyi* in agarose gel with Gel Red, using specific mtDNA primers. Marker; **1/** *F. hepatica* adult; **2/** *C. daubneyi* adult; **3-7/**

G. truncatula experimentally infected with 2 miracidia of *C. daubneyi* slaughtered 2, 3, 6, 8, 9 days p.i., respectively; **8/** Negative control.

Fig. 5. Products of PCR amplification of *Fasciola hepatica* and *Calicophoron daubneyi* in agarose gel with Gel Red, using specific mtDNA primers. Marker; **1/** *F. hepatica* adult; **2**/ *C. daubneyi* adult; **3-7/** Pools made up of DNA *Galba truncatula* non-infected and 5 decimal dilutions from 10 ng to 0.001 ng of *F. hepatica* and *C. daubneyi* adult DNA, respectively. **9/** Negative control.

Fig. 6. Products of PCR amplification of *Fasciola hepatica* in agarose gel with Gel Red, using specific mtDNA primers. Marker; **1/** *C. daubneyi* adult; **2/** *F. hepatica* adult **3-6/** Pools made up of 1 µl (=60 ng) of template DNA from one *Galba truncatula* infected with *F. hepatica* and 9, 19, 49 and 99 µl of DNA from a pool of non-infected molluscs, respectively; **7/** Negative control.

Fig. 7. Products of PCR amplification of *Calicophoron daubneyi* in agarose gel with Gel Red, using specific mtDNA primers. Marker; **1/** *C. daubneyi* adult; **2/** *F. hepatica* adult **3-6/** Pools made up of 1 µl (=60 ng) of template DNA from one *Galba truncatula* infected with *C. daubneyi* and 9, 19, 49 and 99 µl of DNA from a pool of non-infected molluscs, respectively; **7/** Negative control.

Fig. 8. Products of PCR amplification of *Fasciola hepatica* and *Calicophoron daubneyi* in agarose gel with Gel Red, using specific mtDNA primers. Marker; **1/***F. hepatica* adult; **2/** *C. daubneyi* adult; **3-6/** Pools made up of 1 µl (=60 ng) of template DNA from one *Galba truncatula* infected with *Fasciola hepatica*, 1 µl of template DNA from one snail infected with *C. daubneyi* and 8, 18, 48 and 98 µl of DNA from a pool of non-infected molluscs, respectively; **7/** Negative control.

Table 1. Trematode infections detected by microscopy in the *Galba truncatula* snails used for determining the specificity of the mt-PCR multiplex technique. Places in Spain from which these snails were collected.

G. truncatula Origin	**Parasites** found
Corunna Province	
Burres (Arzua)	- *Fasciola hepatica*
San Vicente (Vilasantar)	- *Calicophoron daubneyi* - *Fasciola hepatica* - Plagiorchiidae - Notocotylidae
Vigo (Cambre)	- *Calicophoron daubneyi* - *Fasciola hepatica* - Furcocercous cercariae (unknown Family)
León Province	
Cofiñal	- *Fasciola hepatica* - *Plagiorchis elegans* (Plagiorchiidae)
Orones	- *Fasciola hepatica*
Primajas	- *Fasciola hepatica*
Redipollos	- *Fasciola hepatica* - *Notocotylus neyrai* (Notocotylidae)
Vegaquemada	- *Opisthioglyphe ranae* (Plagiorchiidae)

Fig.1

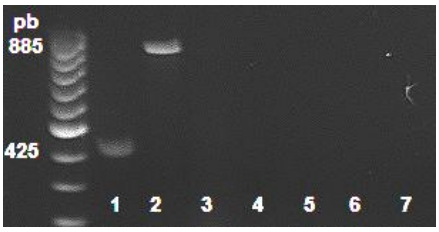

Fig.2

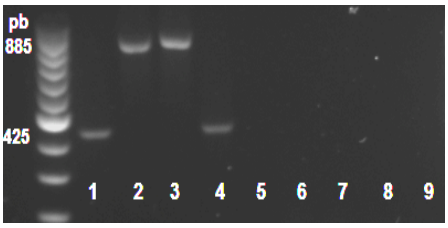

Fig.3

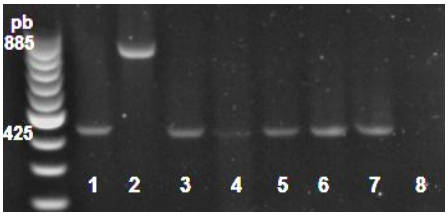

Fig.4

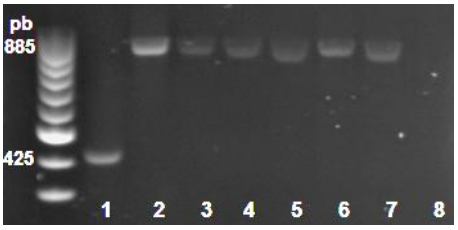

Fig.5

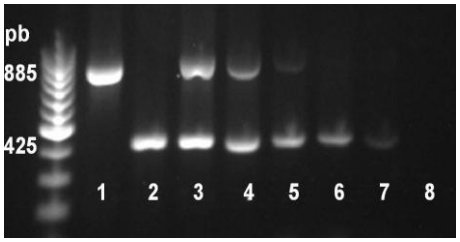

Fig.6

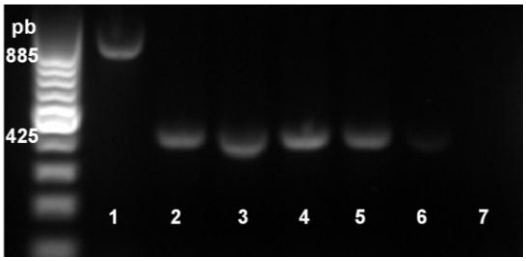

Fig. 7

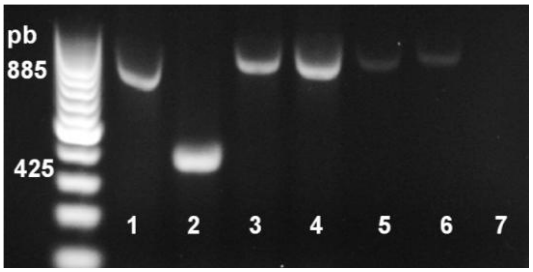

Fig.8