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Short communication

A TaqMan real-time PCR-based assay for the identification of *Fasciola* spp.

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ABSTRACT

Real time quantitative PCR (qPCR) is one of the key technologies of the post-genome era, with clear advantages compared to normal end-point PCR. In this paper, we report the first qPCR-based assay for the identification of *Fasciola* spp. Based on sequences of the second internal transcribed spacers (ITS-2) of the ribosomal rRNA gene, we used a set of genus-specific primers for *Fasciola* ITS-2 amplification, and we designed species-specific internal TaqMan probes to identify *F. hepatica* and *F. gigantica*, as well as the hybrid 'intermediate' *Fasciola*. These primers and probes were used for the highly specific, sensitive, and simple identification of *Fasciola* species collected from different animal host from China, Spain, Niger and Egypt. The novel qPCR-based technique for the identification of *Fasciola* spp. may provide a useful tool for the epidemiological investigation of *Fasciola* infection, including their intermediate snail hosts.

1. Introduction

Digenean trematodes of the genus *Fasciola* (Digenea: Fasciolidae) are the common liver flukes of a range of animals (especially sheep and cattle) with global geographical distribution (Spithill and Dalton, 1998). Fasciolosis caused by *Fasciola* spp. is a significant animal health problem, which causes substantial economic losses worldwide (Spithill and Dalton, 1998). Human infection with *Fasciola* spp. has been reported in a number of countries and millions of people are estimated to be infected, and hundreds of millions of people are at risk throughout the world with Bolivia, Peru, Egypt, the eastern Mediterranean, Vietnam and China being the hyper-endemic areas (Mas-Coma et al., 1999, 2005, 2009).

Of the several species which have been described within the *Fasciola* genus, only *Fasciola hepatica* and *Fasciola gigantica* are commonly recognized as taxonomically valid (Huang et al., 2004; Mas-Coma et al., 2005). While *F. hepatica* mainly occurs in temperate areas, *F. gigantica* occurs in tropical zones, but both species can overlapp in subtropical areas (Krämer and Schnieder, 1998; Mas-Coma et al., 2005; Alasaad et al., 2008). Based on the use of first and/or second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal RNA (rRNA) gene, an intermediate *Fasciola* between *F. hepatica* and *F. gigantica* has been identified in many coun-

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Table 1
Fasciola samples used in the post-optimisation evaluation of the TaqMan qPCR assay for *Fasciola* species identification. Samples were collected from the livers of the infected hosts.

Fasciola species	Geographical origin	Host species	No. of samples
F. hepatica	Spain (Valencia)	Horse (*Equus caballus*)	4
F. hepatica	Spain (País Vasco)	Ovine (*Ovis aries*)	5
F. hepatica	Spain (Lugo)	Bovine (*Bos taurus*)	2
F. hepatica	Spain (Mallorca, Balearic Islands)	Bovine (*Bos taurus*)	5
F. hepatica	Spain (Tenerife, Canary Islands)	Bovine (*Bos taurus*)	4
"Intermediate" *Fasciola*	China (Heilongjiang)	Bovine (*Bos taurus*)	4
F. gigantica	China (Guangxi)	Bovine (*Bos taurus*)	2
F. gigantica	Niger (Tera)	Ovine (*Ovis aries*)	2
F. gigantica	Egypt (Giza)	Egyptian water buffalo (*Bubalus bubalis*)	12

tries, such as China, Vietnam, Korea, Japan, Iran and Egypt (Itagaki and Tsutsumi, 1998; Huang et al., 2004; Ashrafi et al., 2006; Lin et al., 2007; Periago et al., 2008).

Morphological identification of *Fasciola* species requires significant parasitological expertise and is not a definitive method of characterization, especially for the 'intermediate' form (Kendall, 1965; Lin et al., 2007; Le et al., 2008). Hence, different molecular tools have been developed during the last decade for the accurate identification of *Fasciola* spp. (Marcilla et al., 2002; Velusamy et al., 2004; Cucher et al., 2006; Magalhães et al., 2008; Ai et al., 2010; Alasaad et al., in press). All these methods were based on end-point PCR.

Real time quantitative PCR (qPCR) is considered one of the most important molecular tools of the new genetic era (Syvanen et al., 1988). There are numerous applications of this technique in different molecular fields where the use of qPCR has nearly supplanted other approaches (Weksberg et al., 2005; VanGuilder et al., 2008).

Originally designed for gene expression assays (Bustin, 2000), TaqMan probe-based assays found wider applications in other molecular studies (Papli et al., 2010). TaqMan qPCR is characterized by its high specificity and sensitivity in comparison with the normal PCR. This technique requires no sample post-PCR manipulation, and is not only used for PCR amplification but also quantification (Livak et al., 1995). This technique was used by Schweizer et al. (2007) to estimate the prevalence of *F. hepatica* in the intermediate host *Lymnaea truncatula*. The objective of the present study was to develop and validate a new method based on TaqMan qPCR for the identification of *Fasciola* spp.

2. Materials and methods

2.1. Sample collection

Forty samples of adult *Fasciola* were collected from naturally infected horse, sheep, cattle and Egyptian water buffalo from China, Spain (mainland and islands), Nigeria, and Egypt (Table 1). Adult *Fasciola* specimens were washed extensively in a physiological saline buffer before being tentatively assigned to species according to its predilection site and morphological features, using the available keys and descriptions (Yamaguti, 1958). The flukes were then fixed in 70% ethanol until extraction of their genomic DNA. DNA samples representing heterologous species of *Fascioloides magna* (from chamois in Italy), *Schistosoma mansoni* (from mouse in Puerto Rico), *Schistosoma japonicum* (from cattle in Yunnan, China) and *Clonorchis sinensis* (from cat in Guangzhou, China), and DNA samples extracted from cattle and buffalo livers were used as negative control.

2.2. DNA extraction

Genomic DNA (gDNA) was extracted from tissue samples (~1 mm^3) following standard phenol/chloroform procedures (Sambrook et al., 1989). Two blanks (reagents only) were included in each extraction to monitor for contamination.

2.3. Fasciola generic-primers and TaqMan species-specific probes

We used the set of genus-specific primers for *Fasciola* ITS-2 amplification reported by Alasaad et al. (in press) SSCPFa*F*: 5′-TTGGTACTCAGTTGTCAGTGTG-3′ and SSCPFa*R*: 5′-AGCATCAGACACATGACCAAG-3′ (generating 140 bp amplicons), and based on comparison of the known ITS-2 sequences of *Fasciola* species (Huang et al., 2004; Alasaad et al., 2007), we designed novel species-specific TaqMan probes for the identification of *F. hepatica* (ProFh: 5′-ACCAGGCACGTTCCGTCACTGTCACTTT-3′) and *F. gigantica* (ProFg: 5′-ACCAGGCACGTTCCGTTACTGTTACTTTGTC-3′). Probes were designed using Primer3 (v. 0.4.0) (Rozen and Skaletsky, 2000), according to the parameters required for the qPCR applications. Both TaqMan probes were labelled with a BHQ quencher dye (Kapa Biosystems) at their 3′-end, but at the 5′-end ProFh was labelled with an FAM reporter dye and ProFg with an HEX reporter dye.

Amplification reactions contained 0.3 μM of each primer (SSCPFaF and SSCPFaR), 0.1 μM of each probe (ProFh and ProFg), 1× Master Mix (Kapa Probe Fast qPCR Kit), 1 μL of DNA solution (replaced by water in No Template Controls) and nuclease free-water in a final volume of 20 μL. Cycling conditions for the PCR consisted of a 2 min start-up denaturation step at 95 °C, followed by 45 cycles of amplification for 3 s at 95 °C and 30 s at 60 °C. PCR efficiency was considered and tested by the standard curve during primer selection. In all reactions, it lay between 90% and 110%. DNA-extracts were amplified in duplicate assays, and negative control samples and qPCR blanks were added in all assays.

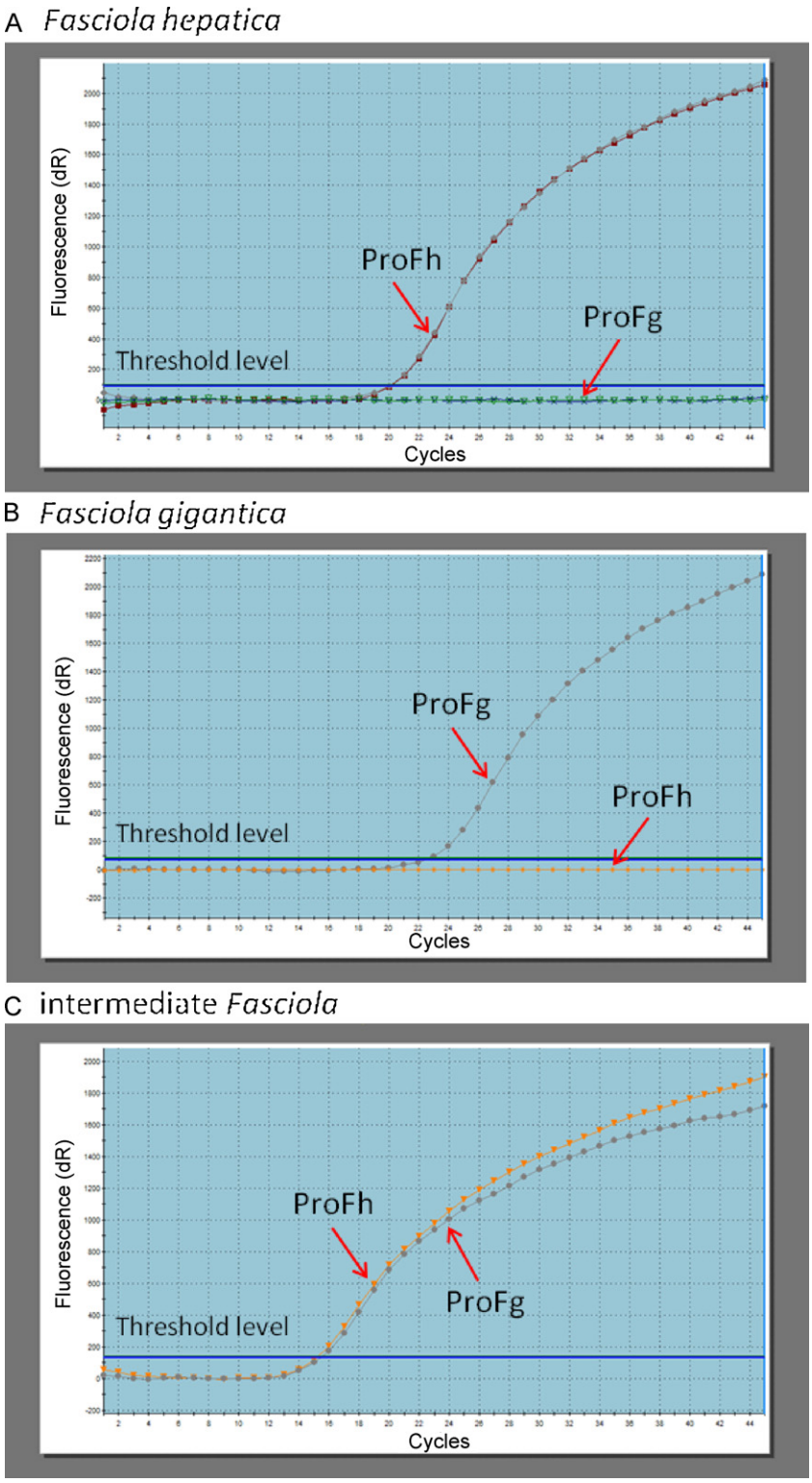

Fig. 1. Representative amplification plots of TaqMan qPCR from *F. hepatica* (A), *F. gigantica* (B) and the 'intermediate' *Fasciola* (C). C_T is the cycle at which the fluorescence exceeds a predetermined threshold level. The threshold level is an arbitrary level of fluorescence chosen on the basis of the baseline variability.

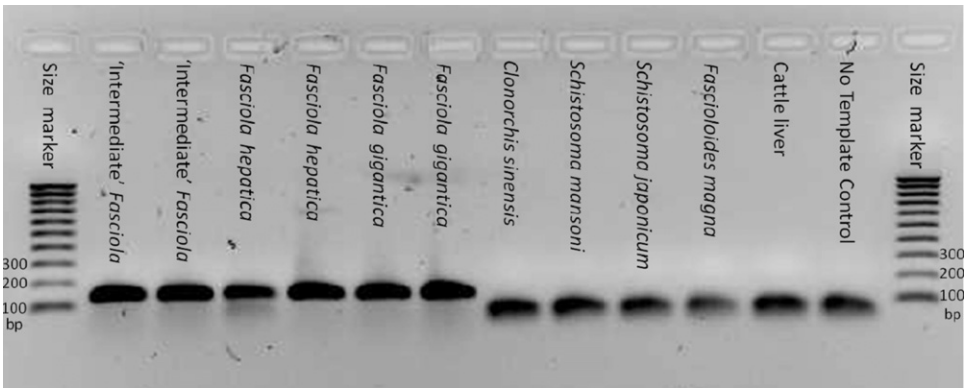

Fig. 2. Agarose gel (2%) showing the normal PCR amplicons produced, using the generic *Fasciola* primers SSCPFaF and SSCPFaR.

The qPCR was carried out in an Mx-3005P cycler (Stratagene). Data were analysed with the software package MxPro v4.00 (Stratagene).

2.4. Assessment of specificity and sensitivity of the TaqMan qPCR assay

The specificity of the generic primers and TaqMan probes for *Fasciola* identification were evaluated using the reference samples of *F. hepatica*, *F. gigantica* and the 'intermediate' *Fasciola*, as well as the heterologous samples of *F. magna*, *S. mansoni*, *S. japonicum* and *C. sinensis*, and DNA samples extracted from cattle and buffalo livers, as negative control. Specificity was verified by DNA sequencing of the PCR product (using SSCPFaF and SSCPFaR primers) of all the 40 samples used in the present study. Normal PCR components, their concentrations, and thermal profile were similar to that reported by Alasaad et al. (2007), with the exception of the substitution the primers by SSCPFaF and SSCPFaR.

The sensitivity of our assay was assessed using a ten-fold dilution series (between 50 ng/μL and 0.5 ng/μL) and two-fold dilution series (between 50 ng/μL and 0.244 pg/μL) of *F. hepatica* gDNA. The limit of detection was based on the final dilution at which the signal of the TaqMan probes was still exponentially amplified. After optimisation, the developed TaqMan qPCR assay was used to investigate the collected 40 samples (Table 1).

3. Results and discussion

The TaqMan qPCR technique was successful in all 40 *Fasciola* samples, evidenced by ProFh and ProFg probe-based identification of *F. hepatica* and *F. gigantica*, respectively and by the identification of the 'intermediate' *Fasciola* using the combined probes approach (Fig. 1). No false positives were generated by our TaqMan probes from those heterologous samples of *F. magna*, *S. mansoni*, *S. japonicum* and *C. sinensis*, or the cattle and buffalo DNA samples. For verification purposes we amplified all fragments in a regular end-point PCR using primers SSCPFaF and SSCPFaR (Fig. 2). Subsequent sequence analysis demonstrated that the *Fasciola* sequences obtained by us were identical to those reported by Huang et al. (2004) (GenBank accession numbers AJ557567–AJ557571).

TaqMan qPCR is characterized by its high specificity in comparison to normal PCR. The specificity of the TaqMan RT qPCR is guaranteed by the double complementarity

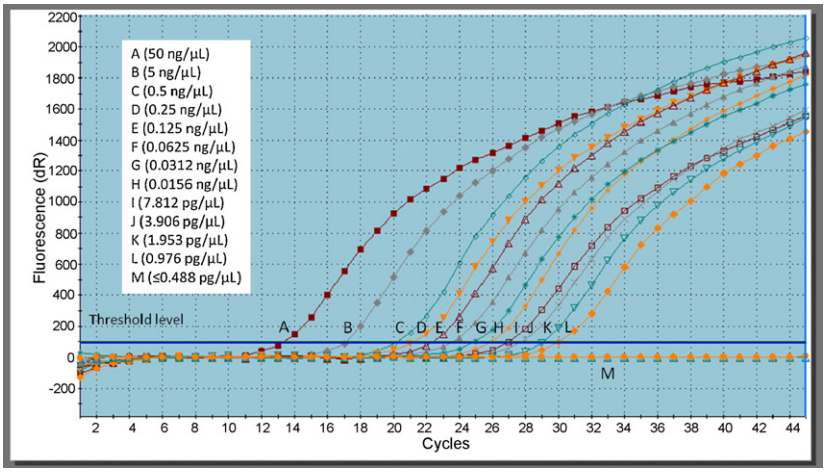

Fig. 3. TaqMan RT qPCR amplification of gDNA from *Fasciola hepatica* several dilutions.

between the set of primers, the internal probes and the targets, being the fluorescent signal generated only by a correct annealing between probes and the target sequence during the PCR amplification (Livak et al., 1995); while in the case of normal PCR, the specificity is guaranteed by single complementarity between the set of primers and the targets (Saiki et al., 1985).

The sensitivity of the TaqMan qPCR method was higher (the minimum amount of *Fasciola* gDNA detected was ~1 pg/μL) compared with end-point PCR (~1 ng/μL *Fasciola* gDNA detected, see Ai et al., 2010). This can be attributed to (i) the short fragment amplified (140 bp), (ii) to the fact that the detection limit in a normal PCR is based on the final dilution at which a PCR product is still visible in agarose gels. In contrast to that the fluorophore's signal of the species-specific TaqMan probes is still detectable at much lower concentrations (Fig. 3), and/or (iii) to the used PCR mixtures/conditions of the TaqMan qPCR and end-point PCR.

An additional advantage of TaqMan qPCR is that it requires no sample post-PCR manipulation of samples. Most DNA-based techniques for parasite identification involve multiple post-PCR manipulations of samples, such as double PCR, nested PCR, semi-nested PCR, sequencing reactions, restriction digests, and/or gel electrophoresis before results can be obtained. These manipulations add time and costs, and increase the chances of human error and contamination (Berry and Sarre, 2007; O'Reilly et al., 2008).

In conclusion, this is the first report of qPCR-based technique for the identification of *Fasciola* spp., which may prove a useful tool for the epidemiological investigation of *Fasciola* infection in humans and animals, including their intermediate snail hosts.

Conflict of interest statement

The authors declare that they have no conflict of interests.

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