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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.