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Rapid identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* by a loop-mediated isothermal amplification (LAMP) assay

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ABSTRACT

The present study developed and validated a species-specific loop-mediated isothermal amplification (LAMP) assay for the rapid detection and discrimination of *Fasciola hepatica* and *Fasciola gigantica*. The LAMP assay is inexpensive, easy to perform and shows rapid reaction, wherein the amplification can be obtained in 45 min under isothermal conditions of 61 °C or 62 °C by employing a set of four species-specific primer mixtures and results can be checked through naked-eye visualization. The optimal assay conditions with no cross-reaction with other closely related trematodes (*Clonorchis sinensis*, *Opisthorchis viverrini*, *Orientobilharzia turkestanicum* and *Schistosoma japonicum*) as well as within the two *Fasciola* species were established. The assay was validated by examining *F. gigantica* DNA in the intermediate host snails and in faecal samples. The results indicated that the LAMP assay is approximately 10^4 times more sensitive than the conventional specific PCR assays. These findings indicate that this *Fasciola* species-specific LAMP assay may have a potential clinical application for detection and differentiation of *Fasciola* species, especially in endemic countries.

1. Introduction

Fascioliasis is a parasitic disease caused by infection with the digenetic flukes of the genus *Fasciola*. These parasites inhabit the hepato-biliary system of the affected hosts, and in rare occasions they can be found in ectopic sites within the host body (Mas-Coma et al., 2005; Nguyen et al., 2009). Despite the major socio-economic importance and considerable zoonotic significance of this disease, the taxonomic classification of the genus *Fasciola* continues to be a challenge for both parasitologists and taxonomists. The two major *Fasciola* species – *Fasciola hepatica* and *Fasciola gigantica* – have been traditionally typified based on morphological features (Mas-Coma et al., 2005; Le et al., 2008; Itagaki et al., 2005, 2009). Recent molecular approaches such as PCR and DNA sequence analysis of the nuclear ribosomal internal transcribed spacers (ITS-1, ITS-2), 28S rRNA genes (Adlard et al., 1993; Itagaki et al., 1998; Marcilla et al., 2002; Itagaki et al., 2005) mitochondrial NDI and

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Table 1
Samples of *Fasciola* spp. used in the present study.

Sample codes	Location	Host	Stage	Identity[a]
FgCAY1	Ayorou, Niger	Cattle	Adult	*F. gigantica*
FgSTE1	Tera, Niger	Sheep	Adult	*F. gigantica*
FgCM1	Malgorou-Gaya, Niger	Cattle	Adult	*F. gigantica*
FgSTO1	Torodi, Niger	Sheep	Adult	*F. gigantica*
FgGXB2	Guangxi, China	Buffalo	Adult	*F. gigantica*
FgGXB6	Guangxi, China	Buffalo	Adult	*F. gigantica*
FgGZB3	Guizhou, China	Buffalo	Adult	*F. gigantica*
FgGZB5	Guizhou, China	Buffalo	Adult	*F. gigantica*
FgGXBegg1-FgGXBegg8	Guangxi, China	Buffalo	Egg	*F. gigantica*
FgGXS1-FgGXS6	Guangxi, China	Snail	Cercariae	*F. gigantica*
FhCM1	Maradi, Niger	Cattle	Adult	*F. hepatica*
FhFG5	France	Goat	Adult	*F. hepatica*
FhAM1	USA	Rabbit	Adult	*F. hepatica*
FhGSG17	Gansu, China	Goat	Adult	*F. hepatica*
FhOS	Bilbao, Spain	Sheep	Adult	*F. hepatica*
FhHS	Valencia, Spain	Horse	Adult	*F. hepatica*

[a] The species identification was based on specific PCR (Ai et al., 2010).

COI genes (Itagaki et al., 2005) have greatly enhanced the ability to differentiate members of the genus *Fasciola*. However, the existing nucleic acid amplification methods have several intrinsic drawbacks, in that they are time consuming (2–3 h), require either a high-precision instrument for amplification or an intricate method for detection of amplified products. More sensitive assays are therefore needed to complement the existing PCR-based assay systems.

Loop-mediated isothermal amplification (LAMP) is a relatively new technology that allows amplification of target nucleic acids under isothermal conditions with high sensitivity, specificity, rapidity and precision (Notomi et al., 2000; Nagamine et al., 2002). Originally developed by Notomi et al. (2000), the LAMP assay is based on the principle of a strand displacement reaction and stem-loop structure that amplifies the target with high degree of specificity, selectivity and rapidity under isothermal conditions, thereby obviating the need for the use of a thermal cycler (Nagamine et al., 2002). The method uses *Bst* polymerase with displacement activity and a set of 4 specifically designed primers that recognize a total of 6 distinct sequences of the target DNA (Sotiriadou and Karanis, 2008). One of the best features of the LAMP method is the extremely high amplification efficiency due to continuous amplification under isothermal conditions, which results in the production of a large amount of target DNA as well as a large amount of the by-product magnesium pyrophosphate, which leads to turbidity (Mori et al., 2001). Therefore, quantitative detection of gene amplification is possible by real-time monitoring of the turbidity in an inexpensive photometer. The higher amplification efficiency of the LAMP method enables simple visual observation of amplification with the naked eye under a UV lamp in the presence of an intercalating dye, such as SYBR Green I. For these reasons, the LAMP assay has emerged as a powerful gene amplification technique for rapid identification of microbial infections and has been applied to the detection and identification of African trypanosomiasis (Kuboki et al., 2003), *Cryptosporidium* (Karanis et al., 2007), and *Schistosoma japonicum* (Xu et al., 2010).

The objectives of the present study were to develop and evaluate a simple and cost-effective LAMP assay based on the ribosomal intergenic spacer (IGS) sequences for the rapid detection and differentiation of *Fasciola* species. Data on the sensitivity and specificity of the method are reported, and the feasibility of use of the technology for the speciation of adult and juvenile stages of *Fasciola* species is discussed. This methodology should enhance and supplement existing procedures to identify *Fasciola* species.

2. Materials and methods

2.1. Parasites and DNA extraction

Adult *Fasciola* spp. samples were collected from different geographical localities in China, Niger, France, USA, and Spain. Also, samples of *Fasciola* eggs and infected snails were collected from Guangxi Province, China. Details of each sample used in the study are provided in Table 1. Several related trematodes were included as 'heterologous control samples' to assess the specificity of the LAMP assay. These include *Clonorchis sinensis*, *Opisthorchis viverrini*, *Orientobilharzia turkestanicum* and *Schistosoma japonicum*. All samples were fixed in 70% molecular grade ethanol, and stored at −20 °C.

Total genomic DNA was extracted from individual worms, snails as well as eggs by sodium dodecyl sulphate/proteinase K treatment, column-purified (Wizard® SV Genomic DNA Purification System, Promega, Madison) as described previously (Zhao et al., 2009; Ai et al., 2010).

2.2. Design of Fasciola-specific LAMP assay primers

The success of the LAMP assay amplification depends on the specificities of the primer sets designed. The *Fasciola* species-specific oligonucleotide primers used for the LAMP assay amplification were selected on the basis of a highly conserved region of the parasite genome. The ribosomal intergenic spacer (IGS) nucleotide sequences of the prototype strains of each *Fasciola* species were retrieved from the GenBank (accession numbers are shown in parentheses), FhCM1-*F. hepatica* (GU903890), and FgCM1-*F. gigantica* (GU903891), and used to design species-specific primers using Primer Explorer V4 software