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A novel monoclonal antibody-based immunoenzymatic assay for epidemiological surveillance of the vector snails of *Fasciola hepatica* (Trematoda: Digenea)

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ABSTRACT

Fasciolosis is a globally distributed snail-borne disease which requires economic consideration due to its enormous impact on veterinary medicine. During recent decades, this parasitosis has also shown increasing prevalence in human populations worldwide. The dissemination and successful transmission of fasciolosis ultimately depends on the existence of susceptible snails that act as intermediate hosts. Therefore, to accomplish effective control of this disease, surveillance and detection of the infected intermediate host would be essential. The screening of trematodes within snails using classical parasitological examination of the larvae can be unreliable (sensitivity and specificity vary depending on the time of infection and the experience of the observer) and relatively costly when using molecular biological methods during large-scale monitoring. Here we propose a novel monoclonal antibody-based immunoenzymatic assay to detect ongoing *Fasciola hepatica* infection in lymnaeid snails. Anti-*F. hepatica* rediae mouse monoclonal antibodies were generated and used to develop a double monoclonal antibody-based ELISA for parasite detection. *Fasciola hepatica*-infected and uninfected laboratory-reared *Galba cubensis* and *Pseudosuccinea columella* were used for assessment of the developed ELISA. Experimentally infected snails were dissected and examined for parasite larvae as the "gold standard" method. Sensitivity results were 100% for both snail species, while specificity was 98% for *G. cubensis* and 100% for *P. columella*. No cross-reactivity was detected in lymnaeids infected with *Trichobilharzia* sp. or *Cotylophoron* sp. The ELISA enabled detection of the infection from day 8 p.i. in *G. cubensis* while in *P. columella* it was noted as early as day 4. To our knowledge no previous immunoassays have been reported to detect helminth-infected snails and the developed sandwich ELISA method is therefore suggested for infection status validation in natural populations of lymnaeid snails.

1. Introduction

Fasciola hepatica (Trematoda; Digenea; Fasciolidae), also known as the liver fluke, is the main etiological agent of fasciolosis worldwide. This disease stands as a re-emerging human infection with increasing prevalence but despite its increasing impact on human health, fasciolosis remains a neglected disease (Mas-Coma et al., 2009). On the other hand, its high prevalence in livestock results in a serious problem not only for veterinary medicine but also for the economies of many countries. Effects on domestic animal health have led to high economic losses due to liver condemnation, reduced milk and meat production, as well as reduced fertility and the high costs of drug therapy (Kaplan, 2001; Mezo et al., 2011).

The prevention of fasciolosis is generally based on time-consuming educational campaigns in communities at risk of fasciolosis (Robinson and Dalton, 2009). Several approaches and molecular candidates have been evaluated for vaccine development (Kennedy et al., 2006; Golden et al., 2010; Jayaraj et al., 2012) but unfortunately a commercially available formulation is still lacking. Alternatively, strategies to control transmission of the parasite are mostly based on anthelminthic treatment of infected humans and livestock. However, this is problematic given the existence and spread of parasite strains resistant to the drug of choice

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(triclabendazole) in several regions of the world (Fairweather, 2011). In addition, the ability of *F. hepatica* to infect a wide range of wild and autochthonous animals (e.g. rodents, lagomorphs, Andean camelids, marsupials) (Mas-Coma et al., 2009; Robinson and Dalton, 2009) combined with occasional inaccurate livestock management (including the application of anti-parasitic treatment) (Arias et al., 2010), complicates transmission control and almost ensures persistence of the parasite wherever infected snails occur. Therefore, in order to accomplish accurate control of this infection, control of the intermediate host populations is essential and requires suitable epidemiological surveillance of these molluscs (Caron et al., 2011).

Detection of *F. hepatica* in infected lymnaeids is subject to the drawbacks of classical parasitological examination of larvae found in snails (variable sensitivity and specificity depending on the time of infection and the experience of the observer), or to the relatively high cost of molecular biological methods employed during large-scale monitoring (see Caron et al. (2008) for a more comprehensive overview). In this sense, monoclonal antibody (mAb)-based assays may be helpful in achieving the sensitivity and specificity needed to detect the parasite within the snails with simple, useful and standardised procedures.

In a previous study, the molecular features of the long-lasting intramolluscan larvae (redia) were explored. The suitability of three different antigenic candidates from *F. hepatica* for further development of specific mAbs was evaluated and the crude extract of rediae was shown to be the most promising (Alba et al., 2014). Here, we present the development of a novel double mAb-based immunoenzymatic assay for the detection of *F. hepatica* in infected lymnaeid snails. To our knowledge, this is the first known report of an immunoassay aimed to identify helminths within their vector snails.

2. Materials and methods

2.1. Fasciola hepatica adults, experimental host snails and mice

Flukes were obtained from cattle abattoirs and kept alive overnight in a solution of 0.85% NaCl (saline solution), 5% glucose (Sigma, USA), for egg laying. Eggs were preserved at 4 °C, in saline solution supplemented with Gentamicin (50 μg/mL) (Sigma), until use. The excretion–secretion antigens (ES Ag) of adults of *F. hepatica* were also obtained according to Espino et al. (1990).

Laboratory-reared snail populations of the Cuban intermediate hosts, *Galba cubensis* and *Pseudosuccinea columella,* were obtained from the Laboratory of Malacology of the Institute of Tropical Medicine "Pedro Kourí" (IPK), Cuba.

BALB/c mice were provided by the Centre for Laboratory Animal Production of Cuba. Animal welfare and all procedures involving experimental animals were in accordance with approvals by the Institutional Animal Care and Use Committee, IPK.

2.2. Experimental infections in snails

Eggs of *F. hepatica* were incubated in distilled water, in total darkness, at 28 °C, for 15 days for complete maturation. At day 15, miracidia were obtained after the induction of egg hatching by exposure to light. Experimental infection of snails was carried out using freshly hatched miracidia, with five miracidia per snail, according to the methodology described by Vázquez et al. (2014).

2.3. Preparation of crude extracts

The crude extracts of rediae and miracidia of *F. hepatica* and the homogenates of *G. cubensis* and *P. columella* were obtained as previously described by Alba et al. (2014). Briefly, *F. hepatica* miracidia were obtained after egg hatching while *F. hepatica* rediae were obtained by dissecting experimentally infected snails. Larvae were washed extensively with saline solution and extracts from both rediae and miracidia were prepared by sonication.

Crude extracts of experimentally infected and non-infected laboratory-reared *G. cubensis* and *P. columella* were prepared in PBS, pH 7.2, using a Potter homogenizer. When required, protein quantification of the extracts was performed with a bicinchoninic acid reaction.

2.4. Development of mAbs

Five 6- to 8-week old female BALB/c mice were immunized with the crude extract of *F. hepatica* redia (Alba et al., 2014). Serum from each mouse was obtained before and 10 days after the last immunization. The serum titre was assessed by indirect ELISA against the crude extract of *F. hepatica* rediae described by Alba et al. (2014).

The mouse showing the highest titre was boosted by an i.v. injection with 30 μg of the crude extract of *F. hepatica* rediae in saline solution. Three days post-booster, the mouse was euthanised and the spleen was removed. Fusions between murine plasmacytomic cells P3X63 (ATCC Number: CRL-1580) and spleenic cells were induced by polyethylene glycol (Sigma). Hybridomas were first screened for anti-*F. hepatica* rediae antibodies by an indirect ELISA (see Section 2.5 for details). All rediae-positive supernatants were then tested for specificity against the crude extracts of non-infected *G. cubensis* and *P. columella.* The hybrids which were specific and highly reactive to rediae were cloned twice using limiting dilution, to recover homogeneous hybridoma cells line. The class and subclass of the mAbs were determined by indirect ELISA according to the manufacturer's guidelines (ISO-2, Sigma). The selected hybrids were inoculated i.p. into BALB/c mice for large-scale production.

IgG antibodies from ascitic fluid were purified on a Protein G Sepharose Fast Flow column (GE, Healthcare Life Science, USA) according to the manufacturer's directions. A portion of the antibodies was chemically coupled to horseradish peroxidase (HRP; Sigma) in order to additionally evaluate those as revealing reagents on the sandwich ELISA. IgM antibodies were purified by affinity chromatography on a Concanavalin A Sepharose Fast Flow column (Amersham-Biosciences, Sweden) followed by gel filtration on a Superose 6 column (GE, Healthcare Life Science).

2.5. Indirect immunoenzymatic assays

Polystyrene microtiter Maxisorp (NUNC, Denmark) plates were sensitised overnight, at 4 °C with different antigens depending on the purpose: (i) crude extract of *F. hepatica* rediae for assessing the reactivity of hybridoma supernatants and of purified mAbs to the target antigen, (ii) crude extracts of *G. cubensis* and *P. columella* for evaluating the cross-reactivity of anti-rediae hybridoma supernatants and of the purified mAbs against lymnaeid antigens, and (iii) ES Ag of *F. hepatica* adults and miracidia for partially characterising the purified mAbs. In all cases, 10 μg/mL of each antigen diluted in carbonate buffer pH 9.6 were used (100 μL/well) (Alba et al., 2014). Afterwards, plates were washed with PBS-0.05% Tween (PBST-T) and wells were blocked to prevent unspecific binding using 5% BSA (Sigma) in carbonate buffer, pH 9.6, for 1 h at 37 °C.

Culture supernatants were added to plates coated with the crude extracts of rediae and of snails (100 μL/well) during the selection procedure of hybridomas. The purified antibodies were diluted in PBS-T and added to the wells at a concentration of 1 μg/mL. A mouse anti-*Trichomonas vaginalis* mAb (Hernández

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