UNIVERSIDADE FEDERAL DO PARANÁ

IZANARA CRISTINE PRITSCH

A MOLECULAR AND EPIDEMIOLOGICAL STUDY OF *Fasciola hepatica* IN BRAZIL

CURITIBA

2019

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A MOLECULAR AND EPIDEMIOLOGICAL STUDY OF *Fasciola hepatica* IN BRAZIL

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Orientador: Prof. Dr. Marcelo Beltrão Molento

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RESUMO

A fasciolose, causada principalmente pela espécie *Fasciola hepatica*, é uma doença comum na pecuária (bovinos, ovinos, bubalinos e caprinos) responsável por perdas econômicas significativas. A doença também tem destaque como uma importante zoonose humana, onde até 17 milhões de pessoas estão infectadas em todo o mundo. O objetivo deste trabalho foi estudar a prevalência da doença em humanos, bovinos e búfalos aquáticos sob diferentes aspectos no Brasil e investigar novas alternativas de tratamento. A presente tese está dividida em introdução, revisão de literatura, objetivos, cinco capítulos e considerações finais. O primeiro capítulo mostra uma recontagem de casos de fasciolose humana (FH) no Brasil nos últimos 60 anos, onde foram encontrados 48 casos na literatura fornecendo um cenário atualizado da doença no país. No segundo capítulo é referente a investigação de FH em Piraquara, Paraná (PR) e Balneário Piçarras, Santa Catarina (SC). Neste capítulo, reportamos o primeiro caso positivo de FH em SC. A partir do nosso relatório, as autoridades locais foram notificadas para investigar outros possíveis casos, áreas de risco e fontes de contaminação. O terceiro capítulo é um estudo sobre a determinação da prevalência de anticorpos anti-*F. hepatica* em vacas leiteiras para avaliar a relação entre os níveis de anticorpos *Fasciola*-específico (*F. hepatica* catepsina L1 recombinante - FhCL1r) em tanques de leite e parâmetros de qualidade do leite em 1492 amostras do PR. A prevalência média no estado foi de 9,72%, enquanto a maior prevalência foi encontrada na região metropolitana de Curitiba (42,86%); Centro Oriental (13,01%); Noroeste (11,76%) e Centro Sul (8,82%). A análise de redundância, usando um modelo de regressão linear multivariado, mostrou que os teores de proteína do leite, lactose e extrato seco desengordurado foram menores nas amostras positivas em comparação com as negativas. O quarto capítulo mostra a prevalência de *F. hepatica* em bubalinos abatidos de 2003 a 2017 no Brasil. A análise dos dados mostrou uma prevalência total de 7187 casos (3%) de 226.561 indivíduos. A doença foi mais prevalente no PR, Rio Grande do Sul e SC (11,9; 7,7 e 3,2% para fígados condenados, respectivamente). Modelos preditivos indicaram uma tendência constante da doença, destacando a necessidade de acompanhamento regular. Finalmente, no quinto capítulo investigamos o mecanismo envolvido na inibição da catepsina L3 de *F. hepatica* (FhCL3) por seu propeptídeo (pp). ppFhCL3 é um inibidor altamente potente de duas catepsinas colagenolíticas de *F. hepatica*, FhCL3 e FhCL2 envolvidas no estabelecimento da infecção por *F. hepatica*. Além disso, propomos o mecanismo *"clamp"* envolvendo resíduos do pp que interagem com a alça de ligação (Tyr46p, Lys47p) e o sítio ativo com o substrato (Leu66p) para a correta ligação e inibição das catepsinas colagenolíticas de *F. hepatica*. Juntos, esses resultados mostraram uma atualização epidemiológica da doença, aproximando-se da prevalência humana e animal. Além disso, novas possibilidades de projetar inibidores seletivos para as catepsinas de *Fasciola* foram explorados.

Palavras-chave: Trematodas. Doenças de origem alimentar. Fasciolose. Imuno-diagnóstico no leite. Catepsina.

ABSTRACT

Fascioliasis, caused mainly by *Fasciola hepatica*, is a common disease of livestock (cattle, sheep, water buffalo and goats) responsible for significant economic losses. The disease also stands out as an important human zoonosis, where up to 17 million people are infected worldwide. The aim of this work was to study the prevalence of the disease in humans, cattle and water buffalos under different aspects in Brazil and to investigate new treatment alternatives. The present thesis is divided in Introduction, Literature Review, objectives, five chapters and Final Considerations. The first chapter shows a recount of human fascioliasis (HF) cases in Brazil over the last 60 years, where, 48 cases were found in the literature providing an updated scenario of the disease in the country. The second chapter shows an investigation of HF in Piraquara, Paraná (PR) and Balneário Piçarras, Santa Catarina (SC). In this chapter, we reported the first HF positive case in SC. From our report, local authorities were notified in order to investigate other possible cases, risk areas and the source of contamination. The third chapter is a study about the determination of the prevalence of anti-*F. hepatica* antibodies in dairy cattle to assess the relationship between *Fasciola*-specific cathepsin L1 (FhCL1r) antibody levels in bulk tank milk and milk quality parameters in 1,492 samples from PR. The average prevalence in the state was 9.72%, while the highest prevalence was found in the Metropolitan area of Curitiba (42.86%); Eastern Center (13.01%); Northwest (11.76%) and South Center (8.82%). Redundancy analysis, using a multiple multivariate linear regression model, showed that milk protein, lactose and defatted dry extract contents were lower in the positive samples compared to the negative ones. The fourth chapter shows the prevalence of *F. hepatica* in buffaloes slaughtered from 2003 to 2017 in Brazil. Data analysis revealed a total prevalence of 7,187 cases (3%) out of 226,561 individuals. The disease was more prevalent in PR, Rio Grande do Sul, and SC (11.9; 7.7; and 3.2% for condemned livers, respectively). Predictive models indicated a constant trend of the disease, highlighting the need for regular monitoring. Finally, in the fifth chapter we investigated the mechanism involved in inhibition of the *F. hepatica* cathepsin L3 (FhCL3) by its propeptide (pp). ppFhCL3 is a highly potent inhibitor of two *F. hepatica* collagenolytic cathepsins, FhCL3 and FhCL2 involved in the establishment of *F. hepatica* infection. Also, we propose that "clamp" mechanism involving residues of the pp that interacting with the propeptide binding loop (Tyr46p, Lys47p) and substrate bind cleft (Leu66p) is necessary for the correct bind and inhibition of *F. hepatica* collagenolytic cathepsin. Together, these results have shown an epidemiological update of the disease, approaching human and animal prevalence. Besides that, new possibility of designing selective inhibitors for *Fasciola* cathepsins were explored.

Keywords: Trematodes. Food-borne Disease. Fascioliasis. Milk immune-diagnosis. Cathepsin.

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1. Introduction

Fascioliasis is a neglected tropical disease caused mainly by the helminth trematode *Fasciola hepatica*. The World Health Organization estimates that at least 2.4 million people are infected in more than 70 countries around the world with several millions at risk (WHO, 2009). Due to its high diversity related to sources of infection, heterogeneity in the disease transmission patterns and susceptibility to the environmental characteristics, underlie the large epidemiological heterogeneity of human and animal fascioliasis in different countries (MAS-COMA et al., 2018).

Fascioliasis is considered a great veterinary problem, affecting various animals to different extents according to the host species and parasite burden (IBRAHIM, 2017). Welfare conditions and clinical signs vary from asymptomatic infections to devastating signs of weight loss, reduced milk yield and reproductive failure, including death (KAPLAN, 2001; SCHWEIZER et al., 2005). In Brazil, the prevalence of *F. hepatica* in condemned cattle livers from 2002 to 2011 was 6.32%, in which the highest prevalence of the disease was observed in the Southern states, with presence of outbreaks of the disease along the coast of Paraná and Santa Catarina and Rio Grande do Sul (BENNEMA et al., 2014).

Progress toward the control of fascioliasis requires better knowledge in different contexts. The identification of major virulence factors is one of the primary goal in parasite research since they can eventually lead to the development of specific control alternatives. When it comes to *F. hepatica* research, this is of special importance in the light of the emerging drug resistance in several countries, especially considering that the best option to treat *Fasciola* infection is triclabendazole due to its unique efficacy against immature and adult flukes (BROCKWELL et al., 2014; KELLEY et al., 2016; MOLL et al., 2000). In Brazil, triclabendazole resistance was reported in Almirante Tamandaré, Paraná. The treatment against *F. hepatica* on a sheep and goat farm failed to relieve the infection with an efficacy of 66.3%. Besides that, there was no record of triclabendazole having been administered previously on the farm (OLIVEIRA et al., 2008).

Another important tool associated with fascioliasis control is the investment in epidemiological studies. When areas with positive cases are identified, it is possible to trigger an investigation based on possible risk factors and sources of disease transmission. In addition, knowledge about the spatial distribution of fascioliasis contributes to the identification of areas for the infection in animals and humans (MAS-COMA et al., 2018).

Fascioliasis is a global disease, even though still poorly investigated in many countries, including Brazil. For this reason, the present study explored *F. hepatica* infection under different contexts. This document is formed by literature review and five chapters, approaching human and animal fascioliasis, and final considerations. All the chapters are edited according to the requirement of each Journal where they were published/submitted or will be submitted.

2. Objectives

2.1 General objective:

To determine the prevalence of fascioliasis in dairy cattle in the state of Paraná and buffaloes in Brazil, as well as to investigate the occurrence of the disease in humans in Paraná and Santa Catarina. In addition, we tested a new treatment candidate against the parasite.

2.2 Specific objectives:

- To carry out a bibliographic survey of the reported cases of human fascioliasis in Brazil over the last 60 years;
- To investigate the occurrence of human fascioliasis cases in Balneário Piçarras, Santa Catarina and Piraquara, Paraná, Brazil;
- To determine the prevalence of anti-*F. hepatica* antibodies in dairy cattle in Paraná;
- To investigate the association between fascioliasis infection and milk quality parameters from 1,492 farms over the state of Paraná;
- To determine the prevalence and geographical distribution of *F. hepatica* in condemned livers of water buffaloes (*Bubalus bubalis*) across Brazil;
- To run a 5-year forecast analysis of the fascioliasis in buffaloes for the states of Paraná and Rio Grande do Sul;
- To determine the selectivity and potency of the *F. hepatica* Cathepsin L3 propeptide;
- To investigate the residues involved in the inhibition of *F. hepatica* Cathepsin L3 by the its propeptide;
- To select a specific and potent synthetic peptide to inhibit *F. hepatica* Cathepsin L3.

3. Literature review

3.1 Etiologic agent

The etiologic agent that causes fascioliasis belongs to Trematoda Class, Fasciolidae Family and *Fasciola* Genus. The two most common species implicated as the etiological agents of fascioliasis are *F. hepatica* and *F. gigantica*. Adult flukes are characterized by brow leaf-shaped, flattened, two suckers and size of approximately 20-30 mm x 15 mm, easily seen with naked eye. The egg of *Fasciola* spp., size of approximately 120-150 mm, has a thin membrane, oval shape and an operculum on one border (KEISER; UTZINGER, 2009).

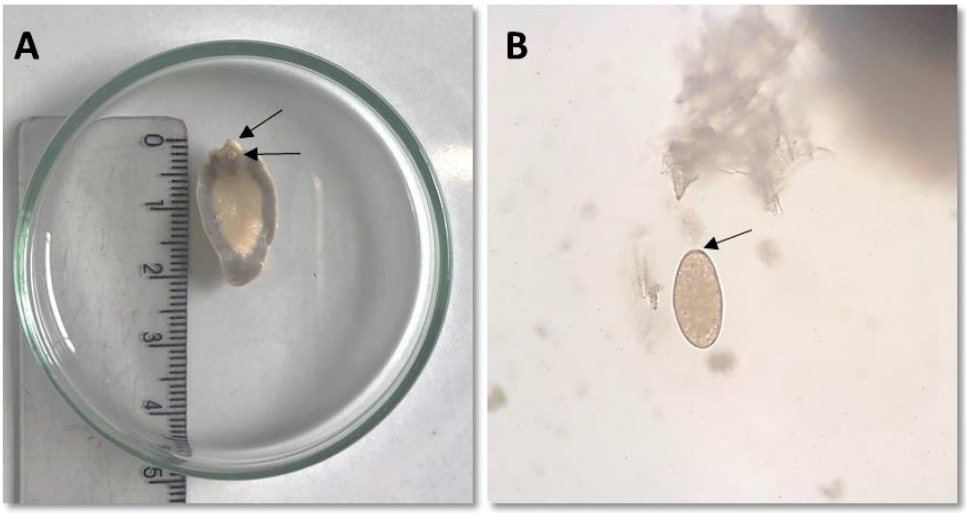

Figure 1. (A) *F. hepatica* adult worm, highlighted oral and ventral suckers, (B) *F. hepatica* egg, highlighted operculum on border, 200-fold increase. Source: The Author.

3.2 Intermediate host

The intermediate host that participates in the biological cycle of *Fasciola* sp. are mollusks belonging to the Lymnaeidae family. They have great parasitological importance, mainly due to the specificity of the *F. hepatica* and *F. gigantica* miracidia to these snails (MAS-COMA; BARGUES; VALERO, 2005).

In Brazil, the main reported species of Lymnaeidae family snails susceptible to infection by *F. hepatica* are: *Pseudosuccinea columella* (Say, 1817), *Galba viatrix* (Orbigny, 1835), *Galba truncatula* (Mueller, 1774) and *Galba cubensis* Pfeiffer, 1839 (MEDEIROS et al., 2014). In Santa Catarina state, the most common species found are *P. columella* and *G. viatrix;* in Paraná state only *P. columella* was identified (AGUDO-PADRÓN; VEADO; SAALFELD, 2013; MEDEIROS et al., 2014).

P. columella is the most common specie in South of Brazil, normally found in banks of small freshwater rivers, preferably clean water with low currents. The optimum temperature for snails' emergence is typically in the range from 14 to 22 °C. These gastropods are hermaphrodites with a shell length of 14-16 mm and the period involving the succession of one generation to the next through reproduction is approximately 46 days. After mating, snails will produce an average up to 1000 eggs, which will be released to the environment (AGUDO-PADRÓN et al., 2013; GUTIÉRREZ et al., 2002).

3.3 Life cycle of *Fasciola hepatica*

The biological life cycle of *F. hepatica* involves two hosts and one vehicle (aquatic plants). The process begins when definitive host (animals or humans) parasitized by adult worms defecate in freshwater sources (REINHARD, 1957). Eggs released with faeces will hatch into larvae called miracidia, which penetrates through the skin of the Lymnaeidae family snails. Inside the snails, the miracidia become sporocyst, which contains germ cells that will generate the first generation of reyes. The reyes migrate to the hepato-pancreas of the snail for further development, generating cercariae (MAS-COMA et al., 1999a; MAS-COMA; BARGUES, 1997). At this point, the cercariae leaves the snail and swim in the vicinity of aquatic or semi-aquatic plants, where they attach to leaves and stems and form small cysts (metacercariae). When plants with small cysts are ingested, a new host infection occurs (MAS-COMA; BARGUES, 1997) (Figure 2). Ingestion of free metacercariae floating in water (independent of carrier plants) and consumption of undercooked liver carrying juvenile flukes may also be a possible mode of transmission (MAS-COMA et al., 1999a; WHO, 2009). Inside the definitive host, the metacercariae excysts in the small intestine, 1h after ingestion, penetrate the host's intestine wall and migrate to the liver within 6 days after excystment. In the liver, they penetrate into the bile ducts where they become sexually mature. The period from the ingestion of metacercariae to the first appearance of the first eggs in the faeces varies according to the host and intensity of the infection, in humans, for example, this period takes around 3 to 4 months (MAS-COMA et al., 2018).

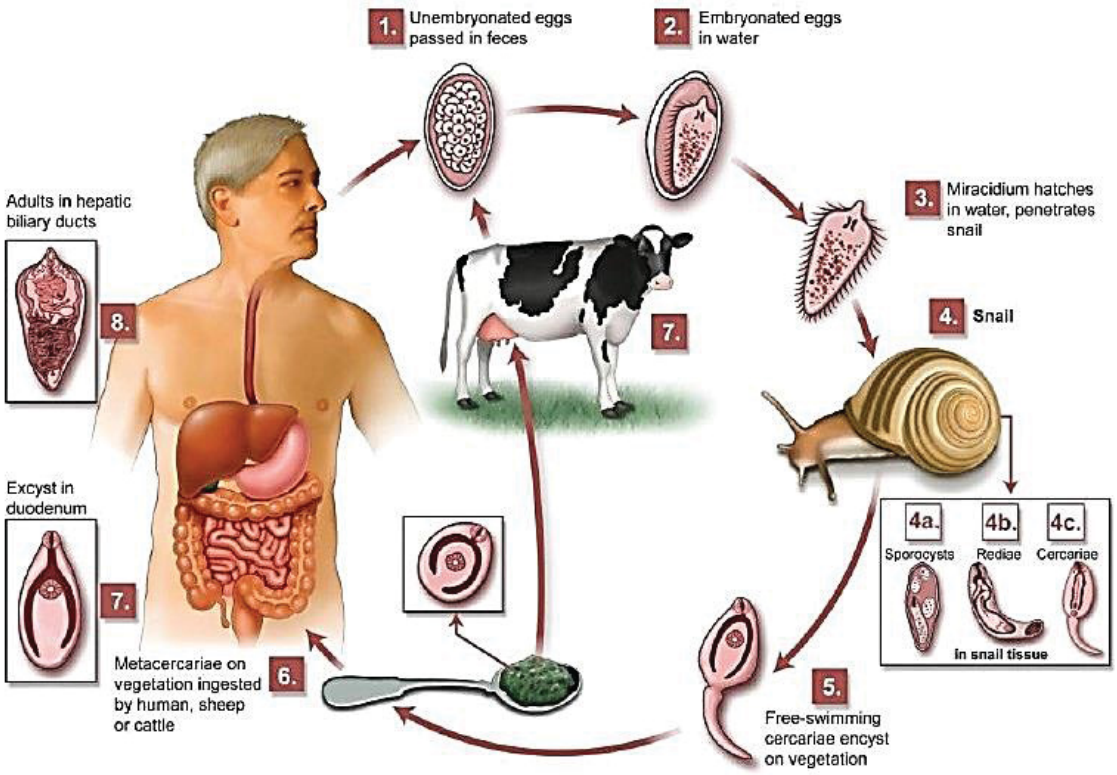

Figure 2. Sequence of the biological life cycle of *Fasciola hepatica* (starting at 1). Source: http://www.md-health.com

3.4 Epidemiology

Fascioliasis is the most widely distributed trematode disease, reported over 81 countries around the world (WEBB; CABADA, 2018) (Figure 3). From the two species involved, *F. hepatica* is widely distributed in all continents, while *F. gigantica* is found in tropical areas, with a more focal distribution in Africa Middle East, and South and East Asia (MAS-COMA et al., 2005). Because of its ability to proliferate in a wide range of different ecosystems, *F hepatica* is probably the vector-borne parasite species with the widest longitudinal, latitudinal, and altitudinal distribution (FÜRST; KEISER; UTZINGER, 2012). Bennema et al. (2017) using the range of isothermality, showed that all regions of Brazil are suitable for *F. hepatica* prevalence.

South America is one of the continents with the highest human fascioliasis prevalence. High prevalence of human liver fluke infection has been described in Bolivia, with prevalence rates of up to 72% and numerous cases reported in children (MAS-COMA et al., 1999b; PARKINSON; O'NEILL; DALTON, 2007). High levels were found amongst indigenous Aymaran people in the basin of Lake Titicaca (ESTEBAN;

BARGUES; MAS-COMA, 1998). Also, hyperendemic areas in Cajamarca province, Peru, were reported with rates ranging from 6.7 to 47.7% (GONZÁLEZ et al., 2011).

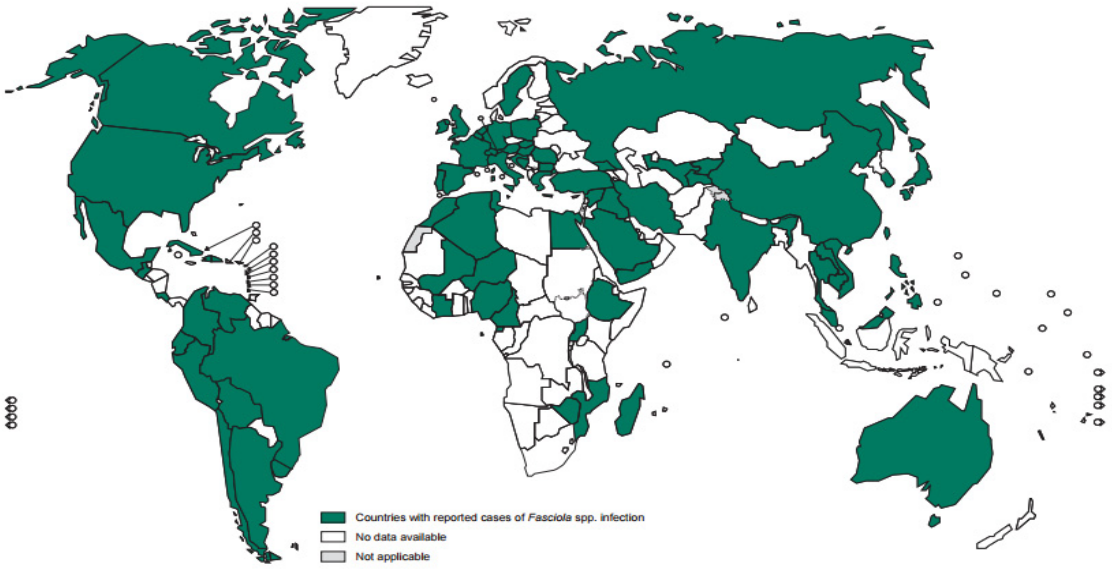

Figure 3. Distribution of fascioliasis according to data from the World Health Organization (2013).

In Brazil, fascioliasis is not a disease of compulsory declaration, and as a result, only 48 cases of human fascioliasis were reported in scientific papers from 1950 to 2016 (PRITSCH; MOLENTO, 2018). Recently, a report coming from the Amazonas state, found that 36 (8.3%) human serum samples were reactive in ELISA, of which 8 (1.8%) were Western Blot reactive, and only one faecal sample was positive using a *F. hepatica* coprological test (MACIEL et al., 2018).

The prevalence of fascioliasis in ruminants have been reported from all continents. In Europe, fascioliasis' prevalence has been reported from 11 countries, where the highest range was found in cattle varying (0.12 to 86.0%). In Africa, the highest range of fascioliasis was also reported in cattle (1.2 to 91.0%). In Americas, studies mentioned the prevalence in goats (24.5 to 100%) and cattle (3.0 to 66.7%). A very high prevalence rate (86%) has been documented in cattle from Argentina (MEHMOOD et al., 2017). In Figure 4 shows the prevalence of *F. hepatica* in cattle in Brazil between 2002 and 2011, in which the positivity index for bovine fascioliasis was 14.39% in Rio Grande do Sul and 4.5% in Santa Catarina (BENNEMA et al., 2014). Gavinho, Kulek and Molento (2008) determined the prevalence of *F. hepatica* in cattle slaughtered in Paraná. The

authors showed that city of Castro had the largest number of positive animals, representing 61.6%.

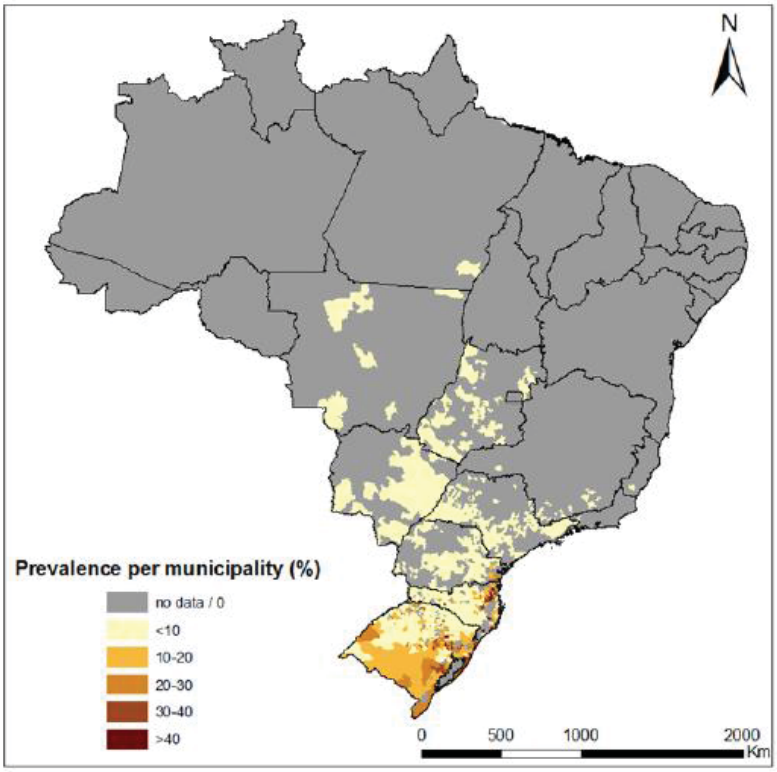

Figure 4. *Fasciola hepatica* prevalence in Brazil in the period of 2002-2011. Source: Bennema et al. (2014).

3.5 *F. hepatica* infection and immune response

The immune response in hosts are characterized by T-cell activation in response to antigen stimulation leading to the differentiation of effector T-cell subtypes leading to specific effector functions (VALERO et al., 2017). During the infection by *F. hepatica*, there is an increase of eosinophils, lymphocytes, interleukin IL-4 and IFN-y, that induces Th2 type responses in the hosts (MULCAHY; DALTON, 2001; VALERO et al., 2017). Besides that, the parasite is able to supress cytokines production that induce Th1 response (MULCAHY et al., 2001; O'NEILL et al., 1999).

In cattle naturally infected with *F. hepatica* a balance is achieved between the levels of IFN-γ, IL-4 and IL-10 expression in liver tissue, which confirm the predominance of the Th2 response in naturally infected animals from endemic areas. This balance helps in the defence of the parasite against antiparasitic drugs and allows the progression of fascioliasis, favouring the permanence of the parasite in the host for prolonged periods of time (MENDES et al., 2013).

The impact of *F. hepatica* on the host's immune system is evident in a study conducted by Claridge et al. (2012). They show a significant negative association between the exposure to *F. hepatica* and the diagnosis of bovine tuberculosis (bTB), in which, the interaction between *F. hepatica* and *Mycobacterium bovis* reduce the sensitivity of the main diagnostic test used for *M. bovis*. This finding has significant implications for the bTB eradication programmes.

3.6 Clinical Aspects

After ingestion of the larvae with contaminated food or water, an incubation period begins (asymptomatic), lasting from days to months. In humans, the clinical manifestations of fascioliasis vary depending on the stage of infection and the amount of metacercariae ingested (ROWAN et al., 2012). The duration of the acute phase may range from 2 to 4 months, and typical symptoms include fever, nausea, liver swelling (hepatomegaly), rashes, and abdominal pain. In the chronic phase, symptoms include intermittent pain, biliary colic, jaundice (yellowing of the patient) and anaemia, cholangitis, liver hardening (fibrosis) may also occur as a result of long-term inflammation (FÜRST et al., 2012; HARRINGTON et al., 2017; ROWAN et al., 2012). During the chronic phase, adult flukes live up to 13.5 years. The severity of the disease in both stages ranges from an asymptomatic infection to an intense hepatic infection (HARRINGTON et al., 2017; ROWAN et al., 2012).

In animals, the disease is also characterized in acute and chronic phases. However, the degree of infection varies according to age, health condition, species susceptibility and amount of metacercaria ingested (IBRAHIM, 2017). The acute phase is more severe in sheep, and may even cause a higher death rate, as the chronic phase is more frequently verified in other larger hosts (SOLIMAN, 2008). The general clinical findings associated with the acute phase are severe anaemia, eosinophilia and hypoalbuminemia. In the chronic phase gradual weight loss, weakness, anemia, hypoproteinemia and subcutaneous edema, particularly in the submandibular region and abdomen may be evidenced (IBRAHIM, 2017).

The most frequent hepatic lesions caused by *F. hepatica* in ruminants are: parasite damage inside the biliary ducts, portal system fibrosis and biliary duct hyperplasia. In bovine and bubaline; lymphocytes, plasmocytes, eosinophils and neutrophils, are characteristics of a chronic disease. In contrast, the presence of eosinophils in sheep is characteristic of the initial phase of a chronic fasciolosis (BOSTELMANN et al., 2000).

Biochemical changes associated with pathological lesions caused by liver fluke infection in ruminants are high ALT and AST in cases of acute parenchymal lesions like eosinophilic hepatitis and necrosis, while ALP is elevated in fibrosis and cirrhosis. The findings of the present study indicated that serum biochemical changes were consistent with KITILA and MEGERSA (2014).

The disease causes economic problems mainly through liver condemnation, reduced beef and milk production, affects fertility, increase expenditures for anthelmintics and mortality (KAPLAN, 2001; SCHWEIZER et al., 2005). Associations between anti-*F. hepatica* antibody levels in milk and productive parameters have been described in literature. Mezo et al. (2011) showed a milk yield reduction of 1.5 kg milk/cow/day in herds positive to anti-*F. hepatica* antibodies in Spain. Charlier et al. (2007) reported a decrease in the annual average milk yield of 0.7 kg milk/cow/day, with a decrease of 0.06% in fat content. Also, Köstenberger et al. (2017) showed a reduction of milk yield of 6% (438 kg/cow/year) and significant effect on butterfat and protein contents, decreasing 0.091% and 0.046% respectively.

3.7 Diagnosis

The diagnosis of human and animal fascioliasis based on the infection history and clinical signs may be difficult to perform, thus, the confirmation of the diagnosis should be associated with laboratory tests. Diagnostic techniques for fascioliasis include: (1) fecal techniques for the detection of *Fasciola* sp. eggs. Coprological tests require the collection of several samples due to the irregular release of the eggs by the parasite and in infections with low parasite numbers. Eggs may not be found in faeces even after multiple faecal exams as these tests have better results when employed in the chronic phase (CABADA; WHITE, 2012; ESTEBAN et al., 1998; WHO, 2009); (2) immunological techniques are used for the detection of specific antibodies against *Fasciola* sp. in serum (blood and milk) or parasite-specific antigens in faecal samples. These tests are generally more sensitive than coproparasitological techniques, however, the antibody positivity does not distinguish between recent and past infections (ESTEBAN et al., 1998; KEISER; DUTHALER; UTZINGER, 2010; KEISER et al., 2009). (3) Molecular techniques, such as the polymerase chain reaction, offer a high sensitivity and specificity, and allow the discrimination between infections caused by different species. However, these techniques are not employed as routine diagnosis (KEISER et al., 2009).

Serological tests are a non-invasive alternative and have excellent advantages, such as practicality in the execution, allowing the analysis of multiple samples concomitantly. However, highly sensitive and specific commercially methods are still not available.

The recombinant form of *F. hepatica* cathepsin, FhCL1r, have been developed to act against total anti-IgG and various subclasses (IgG1, IgG2 and IgG4). See details on cathepsins in item 3.8. FhCL1 is a variant FheproCL1Gly26 (amino acid in the position 26, Cysteine, was substituted to Glycine) in order to avoid auto-catalytic degradation during purification and also cleaved immunoglobulin in the ELISA (SANTANA et al., 2013). The ELISA test employing FhCL1 as an antigen was designed to detect antibodies in people and animals infected with *Fasciola* spp. even if the host carries other parasites (i.e. amebiasis, ascariasis, Chagas disease, cysticercosis, echinococcosis, enterobiasis, filariasis, giardiasis, leishmaniasis, malaria, schistosomiasis, strongyloidiasis, toxocariasis, toxoplasmosis or trichinosis). The test presents a sensitivity of 99.9% and specificity of 99.9% (SANTANA et al., 2013). This diagnostic method can be used in individual samples and in mass screening programs to evaluate the extent of fascioliasis in endemic or suspected regions (SANTANA et al., 2013).

3.8 Treatment

Triclabendazole (TBZ) is the drug of choice for *F. hepatica* and *F. gigantica* infections in humans and animals due to its activity against juvenile and adult forms of the parasite. The treatment recommended is one or two doses of TBZ, 10 mg/kg (FAIRWEATHER, 2009; FÜRST et al., 2012). The treatment success rate is high, though emergent reports of TBZ-parasite resistant is of great concern. One of the most important reason is the massive use of TBZ in livestock as a preventive strategy against production losses (WEBB et al., 2018). Several countries (i.e. Ireland, Spain, Australia, Peru, Brazil and Argentina) have TBZ-parasite resistance in cattle and sheep (KELLEY et al., 2016). Also, the failure of human treatment was also reported (CABADA et al., 2016; GIL et al., 2014). Taking it into consideration, finding new drug candidates is urgently needed. Further research to improve TBZ effectiveness against fascioliasis have great perspective.

3.9 Cysteine proteases

Proteases are enzymes that cleave or degrade peptides catalysing the hydrolysis of peptide bonds (BARRETT; MCDONALD, 1986). According to MEROPS, a peptidase

database, these proteins are classified into families based on the nature of their catalytic residues in: Aspartic (A), Cysteine (C), Glutamic (G), Metallo (M), Asparagine (N), Mixed (P), Serine (S), Threonine (T) and Unknown (U). Among them, cysteine proteases are widely distributed in several organisms, including vertebrates, invertebrates, viruses and plants. The majority of cysteine proteases belong to the papain superfamily (Clan CA, family C1:CA1), which are generally referred as cathepsins (BRÖMME, 2000; CHEN et al., 2017).

Structurally, cysteine proteases consist of an amino terminal domain that is mostly α-helical and a carboxy-terminal domain featuring an antiparallel β-sheet with a cysteine and histidine catalytic residues forming a thiolate-imidazolium dyad (BEVERIDGE, 1996). To protect the cell from the consequences of uncontrolled degradative activity, these proteases are synthesized as inactive enzymes (ROCHE et al., 1997). For this reason, cathepsin L proteases are synthesised as pre-pro-enzymes that consist of a pre-peptide, a pro-peptide and the mature (catalytic) enzyme. The inactive enzyme is subsequently processed into a proenzyme (zymogen) during their passage through the endoplasmic reticulum and Golgi apparatus, where it loses the prepeptide (ROCHE et al., 1997). Although, the catalytic power of the mature enzyme is already present at this stage, it is nearly completely masked by the presence of propeptide (QURAISHI; STORER, 2001; SIVARAMAN et al., 2000). Furthermore, apart from being potent inhibitors of the proteolytic activity of its cognate mature enzyme, the propeptide region of the cathepsins is also fundamental, as it is involved in foldase activity, with the maintenance of stability, intracellular sorting and regulation of enzyme activity (ROCHE et al., 1997; TAO et al., 1994).

Cathepsins from parasites perform key roles to establish infection in the host during their life cycles and have been shown to be key players in host–parasite interactions (STACK et al., 2008). These enzymes are found in excretory-secretory products (ES), as well as in somatic antigens, derived from helminths and can be differentially expressed during the growth and maturation of the parasite within the host. This demonstrate the parasite's ability to change the host interface along the course of infection (CANCELA et al., 2008).

During all life stages of *F. hepatica* different proteases are expressed (Figure 5). Two main classes of cathepsin proteases are expressed and secreted by *F. hepatica*: cathepsin B (CB) and cathepsin L (CL) (CORVO et al., 2013). While all seven identified CB's belong to a single clade (CB1 to CB7), it was verified that *F. hepatica* express a

total of 17 members of CL's that were classified in five clades (papain-like). In addition, while adult worms expressing CL's from clades 1 (CL1), CL2, and CL5, NEJ (Newly excysted juvenile) were shown only to express CL3 and CL4 (CANCELA et al., 2008; CWIKLINSKI et al., 2015; ROBINSON et al., 2008a).

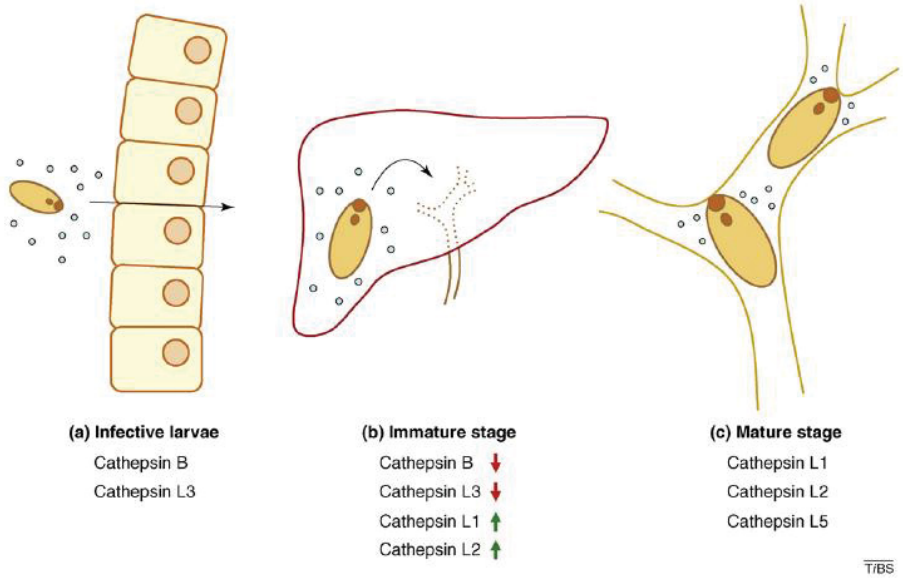

Figure 5. Differential expression of cathepsin proteases in *Fasciola hepatica* development stages. Source: Robinson et al., 2008.

According to transcriptomic and proteomic analysis, *F. hepatica* cathepsin CL3 (FhCL3) and FhCB account for over 80% of the total protease activity detectable in NEJ (ROBINSON et al., 2009). The abundance of FhCL3 could be related to its strong collagenase activity that might be critical to enable the NEJ penetrate the intestinal wall (CORVO et al., 2009). The identification of major virulence factors is one of the primary goal in parasite research since they can eventually lead to the development of specific control measures, either through drugs or immunogens (immune therapy). In this context, cathepsin proteases stand out as good target candidates for new alternatives of treatment.

4. Chapters

4.1 Chapter 1

Contradictory data was found in several published articles during the search for Brazilian literature regarding human fascioliasis. At first, we found discriminative data but there wasn't a correct number of reported cases from many studies. For this reason, we provided a recount of human fascioliasis in Brazil. The article was published at Revista de Patologia Tropical (Federal University of Goiás - Goiânia, Goiás, Brazil). Doi: 10.5216/rpt.v47i2.53636

RECOUNT OF REPORTED CASES OF HUMAN FASCIOLIASIS IN BRAZIL OVER THE LAST 60 YEARS

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ABSTRACT

Fascioliasis is an important anthropozoonotic disease caused by the ubiquitous trematode helminth, *Fasciola* spp. Here, as elsewhere, it is thought that the disease lacks proper reporting, and the available literature does not reflect unreported cases found in the Brazilian population, or new recently reported cases. The purpose of this work was to perform a recount of human fascioliasis (HF) cases in Brazil. For this, we considered all positive cases published in local and international official Journals, from 1950 to 2016. A theoretical-conceptual research method based on a systematic bibliographic review was applied to identify, select and index articles using the Endnote Basic Software. Here, only 48 cases of HF were found, of which 21 (43.7%) occurred in the South of the country. The small number of reported cases reflects the difficulty in diagnosing HF correctly (clinical and fecal tests). This work provides a real figure of HF reported cases in Brazil and has also corrected inaccurate information found in the literature by conducting a historical survey of the disease. *Fasciola hepatica* is highly endemic in ruminants and, hypothetically, the number of human cases should also be considerably higher than that reported in the literature. These findings call for more attention in regard to this neglected disease in Brazil.

KEY WORDS: Trematode; parasites; *Fasciola* spp.; public health; zoonosis.

INTRODUCTION

Fascioliasis, considered an important anthropozoonotic disease and included in the neglected tropical disease list, has re-emerged over the last 25 years (Mas-Coma et al., 2014a). This food-borne trematode infection is also among the most neglected diseases worldwide, having an invasive pathogenicity and advanced chronic phases, mainly in low income and farming communities (Fürst et al., 2012; Mas-Coma et al., 2014a).

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Fascioliasis is well characterized as a veterinary problem, causing significant economic losses, but despite its importance in animal husbandry, human infection by *Fasciola hepatica* has historically been of secondary importance (Chen & Fleury, 1990). Its significance to humans has increased due to the recent emergence of the disease related to climate and global changes (Mas-Coma et al., 2014a).

The World Health Organization has estimated that at least 2.4 million people have been infected in more than 75 countries with several thousand seriously risking contamination (WHO, 2007). South America, Bolivia and Peru present the highest prevalence rates (Esteban et al., 1997a; 1997b; 2002). The disease affects humans by the accidental ingestion of uncooked fresh water plants or drinking water contaminated with metacercariae. Infection is also related to the local distribution of the intermediate host (*Lymnaea* sp. snail) populations (Mas-Coma et al., 1999a).

Human fascioliasis (HF) is normally asymptomatic or non-specific and usually the manifestation of clinical signs occurs long before the parasite eggs appear in the fecal test. Clinically, acute and chronic cases may be distinguished. The mechanical destruction of the liver tissue and abdominal peritoneum by the migrating flukes causes localized or generalised toxic and allergic reactions lasting 2 - 4 months, evidenced in the acute period. The chronic period may develop after months to years of infection, where the adult flukes cause inflammation, hyperplasia of the epithelium, and thickening and dilatation of the bile duct and gallbladder walls (Ashrafi et al., 2014). The incubation or prepatent period goes from the ingestion of metacercariae to the appearance of the first symptoms, which may vary considerably from a few days to 3 months or even more, depending on the number of metacercariae ingested and the host's immune response. While the latent phase of the infection, which involves the maturation of the parasites and initial oviposition, can take months or years, the subsequent chronic or obstructive phase may last for more than 13 years (Mas-Coma et al., 1999b, 2000).

The diagnosis of HF is normally based on the microscopic detection of parasite eggs in feces using coproparasitological methods. Testing is often performed in routine diagnostic laboratories and is based on the sedimentation of parasite eggs (Hoffmann et al., 1934). However, the use of routine parasitological diagnostic methods is not sufficient for diagnosis. Some downsides of the testing include: the absence of eggs in the stool sample due to reduced egg shedding (low infection burden); termination of egg shedding in advanced chronic fascioliasis (old infections); or the total lack of oviposture, as flukes may not attain maturity in human subjects (i.e. ectopic parasite) (Mas-Coma et al., 2014a;

Rojas et al., 2014). The parasitological diagnosis is not helpful in ectopic cases, as the juvenile fluke may deviate while migrating from the intestine to the liver and enter different organs (i.e. subcutaneous tissues, peritoneum, brain and lungs) (Mas-Coma et al., 2014b). Besides, it is important to emphasize that eggs are not detected during prepatent infection, where there is only the presence of juvenile worms. A more proper indication to increase precision in detecting *Fasciola* spp. eggs is to perform multiple tests from the same suspected patient (Rapsch et al., 2006).

Faria et al. (2008) evaluated the commercial test Flukefinder (Richard Dixon, ID, US). This test isolates liver fluke eggs by differential filtration, followed by differential sedimentation, in comparison to other techniques for *Fasciola* egg detection. The test has shown good performance, however it is time consuming and the large amount of sediment in the end of the process hinders egg visualization. Furthermore, its high cost is also an impediment to many laboratories, compared to the four-sieve (Girão & Ueno, 1985) differential filtration technique.

Immunodiagnosis, based on antigen or antibody detection, is another option for the diagnosis of HF. For this there are commercial tests, such as the *Fasciola* IgG Enzyme Immunoassay Kit® (Alpco Diagnostics, NH, USA) and DRG *Fasciola hepatica* IgG ELISA® (DRG International, Inc., USA) which are based on the detection of antibodies; and the MM3-COPRO ELISA and its commercial version BIO K 201® (BIO X Diagnostics, Belgium) based on the detection of antigens in feces. Despite the availability of the above technique, medical doctors do not usually order these tests, and only a few laboratories are using immunodiagnosis for HF. Regular patients with liver diseases may undergo image tests (ultra-sound, X-Ray) with reports that indicate that the patient is a 'suggestive' case. We believe that the diagnosis has limitations and since HF is not a disease of compulsory declaration Brazil, the number of human acute and chronic cases is greater than the cases reported in scientific papers. Several studies are being conducted in South America demonstrating that the disease has a disperse distribution. The highest fascioliasis prevalence is encountered in the northern Altiplano of Bolivia (Esteban et al., 1997a; Esteban et al., 1999) and in the Andean valleys such as the Peruvian valleys of the Cajamarca area (Ortiz et al., 2000; Hillyer et al., 2001; Valero et al., 2012). Esteban et al. (2002) investigated the Asillo irrigation area in Peru, and proved that this region was also hyper endemic for HF with a 24.3% prevalence of *F. hepatica*.

According to the epidemiological importance of HF and the lack of proper diagnosis, we believe that cases of HF in Brazil are underreported. Taking this into

account, our purpose was to recount the available data of reported cases of HF conducting a historical survey of the disease in the country.

METHODS

A theoretical-conceptual research method based on a systematic bibliographic review was applied to perform an accurate and therefore reliable review (Kitchenham, 2004). This technique uses technological resources to identify, select and index articles through Endnote Basic Software.

Two bases were chosen, Pubmed and the Web of Science, for having most articles published internationally. The keywords "*Fasciola*", "human fascioliasis", "fascioliasis", "liver fluke" and subsequently combined "human fascioliasis and Brazil", "fascioliasis and Brazil" and "liver fluke and Brazil" were used in the search engine. The search was made for all publications that contained the key words in the title, abstract and keywords, which resulted in 5,972 publications in Pubmed and 5,171 in Web of Science. 3,066 duplicated articles were found and subtracted, thus defining 8,117 articles for the research portfolio. The articles were selected according to our purpose, including all types of publication (abstracts, original articles, reviews, short communications, case reports and technical notes) available in Portuguese, English and Spanish from 1950 and excluding all other countries and animal reports, therefore reducing results considerably.

The COMUT (Bibliographical Commutation) database was also used to include unavailable online articles. Databases including SID (State Inpatient Databases), Google Scholar and Scopus were also used to search for references from articles included in the research portfolio. Data were extracted from those articles which fulfilled our eligibility criteria.

RESULTS

The full historical data (n = 13), and the distribution of HF cases among Brazilian states is shown in Table 1 and Figure 1. Forty-eight cases of HF were reported in Brazil since 1958, of which 43.7% occurred in the South of the country, 25% in the Southeast, 23% in the North, and only 4.2% in the Northeast and in the Mid-West. The states with the highest number of reported cases were Paraná (20) followed by Amazonas (11) and São Paulo (7).

Table 1. Human fascioliasis reported (Reference) from 1958 to 2016 in Brazil, including the city or region and the method of diagnosis.

Author	Year	City - State	Positive samples	Diagnostic method	Reference
Rey	1958	Campo Grande, MS	1	Hoffman, Pons & Janer	39
Santos	1967	Urucuba, BA Ilheus, BA	2	Hoffman, Pons & Janer	44
Santos & Vieira	1967	Vale do Paraiba, SP	7	Hoffman, Pons & Janer e Faust & col. duodenal tube and intradermal reaction	43
Corrêa & Fleury	1971	Cornelio Procopio, PR	1	Hoffman, Pons & Janer	11
Amato Neto & Silva	1977	Vale do Paraiba, SP	1	Fecal egg and parasite presence	3
Baranski et al.	1978	Curitiba, PR	2	Hoffman, Pons & Janer and duodenal tube	6
Amaral & Busetti	1979	Curitiba, PR	8	Ether sedimentation	2
Andrade Neto et al.	1999	Curitiba, PR	9	Fecal exam	4
Pile et al.	2000	Volta Redonda, RJ	2	Fecal exam	38
Mezzari et al.	2000	MT[1]	1	Endoscopic retrograde cholangiography	33
Igreja et al.	2004	Rural area, RJ	2	Hoffman, Pons & Janer and Kato-Katz	24
Coral et al.	2007	Rural area, RS	1	Choledoscopy	10
Oliveira et al.	2007	Canutama, AM	11	Fecal exam	35
Total			48		

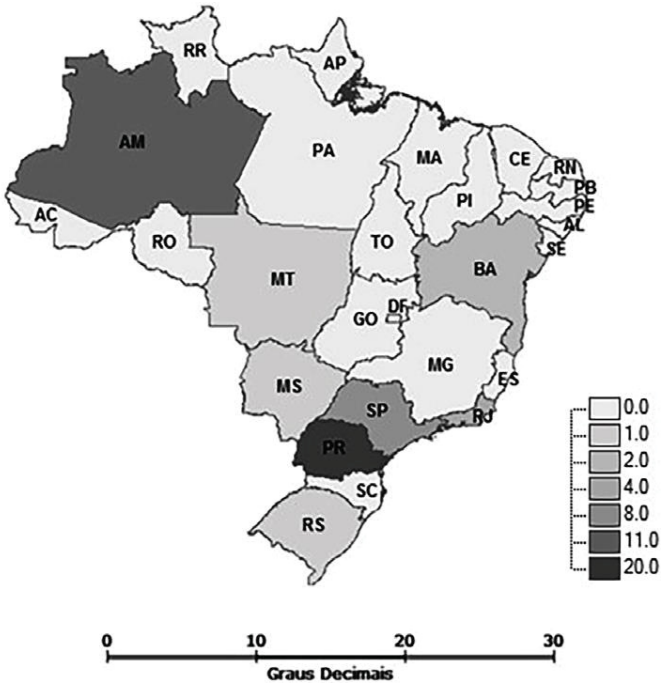

Figure 1. Distribution of human fascioliasis among Brazilian states.

Legend: AC = Acre, AL = Alagoas, AP = Amapá, AM = Amazonas, BA = Bahia, CE = Ceará, DF = Distrito Federal, ES = Espírito Santo, GO = Goiás, MA = Maranhão, MT = Mato Grosso, MS = Mato Grosso do Sul, MG = Minas Gerais, PA = Pará, PB = Paraíba, PR = Paraná, PE = Pernambuco, PI = Piauí, RJ = Rio de Janeiro, RN = Rio Grande do Norte, RS = Rio Grande do Sul, RO = Rondônia, RR = Roraima, SC = Santa Catarina, SP = São Paulo, SE = Sergipe, TO = Tocantins.

DISCUSSION

The majority of HF cases were found in the state of Paraná, in the South of the country; however, we believe that the overall incidence of fascioliasis is largely underdiagnosed. As for the diagnosis of HF, although some studies (Andrade Neto et al., 1999; Pile et al., 2000; Oliveira et al., 2007) have described the use of fecal testing, this technique should not be used to obtain the final diagnosis. Sarkari & Khabisi (2017) reviewed the new achievements in designing and improving diagnostic approaches and the inconveniences in the techniques. Parasitological tests are not useful for the diagnosis of infection during the acute phase of the disease, and have poor sensitivity during the chronic phase. Antibody detection assays also have disadvantages such as the lack of sensitivity and specificity and the inability to differentiate between past and present infections. Accordingly, detection of antigen in stools (coproantigens) seems to be a suitable method, however, the assay still requires proper field evaluation (Sarkari & Khabisi, 2017). It is worth mentioning that none of these methods of diagnosis is performed as a routine technique in laboratories in Brazil, except when doctors have a suspect clinical case.

According to our survey, Mezzari et al. (2000) reported the occurrence of 56 cases of human fascioliasis in Brazil, whereas Pile et al. (2000) and Igreja et al. (2004) reported 44 and 57 cases, respectively. However, we found 48 reported cases in the literature. These contradictory data may have occurred considering the difficulty in obtaining old articles to compare with the references, and cases reported in other countries, for example, cases from Cajamarca and Lima, assuming that they were from Brazil (WHO, 1990). Amaral & Busetti (1979) reported the occurrence of eight cases of human fascioliasis in Curitiba, PR, however, in the discussion they described two other new cases, totaling 10 cases; nevertheless, these two new cases were not found in the literature. The last report is from Oliveira et al. (2007) and Coral et al. (2007), and both groups noted negligence in the diagnosis of HF in Brazil.

The present study was conducted up to 2016, however, a cross-sectional serological survey of HF was recently performed in Amazonas state, where 36 (8.3%) samples were reactive in ELISA, of which 8 (1.8%) were Western Blot reactive and only one sample was positive using *F. hepatica* fecal test (Maciel et al., 2018). Our laboratory is also investigating HF in Brazil using a specific and sensitive ELISA technique (Santana et al., 2013). So far, one new case has been confirmed, associating different diagnostic techniques, such as serology (ELISA), liver imaging tests, and there is a case report undergoing preparation (data not shown).

Some factors may have contributed to the small number of cases, such as the inability of health professionals to diagnose the disease adequately and the lack of specific diagnosis in routine laboratory procedures. The uncertainty about the real situation in the country is due to the lack of a more specific diagnosis and of epidemiological studies, since patients harbouring mild a metabolic (liver) illness often treat themselves with antibiotics or consult a doctor based on clinical signs (i.e. abdominal/liver pain).

The authors have presented a recount of HF reported in scientific papers, however we do not have access to medical records to provide the exact number of HF cases. Furthermore, we have only a few (n = 8) research groups dealing with *Fasciola* sp. and/or Trematodes, according to the National Technological and Scientific Development Council (CNPq) database.

Although the epidemiology of HF still requires better elucidation in Brazil, the present study showed the relevance of performing data mining to check the distribution of fascioliasis infection. The information collected revealed the demand for improvement in the diagnosis of *F. hepatica*, indicating that the disease is spread over a large area with considerable overlapping with the animal form of the disease (Aleixo et al., 2015; Silva et al., 2016, Bennema et al., 2017). Despite the high incidence of *F. hepatica* in livers from beef cattle (Gavinho et al., 2008; Dutra et al., 2010; Bennema et al., 2014) being considered an important risk factor for the

transmission of the disease to humans, there is no data to confirm a relationship between human and animal fascioliasis in Brazil. On the other hand, Perez-C et al. (2016) reported the first case of HF in a bovine fascioliasis endemic region in Colombia, showing that a high prevalence of bovine fascioliasis may affect people who are exposed to risk factors associated with the infection. There is also the human-to-human food-borne transmission due to cultural diets, suggesting that urban fascioliasis is possible, maintaining the parasite life cycle without animal involvement.

We are still a long way from understanding the animal-human disease interaction, but thelarge geographic distribution of the disease poses anenormous challenge (Bennema et al., 2017). The Geographic Information System map query showed that in a large part of Brazil the studied climate variables were within the same range as in the areas where *F. hepatica* infections in cattle were observed. Only the North and North East Regions, with the exception of three states were outside this range (Bennema et al., 2017). Future parasite control programs should be based on the epidemiological understanding of the disease and large diagnostic campaigns. Another concern regarding HF is to provide proper treatment for positive patients. Triclabendazole resistant *F. hepatica* was prescribed on a sheep farm in Paraná (Oliveira et al., 2008), and this can pose a risk to sustainable HF control. Only albendazole and closantel are available to be used in humans in the country, but triclabendazole is the only compound that affects adult and young forms of the parasite (Kelley et al., 2016).

It is very hard to provide realistic estimates given the large number of undiagnosed or unreported cases, the absence of active disease screening and the fact that fascioliasis is not a notifiable disease. We believe that the diagnosis of HF in Brazil may be improved by training laboratory professionals, pre-conference courses and extension programs, approaching the population to the scientific community. Nevertheless, it is clear that the small number of reported cases considerably underestimates the problem due to the large number of subjective cases.

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4.2 Chapter 2

As previous reported in chapter one, although *F. hepatica* is a common parasite, the epidemiological knowledge of human fascioliasis in Brazil remains largely unknown due to the small number of investigations and also because the disease was not listed in the compulsory notification National Health list, resulting in the lack of regular diagnosis. Knowing this weakness, we (Laboratory of Parasitic Diseases of UFPR) have established a partnership project with the Municipal Health Secretary of Balneário Piçarras in the state of Santa Catarina and Piraquara in the state of Paraná in order to investigate human fascioliasis. These two cities were chosen due to the high incidence of fascioliasis in cattle determined by previous studies in our Laboratory (ALEIXO et al.; 2015; BENNEMA et al., 2014; DUTRA et al., 2010; SILVA et al., 2016). The project was approved by the Ethics Committee of the Federal University of Paraná (CAAE number 50984215.0.0000.0102) (see Addendum 3).

To investigate the occurrence of fascioliasis in humans in these cities, we validated an ELISA technique in Brazil, employing a recombinant cathepsin L1, which was provided by Professor John P. Dalton, from the Queens's University, Belfast, Northern Ireland. This diagnosis method has shown 99.9% of specificity and sensibility (Santana et al., 2013). Until now we have collected 90 samples from both places, in which one individual was positive for anti-*F. hepatica* antibodies. This positive case was described as a case report manuscript in this chapter. The manuscript was submitted to the Journal of the Brazilian Society of Tropical Medicine (Uberaba, Minas Gerais, Brazil), as a Case Report, and is currently "Under review".

First reported case of clinical fascioliasis in Santa Catarina, Brazil

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Case report

Title: First reported case of clinical fascioliasis in Santa Catarina, Brazil

Abstract

Fascioliasis is a food-borne anthropozoonotic disease, caused by *Fasciola hepatica* that affects multiple hosts, including humans. We reported the first human fascioliasis case in the state of Santa Catarina, Brazil. A 57-year old female patient was admitted to hospital for a clinical investigation complaining of abdominal pain. The diagnosis of *F. hepatica* was confirmed by ultrasound and indirect ELISA. Authorities in the North Coast of Santa Catarina were notified in order to investigate other cases and risk factors for contamination. The disease is also prevalent in cattle and it is important to investigate this as a potential risk of infection.

Keywords: *Fasciola hepatica*; liver fluke; neglected disease; public health; zoonosis.

Introduction

Human fascioliasis generally occurs by the ingestion of metarcercariae, which is the encysted form of the Trematode parasite *Fasciola* spp. This infective form can be found attached to plants or in the water[1]. After ingestion, an incubation period starts, followed by an acute and chronic clinical phases. The acute phase includes fluke migration up to the bile ducts, for about 2-4 months, and during the chronic phase, adult worms install themselves in the bile ducts, leading to months or years of infection[2,3].

In Brazil, fascioliasis is not a disease of compulsory declaration and for this, only 48 cases of human infection were reported in scientific papers from 1950 to 2016[4]. When added to the difficulties in performing a accurate diagnosis, this results in a small number of reported cases, leading to a considerable underestimate of the real problem with large number of subjective cases[4]. To the best of the authors' knowledge, the last report found in literature in

Brazil was from the Amazonas state, where 36 (8.3%) human serum samples were reactive in ELISA, of which 8 (1.8%) were Western Blot reactive, and only one fecal sample was positive using the *F. hepatic*a coprological test[5].

The difficulty in performing a proper diagnosis, as well as the lack of knowledge by health care professionals with regards of fascioliasis, have both been associated with the under-reporting of human fascioliasis[3,6]. Considering these difficulties, local authorities must be supported through more frequent case reporting, such as this one, which would allow them to take necessary preventive measures. Thus, case reports are important in the local scenario, especially when it comes to such a neglected disease. This work is the first human fascioliasis case to be reported in the state of Santa Catarina, South of Brazil, an area well established for the occurrence of bovine fascioliasis[7].

Case report

A 57-year old woman, originally from Balneário Piçarras, Santa Catarina, Brazil (Figure 1), was admitted to the Health Unit of Balneário Piçarras, complaining of persistent abdominal pain. The patient had a previous medical record, as 15 days earlier she had been admitted to a hospital for 5 days, presenting acute abdominal pain, followed by fever and acute jaundice (yellowing of the skin). An ultrasound examination revealed a hepatic cyst and a small nodule of hemangioma. She was treated with non-specific antibiotics and showed signs of clinical improvement. However, the patient had recurring pain episodes after a few days. For this reason, the patient sought a new consultation, where she was submitted to another ultrasound examination. Although the liver showed a normal parenchyma, a hepatic cyst of 2.6 cm was noticed. The absence of lithiasis was also noted.

Liver function findings were as follows: aspartate transaminase 66 [normal < 31] U/L, alanine transaminase 150 [normal < 32] U/L, alkaline phosphatase 159 [normal 27–100] U/L,

gamma-glutamyl transferase 106 [normal 5-32] U/L and a normal total bilirubin, with persistent eosinophilia and elevated inflammatory tests.

After 4 months, she returned to the hospital, maintaining a recurrent but mild abdominal pain. The possibility of *F. hepatica* infection was suspected after a hepatic capsule rupture in the MRI (Figure 2A and 2B) was observed. For this reason, a blood sample (serum) was collected for an anti-*F. hepatica* antibodies screening, using an indirect ELISA. The ELISA method presents a sensitivity of 99.9% and specificity of 99.9% and employs a *F. hepatica* Cathepsin L1 recombinant protein as an antigen [8]. The immunological test confirmed the presence of anti-*F. hepatica* IgG antibodies.

Unfortunately, after multiples contacts, the patient did not return to receive the required follow ups and treatment. This study was approved by the ethics committee of Federal University of Paraná (CAAE number 50984215.0.0000.0102).

Discussion

The life cycle of *Fasciola hepatica* is heteroxenous, where hosts of different species are required for the entire life cycle to be completed. The intermediary hosts are snails that belong to the Lymnaeidae Family, while the most common definitive hosts are livestock animals and humans[3]. These multiple *F. hepatica* reservoirs make fascioliasis an almost impossible infection to be targeted for eradication[6].

The diagnosis of fascioliasis is still a challenge in humans, mainly in non-endemic regions, given that the disease is not recognized by Health Authorities and that there are numerous nonspecific symptoms observed in infected people. In the acute phase of fascioliasis, eosinophilia is the most common laboratory finding, as we observed. However, eosinophilia may not be observed in all human fascioliasis cases, being only an indication of parasitic infections[3,6]. Although diagnose methods based on coprological exams are routinely used (to

search for parasite eggs), they are time-consuming and have low accuracy. Furthermore, the immunodiagnostic based on ELISA is seldom used, even for fascioliasis validation in Brazil (M. Molento, personal observation).

Triclabendazole (TCBZ) is the drug of choice for human and animal fascioliasis, which effectively kills early immature and adult *Fasciola* liver flukes. Although fascioliasis treatment with TCBZ is well tolerated and easily administered, the patient reported in the present case did not return to the Health Care Unit to receive it. This episode serves as a warning, to show that the community should be better informed about the importance this parasitic diseases, through educational health programs.

Regarding the risk of human infection, some factors need to be considered, such as the areas that have a high incidence of fascioliasis in ruminants[9]. Although there is no data that can confirm an overlapping relationship between human and animal fascioliasis in Brazil, the high prevalence of fascioliasis in cattle in the same region (Joinville, Blumenau, Florianópolis and Itajaí), close to Balneário Piçarras, has been reported[7].

This study reports the first case of human fascioliasis in Balneário Piçarras, an area with a high prevalence of animal fascioliasis[7]. The present case-report is important in order to alert the scientific and medical community and local authorities to the occurrence of the disease in this region. As this liver-affecting infection is a food born disease, it is recommended that specific safety measures be given to the population, orienting the proper sanitation of fresh vegetables (i.e. cress, arugula/rocket) and the consumption of potable water. These important preventive actions would considerably reduce disease risks, improving community welfare.

Figures subtitles

Figure 1: Map locating the city of Balnéario Piçarras, from the state of Santa Catarina, Brazil.

Figure 2. Nuclear Magnetic Resonance of (A) main coronal contrast, (B) axial contrast. Highlighted the lesions formed by the presence of *Fasciola hepatica* adult worm.

Declarations of interest

None.

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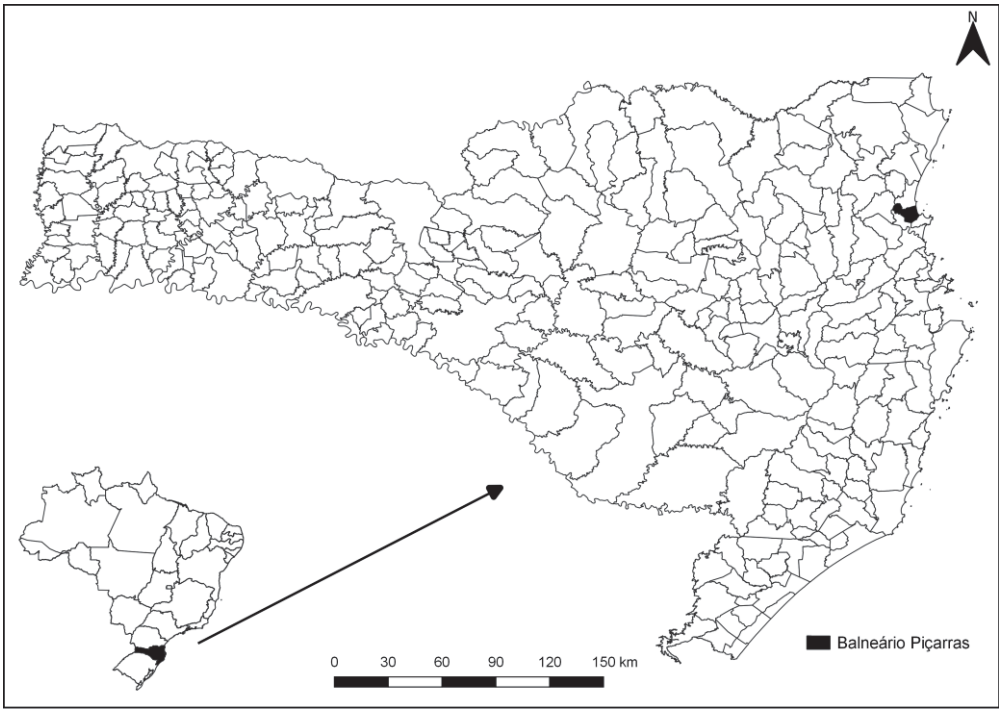

Figure 1: Map locating the city of Balnéario Piçarras, from the state of Santa Catarina, South of Brazil.

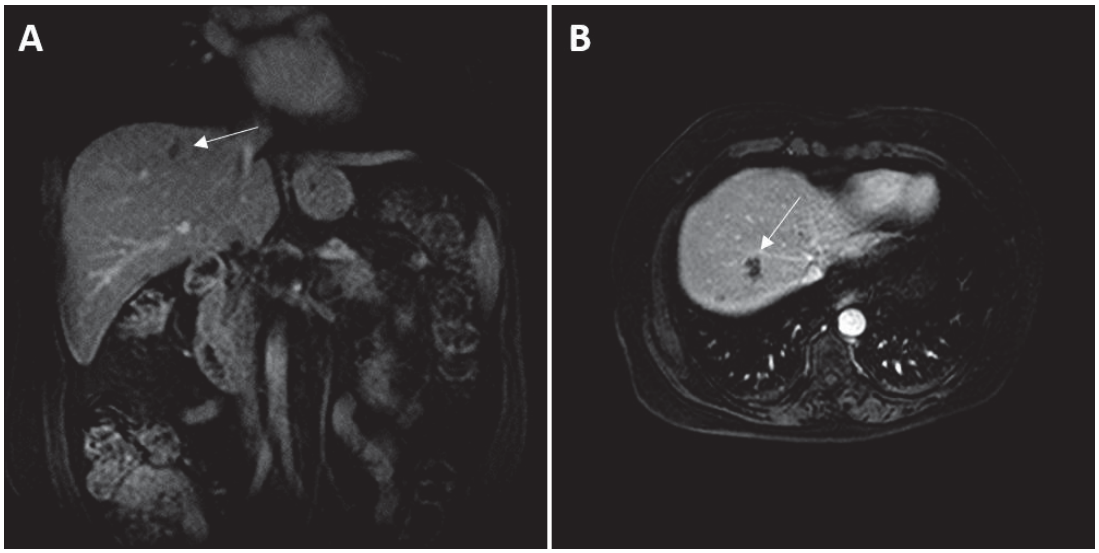

Figure 2. Nuclear Magnetic Resonance of (A) main coronal contrast, (B) axial contrast. Highlighted the lesions formed by the presence of *Fasciola hepatica* adult worm.

4.3 Chapter 3

The chapters one and two approached fascioliasis disease regarding human infection in Brazil. However, this disease is also neglected from the veterinary point of view. Based on the lack of studies regarding the epidemiology of animal fascioliasis and also the problems (liver condemnation, reduced production of meat and milk, affects the herd fertility, expenditures for anthelmintics, and mortality), caused by the infection to the welfare of livestock, this chapter aims to determinate the prevalence and association of anti-*Fasciola hepatica* antibody levels in bulk tank milk and milk quality in dairy cows in Paraná.

The prevalence and the impact of the disease on milk quality has never been studied in Brazil until now. The manuscript will be submitted to Parasitology (Cambrigde University, Cambridge, United Kingdom).

Association between anti-*Fasciola hepatica* antibody levels in bulk tank milk and production parameters in dairy cows

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Running title: Anti-*Fasciola hepatica* antibody levels in bulk tank milk

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ABSTRACT

The liver fluke *Fasciola hepatica* is a helminth parasite responsible for a significant impact on the health of livestock. The objective of this work was to assess the relationship between *Fasciola*-specific cathepsin L1 antibody levels in bulk tank milk and milk quality parameters in Paraná (PR) State, Southern Brazil. Bulk tank milk samples were collected from 1,492 dairy farms representing the 10 mesoregions of PR. The average prevalence was 9.72%, while the highest rates were found in the Metropolitan area of Curitiba (42.86%); Eastern Center (13.01%); Northwest (11.76%) and South Center (8.82%). The redundancy analysis using a multivariate linear regression model, showed that milk protein, lactose and defatted dry extract contents were lower in the positive samples, compared to the negative ones. This is the first study focusing on *F. hepatica* distribution in dairy cattle covering such a large area in Brazil, revealing an important variance in disease distribution. We found milk quality differences that could have an impact on the producers' income, as fat and protein may receive a payment differentiation by the dairy industry. Parasite control program in dairy cows is discussed.

Keywords: Trematode; Liver fluke; Milk quality; Milk immune-diagnosis.

KEY FINDINGS

Anti-*Fasciola hepatica* antibodies in bulk tank milk samples of Parana was 9.72%.

Milk fat, milk solids contents and somatic cell count were higher in the positive samples.

Milk protein, lactose and defatted dry extract contents were lower in positive samples.

1. INTRODUCTION

Fascioliasis, a widespread parasitic disease of ruminants and other animals including humans (Mas-Coma et al., 2005), is caused by the helminths *Fasciola hepatica* and *F. gigantica*. In cattle, fascioliasis can be acute in calves, but is more typically subclinical in adult

animals (Ibrahim, 2017). The disease causes problems mainly through liver condemnation, reduced beef and milk production, affects fertility, increase expenditures for anthelmintics and mortality (Kaplan, 2001; Schweizer et al., 2005). In Brazil, the infection also causes a loss of US$ 210 million annually to the beef cattle sector (Molento et al., 2018). The study of the prevalence of *F. hepatica* has increased in importance since Claridge et al. (2012), reported a significant association between the exposure to *F. hepatica* and the misdiagnosis of bovine tuberculosis (bTB).

The prevalence of *F. hepatica* in beef cattle in Brazil is on average 6.32% where the highest prevalence is observed in Southern states, specifically along the Parana (PR) and Santa Catarina (SC) coast, and the entire Southern part of Rio Grande do Sul (RS) (Bennema et al., 2014). Dairy cattle infected with *F. hepatica* studies have demonstrated an association between reduced milk yield and milk fat in Spain (Mezo et al., 2011) and Belgium (Charlier et al., 2007). The milk yield reduction of 1042 kg/year represented a financial loss of £333.00 per cow to the United Kingdom dairy industry (Howell et al., 2015). The authors reported that the magnitude of these effects would depend on the breed of the cow (i.e. Holstein and Jersey) and husbandry systems (intensive and semi-extensive production).

The bulk tank milk enzyme-linked immune sorbent assay (ELISA) is a rapid and inexpensive test that relies on the detection of host antibody as an indicator of infection (Santana et al., 2013). One example of its potential use comes from the European dairy farm improvement program, with routine collection of on-farm milk samples to monitor animal productivity, milk quality and some diseases (Mezo et al., 2010). Production quality parameters include the evaluation of the milk nutritional value, which would reflect on the producer's income and to the dairy industry (Reis et al., 2007). Of note, milk yield and its physicochemical composition can vary according to the bovine breed, age, stage of lactation, nutrition, physiological and pathological factors, as well as milking interval (Costa et al., 1992; Weiss et

al., 2002). Considering pathological factors, studies have shown the negative association between milk quality and fascioliasis in dairy cattle, resulting in a significant reduction of volume and milk composition (Charlier et al., 2007; Köstenberger et al., 2017; Mezo et al., 2011). The present study had the objective of determining the prevalence of anti-*F. hepatica* antibodies in bulk tank milk and the impact of infection on milk quality from 1,492 farms, over the state of PR, Brazil.

2. METHODS

2.1. Study area

State of Paraná is located in the South of Brazil. The state area has 199,709 square km, distributed in 399 municipalities with a predominant humid subtropical climate. The geographical distribution consists of low altitudes in the coastal region, plateaus to the west and east (max. 1,300 m) and depression in the central region (max. 450 m). The dairy cattle population is about 1,641,000, producing an average of 4.66 billion litters/year (IBGE, 2015).

2.2. Sample collection

The laboratory of the Holstein Association of Paraná, located in Curitiba receives milk samples from the entire state. Each milk sample represent an individual farm. Each sample is taken from a bulk tank. The state of Paraná has approximately 120,000 dairy farms, distributed along 10 mesoregions: Southeast (SE); North Central (NC); Western Center (WC); North (NO); Southwest (SW); Eastern Center (EC); South Center (SC); Northwest (NW); West (WE) and metropolitan area of Curitiba (MA). At the laboratory, a total of 1492 milk samples were randomly collected based on a homogeneous distribution of the farms among all mesoregions. The samples included in the study were obtained from Holstein cows and herd sizes varied from small (approx. 12 animals/farm) to large (approx. 120 animals/farm). Samples from

individual animals were not included in the study. The sample size calculation had a margin of error of 2.5%. The milk serum was obtained using the coagulant Estrella (Chr. Hansen, A/S, São Paulo, Brazil), and the serum was stored at -20°C until analysis.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The ELISA test was standardized using positive and negative bovine milk serum controls. The positive control was confirmed by the presence of *F. hepatica* eggs in the faeces of dairy cattle. The negative control was confirmed by the absence of *F. hepatica* eggs in the faeces and the animals were from an area with no reports of fascioliasis. The samples were tested at different dilutions (0, 1:3, 1:9, 1:27, 1:81, 1:243 and 1:729) with peroxidase-conjugated anti-bovine IgG (Sigma Aldrich, Inc., USA) at 1:4000, 1:6000 and 1:8000. The test was performed using 0.5 µg/ml of *F. hepatica* cathepsin L1 (FhCL1r) as antigen per well, sample dilution at 0 and anti-IgG conjugate at 1:6000. The stable recombinant form FhCL1r was supplied by Professor J. P. Dalton from Queen's University (Belfast, Northern Ireland), which has high sensitivity and specificity, with no cross-reaction to other parasitic diseases. The antigen was validated by Gonzales Santana et al. (2013). Using a 96-well plate, FhCL1r was dissolved in carbonate coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$) at pH 9.0. This solution (100 µl) was added to each well and incubated overnight at 37 °C. After washing three times, excess protein binding was blocked at 37 °C for 30 min by adding 100 µl of 5% milk diluted in phosphate-buffered saline (PBS) 0.1% Tween 20. After a further washing procedure, 100 µl of milk serum was added and the plate was incubated for 30 min at 37 °C. Following another wash, 100 µl of peroxidase-conjugated anti-bovine IgG (diluted 1:6000) was added to the wells and the plates were incubated for 30 min at 37 °C. After a final washing step, bound antibodies were detected by the addition of 100 µl of TMB (3,3', 5,5;-tetramethylbenzidine). The color was developed for 10 min and the reaction was stopped with 100 µl of 0.1 M

sulphuric acid. The optical density (OD) was read on an ELISA plate reader at 450 nm (TP-Reader NM, Thermo Plate, USA).

The OD value of each sample was calculated OD1–OD2, where OD1 is the mean value obtained for the two Ag+ wells, and OD2 is the mean value obtained for the two Ag-wells. The cutoff value was 0.1188, which was taken from the average absorbance of the negative controls plus four standard deviations. Positive samples were double tested. The cut-off methodology determination employed in this study is in accordance to Mezo et al. (2010) for the detection of IgG antibodies against *F. hepatica* in lacteal secretions.

2.4 Milk parameters

All samples were analyzed for milk quality. The milk parameters included were: fat (FAT), protein (PRO), lactose (LAC), solids (SOL), and defatted dry extract (DDE) contents, somatic cell count (SCC) and standard plate count (SPC). The samples were preserved in azidiol or bronopol. The sample analyses were performed at the laboratory of the Holstein Association of Paraná. All the parameters were determined by infrared spectrophotometry with the automated equipment Somacount FC, Bentley 2000 and BactoCount (Bentley Instruments Inc., USA).

2.5 Geographic Information System (GIS)

Kernel Intensity Estimator (QGIS 3.0.3 software) was used to perform the density analysis of anti-*F. hepatica* in bulk tank milk samples according to the municipalities of the farms with an 8-km scale.

2.6 Statistical analysis

The data were organized in a matrix of response variables (milk parameters) and the predictors: mesoregions and OD. Since the goal of the study was to assess the effects of *F. hepatica* on milk parameters, we considered all predictors and response variables simultaneously, through a single statistical analysis as the best analytical choice for reducing biases and achieving the model that best approached farm situations. Therefore, the data were analyzed by a redundancy analysis (RDA), which is an extension of multiple linear regression models for a scenario that included multiple response variables (Legendre and Legendre, 2012). The homogeneity of the variances (homoscedasticity) was evaluated by the Fligner-Killeen test (Killeen and Fligner, 1976). No collinearity was detected among the variables included in the model (FIV> 5; Zuur et al., 2007). Three permutation tests were performed (n = 999) to evaluate the overall significance of the model, to each canonical axis and to each term (i.e. predictor variables). The selection of variables included in the model was done by a Stepwise Forward analysis based on the Akaike Information Criterion (AIC). Analyses were performed in the R v. 3.4.2 environment (R Core Team, 2018) with the vegan packages (Oksanen et al., 2018) and car (Fox and Weisberg, 2011).

3. RESULTS

3.1 Prevalence of antibodies anti-F. hepatica in Paraná State

The number of milk samples collected and the positive serum samples by mesoregion is presented in Table 1. The average prevalence of anti-FhCL1r antibodies in milk serum was 9.3% and the disease is spread in all mesoregions (Figure 1). The mesoregions with higher prevalence were MA, EC, NW and SC, with 42.86, 13.01, 11.76, and 8.82%, respectively.

3.2 Correlation between OD values and milk parameters

RDA analysis is a complete and descriptive analysis and is appropriate for this case, as the variables were analyzed in a set. Variable selection through the Stepwise analysis revealed a non-random relationship between milk parameters and the predictor variables (mesoregions and positive/negative group; ($F_{10, 1481}$=7.98, p=0.001). However, the model had a low adjusted correlation coefficient (R^2adj=4.5%). The data were significantly structured only in relation to the first canonical axis ($F_{1, 1481}$=62.95, p=0.001), with both variables being significant (OD: $F_{1, 1481}$=4.03, p=0.008; mesoregion: $F_{9, 1481}$=8.42, p=0.001). Therefore, parasitism by *F. hepatica* and the farm location within the mesoregion had some effect on milk quality parameters. In this sense, FAT, SOL, and SCC (Figure 2), had higher values (FAT mean ± SD = 3.80 ± 0.97) in the positive samples (PG centroid in Figure 2), compared to the negative ones (FAT 3.73 ± 0.75). In contrast, vector directions in Figure 2 show that PRO (3.10 ± 0.25), LAC (4.34 ± 0.23) and DDE contents (8.38±0.34) were lower in the positive samples than in the negative ones (respectively: 3.21 ± 0.26; 4.35 ± 0.26; and 8.52 ± 0.44).

It was possible to verify that milk parameters also varied according to mesoregion. This factor can be noticed by the distinct relationship between parameter vectors and the mesoregion centroid in Figure 2. Higher values of FAT and SCC were found in SE; where PRO, DDE and LAC in NW, NC and NO. The EC mesoregion, known as the most specialized dairy region in PR, showed a similar pattern for the milk parameters to *F. hepatica* positive areas.

4. DISCUSSION

Our data showed the prevalence and distribution of *F. hepatica* infection in dairy cattle in PR, based on the presence of anti-*F. hepatica* antibodies in milk samples. Although the adjusted correlation coefficient was low (4.5%), it was possible to notice the influence of the infection to milk parameters. The MA mesoregion had the highest prevalence with 42.86% (42

of 98) positive samples. Bennema et al. (2014), analyzed data from beef cattle livers condemned as a consequence of *F. hepatica* infection and found 14.39% positivity in Rio Grande do Sul, 4.5% in Santa Catarina, and only 0.08% in PR. From these data, PR was not in the list of the highest affected states, unlike the data obtained in the present milk analysis. This large discrepancy may be attributed to differences in heard health management, including the access to intermediate hosts in small marsh areas, the lack of diagnosis and most importantly the differences in diagnosing the disease (Munita et al., 2016; Olsen et al., 2015). In the case of beef cattle, data were collected from abattoirs (physical infection), as the present data set were analyzed based on bulk tank milk (immune marker). Dairy farmers in Paraná produce more than 4.6 billion L/milk/year in a milking herd of 1.4 million animals.

To date, no study had correlated milk parameters and the presence of antibodies anti-*F. hepatica* in dairy cows in Brazil. RDA analysis considered all three variables (anti-*F. hepatica* antibodies, milk quality parameters and mesoregion) in a multidimensional plane set. Accordingly, *F. hepatica* positive samples influenced the parameters of milk quality compared to negative samples. FAT and SOL and SCC were higher in the positive samples than in the negative ones. The FAT reduction may be attributed to the farm's size, e. g. small farms tend to offer more pasture period to the cows, as observed in farms in the MA mesoregion (mesoregion with the highest prevalence rate). This diet source increases the fat content of the milk (BERCHIELLI, 2006). In contrast, high production farms include animal ration/concentrate to the cows, due to their high metabolism demand for (higher) milk production. In the MA mesoregion, we observed the lowest value ($5.9x10^5$ cells/ml) for SCC. However, positive ($7.7\ x10^5$ cells/ml) and negative ($6.1\ x10^5$ cells/ml) samples for the entire state showed values higher than the maximum acceptable values ($5x10^5$ cells/ml) established by the regulatory instruction IN-62, published on December 29 2011, by the Ministry of Agriculture.

Nowadays, even such minor shifts in milk parameters due to *F. hepatica* may have economic effects. According to Pool Leite (2016), a farmer association operated by Castrolanda Frísia and Capal Inc., in PR, each 0.1% above 3.05% of protein in the total monthly volume of milk, adds another 6% in (Reais R$) gains to the producer. Therefore, according to our results of 3.1% in the positive group and 3.73% in the negative group), there would be a loss of ~36% (taken from the bonus). The association between anti-*F. hepatica* antibody levels in milk and productive parameters have been previously reported. Mezo et al. (2011) showed a milk yield reduction of 1.5 kg milk/cow/day in herds positive to anti-*F. hepatica* antibodies in Spain. In Belgium, Charlier et al. (2007) reported a decrease in the annual average milk yield of 0.7 kg milk/cow/day, with a decrease of 0.06% in fat content. Also, Köstenberger et al. (2017), showed a reduction of milk yield of 6% (438 kg/cow/year) and a significant effect on butterfat and protein contents, decreasing from 0.091 to 0.046%, respectively.

PRO, LAC and DDE contents are interconnected traits, as well as FAT and SOL. Accordingly, our results showed a consistent correlation among them, when considering that the parameters remained high or low in the same proportions. DDE is dependent of PRO, LAC and the mineral contents of the milk, and LAC corresponds to 52% of DDE. LAC is the most stable milk component. In addition, LAC is directly related to the volume of milk produced (Cruz et al., 2014). In Switzerland, the prevalence of *F. hepatica* infection in dairy cattle was over 16% and Schweizer et al. (2005) estimated an average loss of € 299/infected animal due to loss of milk yield, extended calving to conception, additional artificial insemination services per pregnancy, loss by condemned livers and treatment costs. The constraint in our model does not allow us to numerically estimate the influence of the disease to the above parameters, as the model could be improved by the addition of data from farm husbandry and herd management, mammary gland health, nutrition and animal breed/genetic (Cruz et al., 2014; Novaes et al., 2017; Petrini et al., 2016). We are collecting more information to develop another

model that would incorporate these factors into herd management and individual milk parameters.

Although Mas-Coma et al. (2005), suggested that there is no data to confirm the correspondence of animal and human fascioliasis from the same area, it is important to mention that 41% of the human cases in Brazil were reported in PR, and from those, 95% occurred in the MA mesoregion (Pritsch and Molento, 2018). The present data indicate that the disease is spread all over PR, overlapping with the human form of the disease in the MA region. These leads us to believe that the prevalence of *F. hepatica* obtained from beef (Dutra et al., 2010; Bennema et al., 2014) and dairy cattle could be considered as important risk factors for the transmission of the disease to humans.

The present data describes the hot-spots of fascioliasis in dairy cows in PR based on the use of an immunological test. Afshan et al. (2013), Martins et al. (2008), and Nguyen et al. (2012), considered the use of milk serum as a more specific and accurate diagnose to study parasite prevalence, when compared to coprological exams to be used in dairy cattle. The importance of fascioliasis treatment is reported by Köstenberger et al. (2017), showing a positive impact on milk production (no influences on milk yield and butterfat) and a reduction of €65 per cow/year comparing the financial loss caused by the infection and the treatment costs. The challenge now is to stablish a parasite control program for the specific areas to help producers and local authorities, to improve herd health status and animal welfare.

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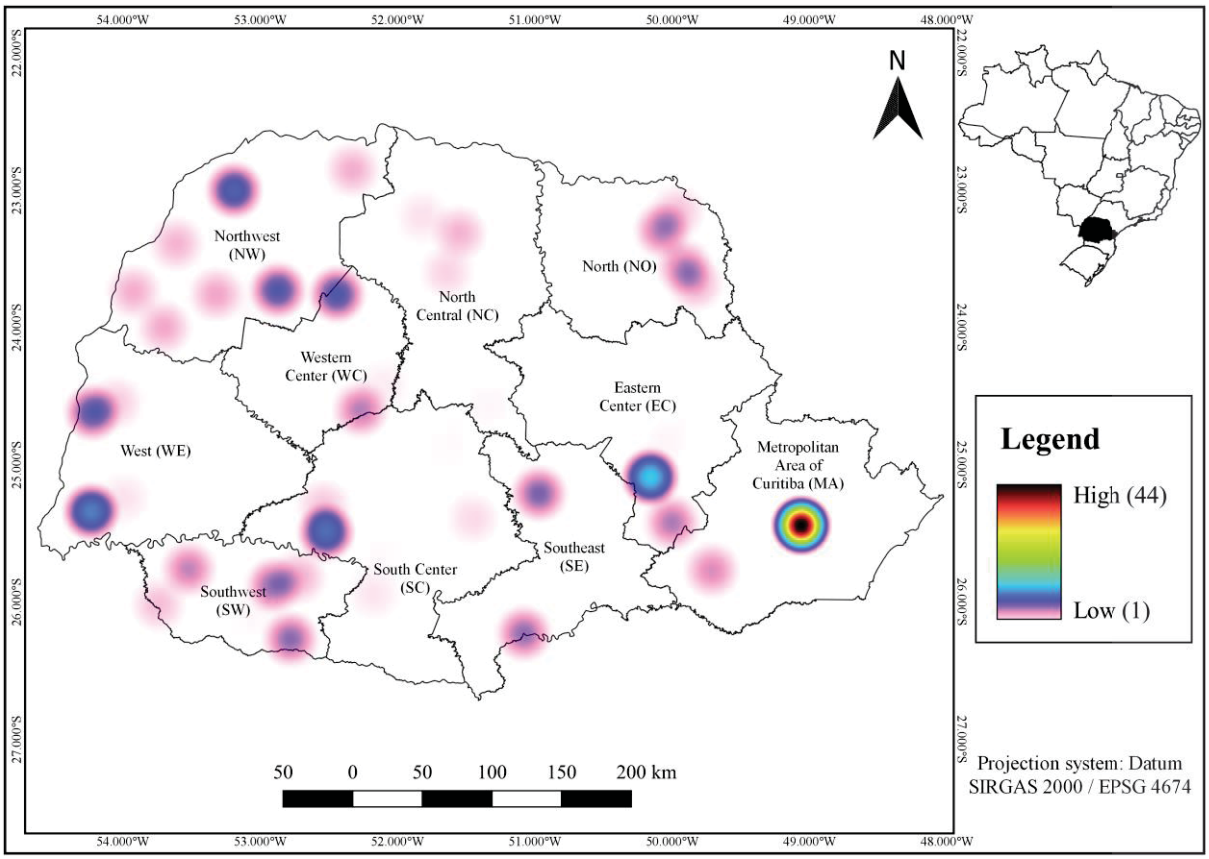

Figure 1. Distribution of anti-*F. hepatica* antibody density in dairy cattle farms in the state of Paraná, Brazil, using Kernel Intensity Estimator. Low (1) = minimum number of positive farms to anti-*F. hepatica* antibody within a radius of 8-km scale. High (44) = maximum number of positive farms to anti-*F. hepatica* antibody within a radius of 8-km scale. The white areas in the map represents negative farms (to anti-*F. hepatica* antibody) or farms that were not investigated.

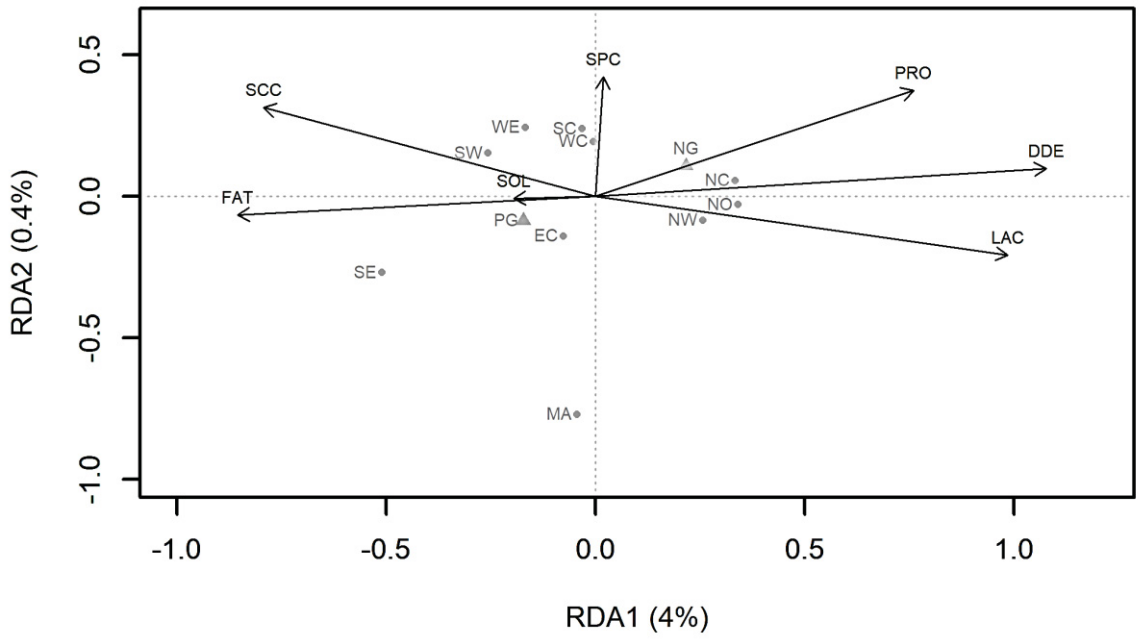

Figure 2. Order of redundancy analysis (RDA) among milk quality parameters (SOL: solids, FAT: fat, PRO: protein, LAC: lactose, DDE: defatted dry extract, SCC: somatic cell count, SPC: standard plate count, PG: positive group to antibodies anti-*F. hepatica*, NG: negative group to antibodies anti-*F. hepatica*, and the mesoregions of the state of Paraná, Brazil (SE: Southeast, NC: North Central, WC: Western Center, NO: North, SW: Southwest, EC: Eastern Center, SC: South Center, NW: Northwest, WE: West and MA: Metropolitan Area of Curitiba). The values in parentheses in the x and y axis, indicate the percentage of variance explained by each canonical axis. The variables were scaled for unit variances and their eigenvalues were resized symmetrically for the representation in the diagram.

Table 1. Distribution and positivity (%) of *Fasciola hepatica* using anti-*F. hepatica* antibodies in dairy cattle and its distribution over the mesoregions* of the state of Paraná, Brazil.

	SE	**NC**	**WC**	**NO**	**SW**	**EC**	**SC**	**NW**	**WE**	**MA**	**Total**
Collected	119	170	130	148	169	123	170	187	178	98	1492
Positive	5	6	6	10	8	16	15	22	15	42	145
Negative	114	164	124	138	161	107	155	165	163	56	1347
Positivity/total	0.34	0.40	0.40	0.67	0.54	1.07	1.01	1.47	1.01	2.82	9.72
Positivity/mesoregion	4.20	3.53	4.62	6.76	4.73	13.01	8.82	11.76	8.43	42.86	---

*mesoregions: SE: Southeast, NC: North Central, WC: Western Center, NO: North, SW: Southwest, EC: Eastern Center, SC: South Center, NW: Northwest, WE: West and MA: Metropolitan Area of Curitiba.

4.4 Chapter 4

We have noticed that there were few data about the prevalence of fascioliasis in water buffalos (*Bubalus bubalis*), and we found no available data in Brazil. Therefore, this chapter aims to determine the prevalence of *F. hepatica* in the liver of buffaloes, slaughtered in the last 15 years (from 2003 to 2017) and to perform a forecast analysis of the disease for the next 5 years. The manuscript has been submitted to the Revista Brasileira de Parasitologia Veterinária (FCAV/UNESP - Jaboticabal, São Paulo, Brazil) and is currently under review.

Fascioliasis in buffaloes: A 5-year forecast analysis of the disease based on a 15-year survey in Brazil

Fasciolose em búfalos: uma análise de previsão de 5 anos da doença com base em uma pesquisa de 15 anos no Brasil

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Running Head:

5-year model for Fascioliasis in water buffaloes

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Fascioliasis in buffaloes: A 5-year forecast analysis of the disease based on a 15-year survey in Brazil

Abstract

In South America, fascioliasis caused by the trematode *Fasciola hepatica* is an anthropozoonosis disease associated with significant economic losses and poor animal welfare. Despite its importance to ruminants, there are few reports on the occurrence and impact of the disease in buffaloes in the region. The objective of this study was to determine the prevalence of *F. hepatica* in the liver of buffaloes slaughtered from 2003 to 2017 in Brazil, and to performed a forecast analysis of the disease for the next 5 years using the Autoregressive Integrated Moving Average (ARIMA) model. Data analysis revealed an incidence of 7,187 cases out of 226,561 individuals. There was a considerable interannual variation in the national prevalence over the years, from 6.8% in 2012 to 1.5% in 2015 ($p<0.005$). The disease was more prevalent in the southern states of Brazil; Paraná, Rio Grande do Sul, and Santa Catarina, presenting 11.9, 7.7, and 3.2% of infected livers, respectively. The high frequency of liver condemnation in Paraná was influenced by weather conditions. The predictive models indicated a constant trend of the disease, depicting an average, a worse-case and a positive-case scenario. As we report, there is an urgent need for regular diagnostic in the animals (fecal and immune diagnose) and the environment (intermediate host), in order to avoid the high rates of animal infection. This study identified the geographical distribution of the parasite occurrence in buffaloes, and the regions in which the disease is well established. The ARIMA models may serve as an example to other endemic regions.

Keywords: Fasciola hepatica; Trematoda; Ruminants; Epidemiology.

Resumo

Na América do Sul, a fasciolose causada pelo Trematoda *Fasciola hepatica* é uma antropozoonose associada a perdas econômicas significativas e baixo grau de bem-estar animal. Apesar de sua importância para os ruminantes, existem poucos relatos sobre a ocorrência e impacto da doença em búfalos na região. O objetivo deste estudo foi determinar a prevalência de *F. hepatica* no fígado de búfalos abatidos entre 2003 a 2017 e realizar uma análise de previsão da doença para os próximos 5 anos, utilizando o modelo auto-regressivo integrado de médias móveis. A análise dos dados revelou uma incidência total de 7.187 casos em 226.561 indivíduos. Houve um acentuado grau de variação interanual nas taxas de prevalência nacionais ao longo dos anos; de 6,8% em 2012, para 1,5% em 2015 ($p<0,005$). A

doença foi mais prevalente nos estados do sul do Brasil; Paraná, Rio Grande do Sul e Santa Catarina, com 11,9; 7,7; e 3,2% de fígados condenados, respectivamente. A alta incidência de condenação de fígado no Paraná foi influenciada pelo fator climático. Modelos preditivos (ARIMA) indicaram uma tendência constante na ocorrência da doença, destacando um padrão, assim como cenários de piora e de possível melhoria. Como verificamos a doença no Brasil, existe urgência em regularizar um diagnostico nos animais e no ambiente, para que se evite os altos índices de infecção animal. Este estudo identificou a distribuição geográfica da ocorrência do parasito em búfalos e regiões onde a doença está bem estabelecida. Os modelos ARIMA podem servir de exemplo para demais regiões endêmicas.
Palavras-chave: *Fasciola hepatica*; Trematoda; Ruminantes; Epidemiologia.

Introduction

Fascioliasis is a worldwide anthropozoonotic food and water-borne disease, caused by the trematodes *Fasciola hepatica* and *F. gigantica*. The common liver fluke, *F. hepatica*, is mainly found in large ruminants in the Americas and requires fresh water Gastropoda snails, such as *Lymnaea* spp., as an intermediate host (BEESLEY et al., 2017; IBRAHIM, 2017). The severity of the disease affects various animals to different extents, depending of the host species and parasitic burden. The welfare condition and clinical signs can also vary from an asymptomatic infection to a devastating disease (weight loss, reduced milk yield, and reproductive failure), including death (EL-TAHAWY; KWAN; SUGIURA, 2018; IBRAHIM, 2017; KAPLAN, 2001; SCHWEIZER et al., 2005). In some South American countries, fascioliasis is highly endemic in humans (RODRÍGUEZ-ULLOA et al., 2018), but essentially neglected in others (PRITSCH et al., 2018), with more than 60 million people living in affected areas.

Considering the several characteristics of fascioliasis lifecycle, it can be classified as a disease that is susceptible to the impacts of climatic variations. Studies have shown the influence of the weather on fascioliasis infection in cattle (Bennema et al., 2014, 2017; Dutra et al., 2010). An increase in rainfall was strongly correlated with a higher occurrence of the disease in southern Brazil (DUTRA et al., 2010b). Also in Brazil, Silva et al. (2016) reported that a La Nina event affected the positivity index of bovine fascioliasis through significant changes in air temperature and rainfall regimens.

The correlation between a disease and its geographic and climatic location is possible with the use of geographic information systems, creating epidemiological maps for viewing and evidencing risk factors. Based on these tools, Bennema et al. (2017), using a range of

isothermality, showed that a large part of Brazil is suitable for *F. hepatica*. Aleixo et al. (2015), using a kernel analysis, determined the density of livers infected with *F. hepatica* in Brazil from 2003 to 2008, identifying the localities with higher prevalence of fascioliasis in cattle. Geo-environmental analysis system, considering precipitation, temperature, elevation, slope, soil type and land use was used in Espírito Santo, Brazil for mapping the risk of fascioliasis indicating that over 50% of the southern of the state is either at high or very high risk for fascioliasis (MARTINS et al., 2012).

Domestic buffaloes (*Bubalus bubalis*), also known as river/water buffaloes, are important to local economies and are present in a small segment of the society. In South America, the most common buffaloes are the Murrah, Mediterranean, Jaffarabadi, and their crosses, which are used for milk and meat production. Only 2% of the world's water buffalo population (168 million) is in South America, and of this, 77% (aprox. 1,190 million) is in Brazil (Food and Agriculture Organization of the United Nations, 2018; Instituto Brasileiro de Geografia e Estatística, 2006; Ministério da Agricultura, Pecuária e Abastecimento, 2018). Reports of *F. hepatica* occurrence in buffaloes were found with positivity rates of 24.7% (SERRA-FREIRE; NUERNBERG, 1992), 2.5% (PILE et al., 2001), 46.7% (CARNEIRO et al., 2010) and 23.8% (CARNEIRO et al., 2013), when using fecal exams in Brazil. The occurrence of *F. hepatica* in condemned livers of buffaloes was determined in the state of Rio Grande do Sul (RS). The occurrence rate was 20%, and from that, 81% of the animals had up to two years of life (MARQUES; SCROFERNEKER, 2003).

The objectives of the present study were, (1) to determine the prevalence and geographical distribution of *F. hepatica* (from infected livers) in buffaloes across Brazil, and (2) to run a 5-year forecast analysis of the disease for the states of Paraná (PR) and RS.

Materials and methods

Study area

Brazil is geographically located between the parallels latitude 5°16'19" north, 33°45'09" south and longitude 34°45'54" east, and 73°59'32" west. The country's total area is 8,515,759.090 km^2, distributed among the 5,570 municipalities of the 26 states and one Federal District (IBGE, 2015).

Data collection

The data was collected from a Brazilian slaughterhouse database of the Meat Inspection Department, under the Federal Inspection Service of the Ministry of Agriculture (MAPA). This data included the total number of slaughtered buffaloes and the livers condemned by *F. hepatica* (parasites had to be visualized after incisions in the organ), from all Brazilian municipalities from 2003 to 2017.

Climate data were collected from the National Institute of Meteorology (NIM) database, for the states of PR and RS, for the entire period. Three weather stations per state were selected to collect the monthly rainfall and mean temperature.

Data analysis

A frequency analysis of the municipalities was carried out on the collected data, encoded in Microsoft Excel, using SPSS Version 22.0 (IBM, New York, USA). The frequency of liver condemnation was calculated using Software R 3.5.1 based on the following formula, for each state and year:

$$\text{Frequency} = \left(\frac{Liver\ condemnations}{Slaughtered\ animals}\right) \times 100$$

Kernel density estimation (6-km scale) (Aleixo et al., 29015) and point cluster analyses were used to calculate the density and space distribution of infected livers, respectively, using QGIS 3.2.2 software.

Linear regression models, using Software R 3.5.1, were used to determine the possible influence of the mean values of abiotic factors (temperature and accumulated precipitation) for each of the four calendar seasons on the number of liver condemnations due to fascioliasis in each respective period.

Forecast model

The Autoregressive Integrated Moving Average (ARIMA) was adopted for the development of the models. The variables "slaughtered animals" and "condemned livers" were used to calculate the infection ratio to model the incidence of *F. hepatica* for PR and RS. The variables "year" and "month" were created, and seasonal decomposition was performed according to the observed sequences of data, using the equation:

$$\mathrm{Yt} = \mathrm{TCt} \times \mathrm{St} \times \mathrm{It}$$

Where: Yt is the time series (slaughtered animals, condemned livers, and forecasting ratio); TCt is the trend-cycle component; St is the seasonal component; and It is the irregular or random component. The equation's best fits were chosen according to the R^2 value. For the forecast analysis, IBM SPSS Statistics software version 21 was used.

Results

Even though all 26 Brazilian states were included in the study, according to the MAPA database, only 15 states provided records of slaughtered buffaloes during the last 15 years. Out of these, most of the states (n=11) had low incidence and discontinuous official records for the disease (data not shown). Figure 1 describes the location of municipalities and intensity in which the condemned livers were consistently registered. The average prevalence of *F. hepatica* infection in slaughtered buffaloes was approximately 3% (7,187/226,561), ranging from 1.5% in 2015 to 6.8% in 2012 ($p<0,005$). The highest levels of infected livers were in PR, RS, Santa Catarina (SC), and São Paulo (SP), with prevalences of 11.9, 7.7, 3.2 and 1.42%, respectively. The frequency of liver condemnations in these states during the last 15 years is shown in Figure 2. The highest rates of condemned livers were observed in PR, 43.7% in 2016 and 33.6% in 2012, and in RS (32.8% in 2010). SC and SP presented their highest prevalence rates in 2006 (15.8 and 1.9%, respectively).

The absolute frequency analysis per city from the four states described the number of repetitive occurrences of infected livers over the 15-year period. The cities with the highest repeatability frequencies are in PR: Adrianópolis (14/15), Guaraqueçaba (12/15), Antonina (11/15), Cerro Azul (10/15) and Doutor Ulysses (10/15). Three of these cities (Adrianópolis, Cerro Azul, Doutor Ulysses) are situated in the Ribeira Valley, east of PR, which is one of the most important locations for *F. hepatica* occurrence with a rainfall of 180 mm during the wettest months.

Regarding climatic conditions, *F. hepatica* infection in buffaloes was found to be most influenced by rainfall in the autumn ($F=8.305$, $p<0.05$, R^2: 0.419), and temperature in spring ($F=4.85$, $p<0.05$, R^2: 0.35). Both factors presented a statistically significant relationship ($p<0.05$) with liver condemnation of fascioliasis in PR. None of the abiotic factors were significantly linked to liver condemnation in RS (Figure 3).

The forecast of the disease prevalence for the next 5 years is shown in Figure 4a for RS and 4b for PR. The best fit ARIMA model for RS was ARIMA (0,1,2)x(2,0,2), presenting: $R^2 = 0.751$; Stationary $R^2 = 0.701$; RMSE = 0.057; MAPE = 98.987; and a Normalized BIC -5.395. The best fit ARIMA model for PR was ARIMA (0,1,2), presenting an $R^2 = 0.847$;

Stationary $R^2 = 0.847$; RMSE = 0.087; MAPE = 92.365; and a Normalized BIC = -4.356. Both models are presented with future projections for the occurrence/prevalence of *F. hepatica* in livers.

Discussion

The present study shows a nationwide average prevalence of 3% in buffaloes with its highest frequencies occurring in PR (11.9%) and RS (7.7%). Buffaloes in PR were observed to be raised in different pasture areas from cattle (e.g. marshy pastures vs. seeded). While epidemiological studies in livestock and humans have been overlooked in many countries, the present work seeks to raise awareness of this important disease, particularly to the large population of buffaloes in Brazil. In cattle, previous studies have also shown that the prevalence of fascioliasis was mainly in the southern states (ALEIXO et al., 2015; DUTRA et al., 2010b; SILVA et al., 2016), with an economic loss of approximately US$ 210 million/year (MOLENTO et al., 2018). Bennema et al. (2017) have also determined that the best suitability area for *F. hepatica* development, based on a range of isothermality data was the south region of the country. Other studies have also investigated the weather and climate effects on *F. hepatica* infection (Caminade et al., 2015; Novobilský et al., 2015).

Dracz and Lima (2014) reported the first case of naturally infected indigenous buffaloes in the state of Minas Gerais. The study emphasized the ability of the parasite to disperse to municipalities in this state that previously were considered to be free from infection. Similarly, Pile et al. (2001), published the first report of fascioliasis in buffaloes in the state of Rio de Janeiro, registering an occurrence rate of 2.5%. Although the largest buffalo herds in Brazil are located in the states of Pará and Amapá, North of Brazil (MAPA, 2015), the lack of data from these and other states, complicates a broader analysis. This is mainly due to the scattered data collection and our deficient databank, which should combine the different levels of slaughterhouse inspection (federal, state and municipal) (BENNEMA et al., 2014). According to Collins (1996), the abattoir surveillance has also some limitations such as inspection line speed, light intensity, the inspector's experience and motivation.

PR has two major climate classifications: Cfa with hot summers; and Cfb with mild temperate summers (ALVARES et al., 2014). The disease occurrence falls almost entirely under the Cfb area of PR, and according to the absolute frequency analyzed for the buffalo infections, the cities with a highest fascioliasis repeatability were in that area (Figure 1 and 3), suggesting that the biological lifecycle of the parasite is well stablished. Whereas the prevalence cases in RS occurred almost entirely under the Cfa climatic conditions. Both states

had similar annual rainfall (PR: 1300 - 2200 mm/year; and RS: 1600 - 2200 mm/year). Annual air temperature was also comparable between the two regions (data not shown), but there was a considerably larger difference in land elevation between PR (100 to 1200 m.a.s.l.) and RS (100 to 400 m.a.s.l.).

We believe that *F. hepatica* is well stablished in buffaloes in PR and RS, even considering all the environmental (climate over the years) and topographical differences between the regions. The influence of rainfall and temperature on the percentage of condemned livers during the period of the study was evaluated according to the seasons of the year. Due to the relatively high prevalence observed in the states of PR and RS, the influence of the weather was further investigated in these areas. For PR, the data revealed that *F. hepatica* infection in buffaloes was influenced by the increase in rainfall and temperature in autumn and spring, respectively, since the increase of these factors tended to decrease the number of liver condemnations. On the other hand, no weather influence was related to the liver condemnation in RS. Dutra et al. (2010) reported that temperature did not reveal significant changes in high risk areas for *F. hepatica* infection in cattle, while altitude was found to be a significant determinant of the disease prevalence. Byrne et al. (2016), also found variable associations between long-term climate conditions and condemnation rates of liver infection in cattle. Byrne et al. (2018) found a significant seasonal variation of *F. hepatica* antibodies in dairy herds in Northern Ireland, reporting the highest infection rate in winter, whilst the lowest rates were found during the summer. In this line, Gosling et al. (2011), estimated that an increase in temperature of up to 3.5ºC in Brazil, and based on the above facts, there will be a large chance that risk areas for fascioliasis will spread around new areas. The International Panel on Climate Change (Cline, 2007) panel reported that Latin America may observe an increase in disease incidence. Therefore, the increase in temperature and precipitation will change the prevalence of Fascioliasis, as was also determined in Brazil by Bennema et al. (2017) and Dutra et al. (2010). Silva et al. (2016) noted that La Nina years affected bovine fascioliasis (temperature and rainfall).

Our predictive models developed for this study forecasted a constant trend of the disease over the next 5 years, indicating that intervention measurements must be adopted to reduce parasite prevalence, liver lesions, and economic losses, improving animal welfare. Otherwise, the negative pattern of the disease will remain above the 10% prevalence in endemic areas, or in the worse-case scenario, the disease could be more intense, further aggravating the situation. The model also predicted a possible positive scenario, which would represent an improvement in nationwide herd health conditions.

After determining the high prevalence of *F. hepatica* in the south of the country, we urge to suggest that preventive measures (i.e. diagnosis, disease monitoring) must be regularly made. The negative impact of the disease could then be reduced, while also decreasing the threat of spreading fascioliasis to humans. Furthermore, we see that our models can be used as the basis for monitoring fascioliasis in other endemic areas in South America and the Caribbean, implementing a disease surveillance program. This is an urgent requirement, as the collected data has demonstrated a potential risk to millions of people and animals.

Conclusions

Our study has determined the spacial distribution and the occurrence rates of fascioliasis in buffaloes in Brazil. The data revealed high prevalence rates in the states of PR and RS, demonstrating that the disease is widespread in two distinct geographical and climatic areas. The forecast models predicted three main conditions, ranging from a positive scenario (lower part of Figure 4/low incidence) up to a worrisome future trend (higher part of the same Figure/high incidence). A condition to understand the present data is the need to recognize the existing low sanitary condition of water buffaloes in Brazil, and the necessity to improve the herd health. The use of the ARIMA models may work as an important instrument to help establish future preventive strategies for *F. hepatica* infection in water buffaloes under similar management.

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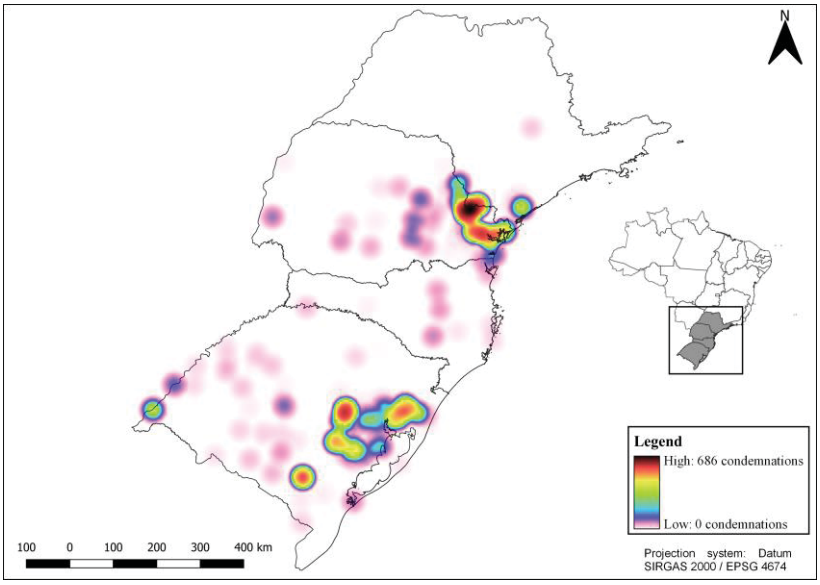

Figure 1. Map of the South of Brazil, including the state of São Paulo, and the density of condemned livers by *Fasciola hepatica* using Kernel analysis. Data provided by the Federal Inspection Service, MAPA from 2003 to 2017, according to the municipalities of Brazil. 0 = Absence of condemned livers. 686 = maximum number of condemned livers.

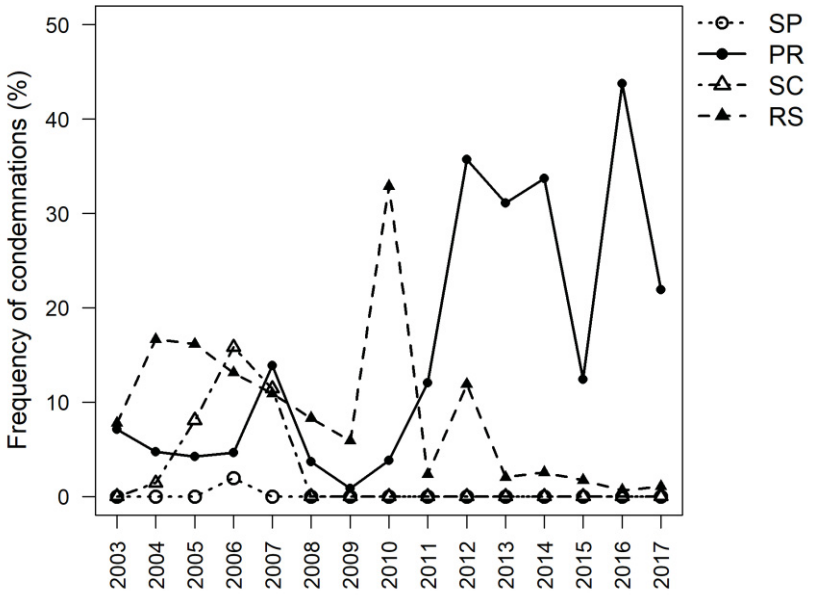

Figure. 2. Frequency of liver condemnations (7,187 thousand) by *Fasciola hepatica* of slaughtered water buffaloes from 2003 to 2017 in Brazil.

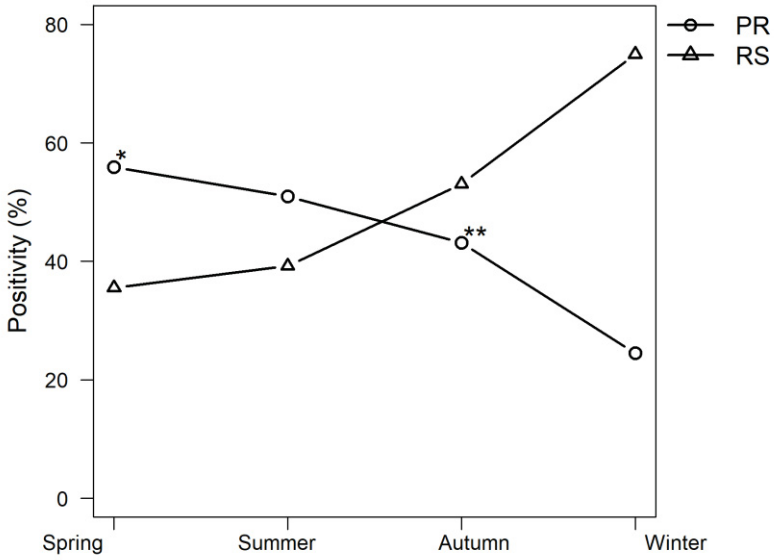

Figure. 3. Influence of rainfall and temperature on the percentage of condemned livers by *F. hepatica* in Paraná (PR) and Rio Grande do Sul (RS) states from 2003 to 2017, according to the Southern hemisphere seasons.

*Temperature ($p<0.05$); **Rainfall ($p<0.05$).

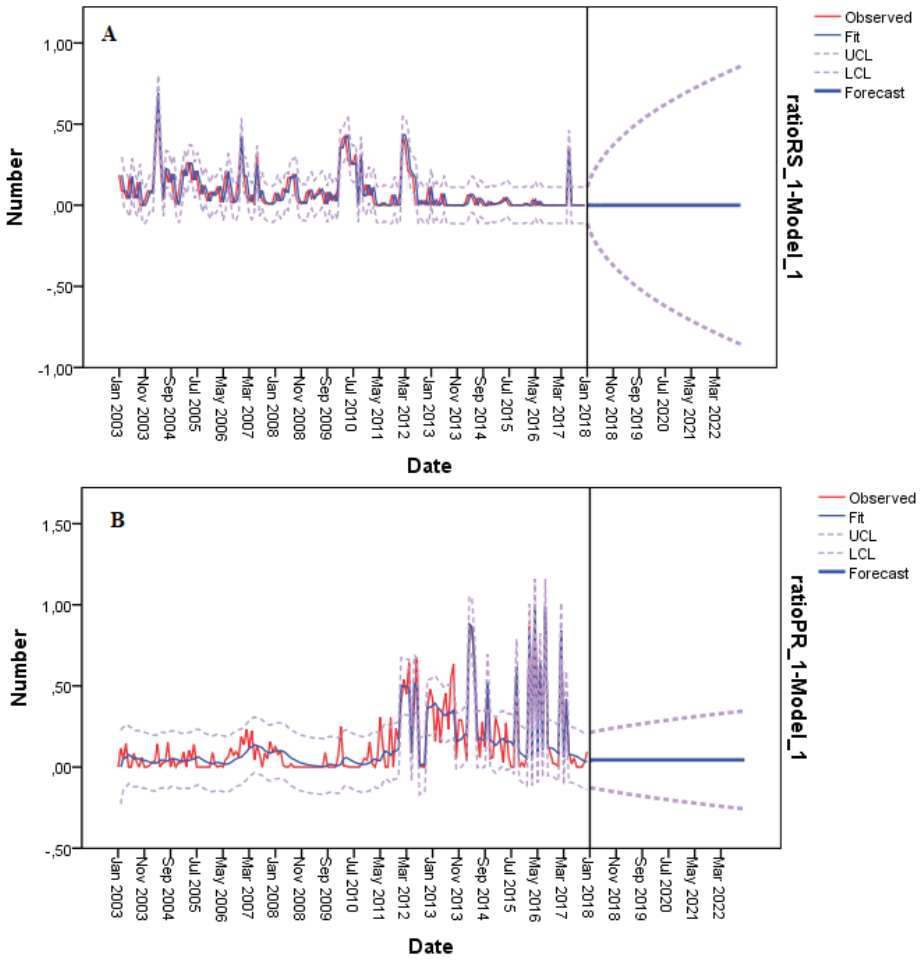

Figure. 4. Observed sequence of the condemned livers from 2003 to 2017 and forecast models until 2022 (thick blue line) for Rio Grande do Sul (A) and Paraná (B), Brazil. Fit: adjusted model; UCL: upper confidence limit; LCL: lower confidence limit.

4.5 Chapter 5

Cathepsins had proved their potential as excellent specific antigens for diagnosis techniques, such as ELISA. The prevalence studies of two previous chapters (second and third) were performed using cathepsin FhCL1 as an antigen for human and cattle fascioliasis diagnosis. Previous studies of Prof. John Dalton's group from Queen's University Belfast (QUB) revealed the importance of these proteases during the establishment of *Fasciola* infection. Also, cathepsins have shown great progress as new alternative methods for the control of fascioliasis. For this reason, in this chapter we investigated the specificity and potency of *F. hepatica* Cathepsin L3 propeptide (ppFhCL3) as a protease inhibitor to disrupt the infection in the initial stage.

This chapter was developed at QUB - Northern Ireland, period of 8 months, during my doctoral sandwich PSDE – Selective Process n° 19/2016, CAPES. The document presented to the QUB containing the whole detailed experiments is shown in the addendum 1. This chapter in a manuscript format is a result from the document presented to the QUB. The manuscript is in preparation and will be submitted to The Journal of Biological Chemistry (Maryland, United States) as a Full Research Article.

A PROPEPTIDE 'CLAMP' MECHANISM IS REQUIRED FOR INHIBITION OF *Fasciola hepatica* COLLAGENOLYTIC CATHEPSIN L3

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The clamp mechanism of cathepsin L's inhibition by propeptide

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Keywords: Cathepsin L, collagenolytic, cysteine proteases, propeptide, prosegment, inhibitor, protease inhibitor, *Fasciola hepatica*

ABSTRACT

Cysteine proteinases are important *Fasciola hepatica* virulence molecules. Among the various cathepsins that *F. hepatica* expresses, cathepsin L3 (FhCL3) secreted by the newly excysted juveniles (NEJs) is of special interest due to its central role in host invasion. Its unique collagenolytic activity facilitates the rapid passage of the NEJs through the host gut wall. To protect cells and tissues integrity cathepsins are initially produced as inactive zymogens whereby the enzyme-specific N-terminal propeptide (pp) is responsible for regulating the catalytic activity. Accordingly, it has been suggested that propeptides represent a structural template on which to develop specific cathepsin inhibitors. Here, differential immunolocalization of the FhCL3 zymogen and its pp in NEJs and immunoblotting of NEJ's excretory-secretory products indicates that most of FhCL3 zymogen is cleaving the pp and becoming active proteases in the parasite gut. After recombinantly produce FhCL3 propeptide (ppFhCL3) we assayed its inhibitory properties and mechanism of inhibition, unveiling a highly potent and selective inhibitor of *F. hepatica* cathepsin L's. Further, using 3-D structural data we made amino acids substitutions in the ppFhCL3 at residues predicted to make crucial interactions to the mature protein. Complementary inhibitory assays with the variants and wild-type pp showed that substitutions in pp residues that interacting with the propeptide bind loop (PBL) ($Tyr^{46}Lys^{47}$/$Ala^{46}Ala^{47}$) or with the active site (Leu^{66}/Gly^{66}) of the mature cathepsins culminate in pp with no inhibitory properties. Althogether, our enzyme kinetics and inhibitory studies unveiled a 'clamp' mechanism that is required for the proper binding and inhibitory activity of the pp to FhCL3. In summary, our results give remarkable insights regarding the propeptide-cathepsin interaction and open up the possibility of exploring these features in order to design new and selective inhibitors for *F. hepatica* cathepsins.

INTRODUCTION

Fasciola hepatica flukes express and excrete-secrete two different families of cathepsin like cysteine proteases, cathepsins L and cathepsins B, which evolved to generate members with specific peptidase activities (IRVING et al., 2003; TORT et al., 1999). By varying the set of cathepsins along their development flukes are able of overcoming the host defences encountered at different stages of the infection, whether physical or immunological. Hence, these proteases are recognized as major players in host–*Fasciola* interaction, enabling the parasite to cleave proteins such collagen, haemoglobin and immunoglobulins that favours migration through host tissues, feeding and immune evasion (IRVING et al., 2003; TORT et al., 1999). The secretory-excretory products of the invasive newly excysted juveniles (NEJ's) cathepsin L3 (FhCL3) and cathepsin B (FhCB) are the most abundant proteins and account for over 80% of total protease activity detectable in the extract (Robinson et al., 2009; Smith et al., 1985). In fact, FhCL3 was verified to play a central role in the initial steps of mammalian host infection, including metacercariae excystment and NEJs invasion through the intestine, which is mainly associated to the unusual collagenolytic activity of the enzyme (CARMONA et al., 1993; CORVO et al., 2009; DALTON et al., 2003; MCGONIGLE et al., 2008; MEEMON et al., 2010; MOLINA-HERNÁNDEZ et al., 2015; TKALCEVIC; ASHMAN; MEEUSEN, 1995). Indeed, since collagenolytic activity was observed to only few enzymes, no name matrix metalloproteinases, human cathepsin K and *F. hepatica* cathepsin L2, it appears that the maintenance of such activity in fluke is critical to the larvae break through the host connective tissue matrix during the migratory stages (ROBINSON et al., 2011).

Since cathepsins are proteases with a wide activity, they are initially produced as inactive zymogens, or procathepsins, that become active when encounter specific conditions. Structurally, a procathepsin differs from its mature form by the presence of a N-terminal segment, the propeptide (pp), which main role is inhibiting the proteolytic activity of the enzyme. Although little is known regarding to the structure of pp of the *F. hepatica* cathepsins, studies using human cathepsin L (HsCL) pp providing important details. The HsCL pp, for example, consists of 96 residues, the first ~79 forming the N-terminal part (P-domain), a globular domain with three α-helices that anchors in the corresponding propeptide binding loop (PBL) of the mature enzyme and forces the adjacent extended C-terminal of the pp into the active site cleft, blocking its access (CANCELA et al., 2010; COULOMBE et al., 1996). A cathepsin protease, including the fluke ones, only become active when cleaves off its own pp via an intramolecular mechanism under acid conditions. In *F. hepatica* this condition is found

in the gut, which was demonstrated to be the source of cathepsins secreted into the host tissues (Ogino et al., 1999; Sivaraman et al., 2000).

Liver fluke disease is one of the utmost economically important helminth diseases in the world, affecting more than 700 million livestock animals worldwide. The number of human cases of fascioliasis is escalating in the recent years and the disease is currently recognized as an emerging zoonotic disease by the World Health Organization (CWIKLINSKI et al., 2016; MAS-COMA et al., 2005). On the top of that, cases of *F. hepatica* resistant to triclabendazole, the drug of choice to treat the *Fasciola* infections, have been reported in several countries in Europe (KELLEY et al., 2016). Consequently, especially considering the lack of alternative treatments that effectively kill NEJs, which could prevent damages caused during invasion and migration of the fluke through tissues, there is an urgent need of identifying new drugs or vaccine to control liver flukes.

In this context, targeting molecules associated to juvenile stages, such as FhCL3, seems to be a well-reasoned strategy (DALTON et al., 1996; PIACENZA et al., 1998; PIEDRAFITA et al., 2010). It has been suggested that propetides represent a structural template on which to develop specific cathepsin inhibitors, mainly due to their overall diversity that could allow selective protease inhibition even of those cathepsins considered to have high homology (COULOMBE et al., 1996; ROCHE; TORT; DALTON, 1999; TURK et al., 2012), probably due differential interactions between pp and mature enzymes from different clades and organisms, which lacks information since most of the studies focus in human cathepsins. Thus, to use the ppFhCL3 to target specifically FhCL3 activity could result in disruption of the migration of NEJs, hindering their ability to establish an infection. In the present study, we investigate the mechanism involved in FhCL3 inhibition by its own pp. We demonstrate that the ppFhCL3 is a highly potent and selective inhibitor of the two *F. hepatica* collagenolytic cathepsins, FhCL3 and FhCL2. Based on 3-DE analysis of the FhCL3 structure we generated variants of the ppFhCL3 to decipher the importance of specific residues to the inhibitory function of the pp. Given the dramatic changes that specific amino acid substitutions, $Tyr^{46}Lys^{47}/Ala^{46}Ala^{47}$ and Leu^{66}/Gly^{66}, had in the inhibitory activity of the ppFhCL3 we propose that "clamp" mechanism involving residues of the pp that interacting with the BPL (Tyr46p, Lys47p) and substrate bind cleft (Leu66p) is necessary for the correct bind and inhibition of *F. hepatica* collagenolytic cathepsin. Together, these results have opened up the possibility of designing new selective inhibitors for fluke cathepsins, a novel approach towards the treatment of fascioliasis.

RESULTS

1. Functional expression of *F. hepatica* ppFhCL3

In order to characterize the inhibitory mechanisms of the ppFhCL3 and biochemical properties, we have cloned and functionally expressed the propeptide from *F. hepatica*, as the sequence given (Fig 6), in *E. coli* cells. A 13-kDa protein band corresponding to the propeptide was purified from the cell cultures induced with IPTG (Fig 1).

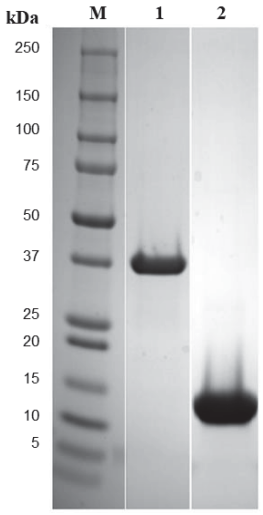


Figure 1. SDS-PAGE gel of the recombinant FhCL3 (lane 1) and ppFhCL3 (lane 2) after purification. M, Molecular weight markers.

2. Immunolocalization of ppFhCL3 and FhCL3 in NEJs indicates a fast production, activation and secretion-excretion of FhCL3 in host tissues

The local of FhCL3 and ppFhCL3 expression within the NEJs was determined by confocal microscopy carried out in 3 and 24 h post-excystment NEJs. The NEJs were probed with polyclonal anti-FhCL3 or anti-ppFhCL3 antibodies and counter-stained with TRITC-labelled phalloidin. Higher fluorescence intensity was observed in NEJs 3 h post-excystment using anti-ppFhCL3 and anti-FhCL3 (Fig 2, panels 2A and 2B) when compared in NEJs 24 h post-excystment (Fig 2, panels 3A and 3B). An enhanced presence of pp in the gut in the first hours post-excystment is consistent with the fact that most of the enzymes are activated within the parasite gut and released in a processed and active mature form in to host's tissues to assist in the invasion process. Low and diffuse background fluorescence was observed in negative control NEJs probed with pre-immune sera, and was considered non-relevant (Fig 2, panels 1A and 1B). Complementary, the western blots indicate the high specificity of anti-FhCL3 antibody since ppFhCL3 was weakly recognized by the antibody (Fig 3A). Moreover, the

recognition of a band at ~37 kDa in the ES and somatic extracts from NEJs is in line with our immunolocalization results (Fig 2), and the presence of several bands lower than 37 kDa in the ES from NEJs 24h post-excystment (Fig 3A and B) indicates that FhCL3 procathepsin present in ES is being partially processed.

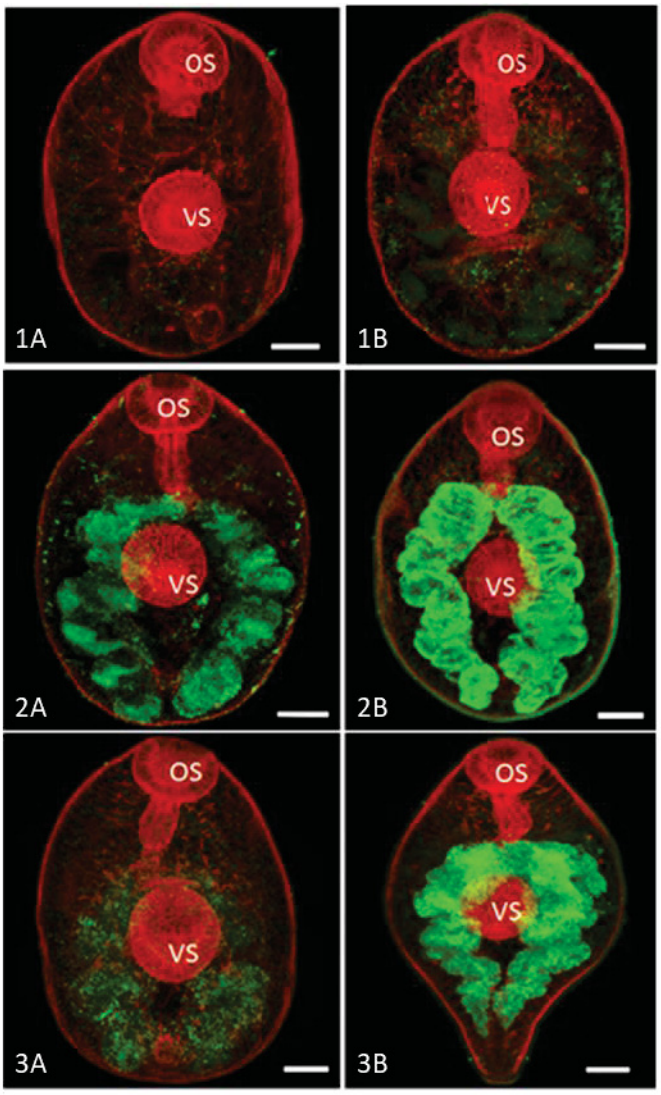


Figure 2. **Immunolocalization of FhCL3 and ppFhCL3 in newly excysted juveniles (NEJs).** Three hours (panels A) and 24 h (panels B) NEJs were probed with rabbit pre-immune antiserum (panels 1A, 1B), and polyclonal antibodies prepared to ppFhCL3 (panels 2A, 3A) and FhCL3 zymogen (panels 2B, 3B). Tissue location of proteins was visualised using secondary antibody fluorescein isothiocvanate (FITC)-labelled goat anti-rabbit IgG under confocal microscopy. To provide background structure, NEJs were counter-stained with phalloidin-tetramethylrhodamine isothiocyanate (TRITC) to stain muscle tissue (red fluorescence) and. *OS*, oral sucker. *VS*, ventral sucker. *Scale bars*, 20 μM.

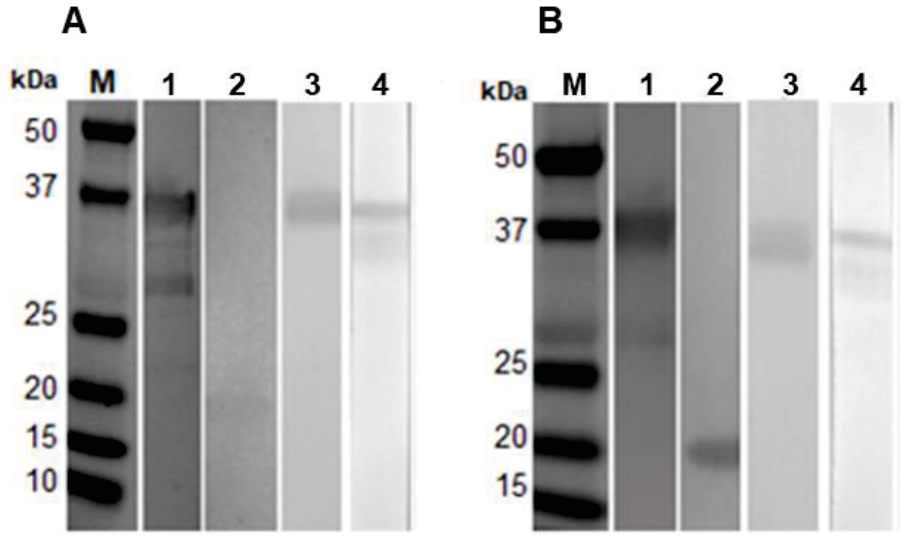


Figure 3. Immunodetection of ppFhCL3 and the FhCL3 zymogen in somatic extracts and secretions of NEJS. Rabbit polyclonal antibodies prepared to ppFhCL3 (panel A) and to the FhCL3 zymogen (panel B) were used to probe samples of recombinant FhCL3 zymogen (lane 1), recombinant ppFhCL3 (lane 2), somatic extract of NEJ 24h post-excystment (lane 3) and ES products of NEJ 24h post-excystment (lane 4). M, Molecular weight markers.

3. ppFhCL3 binds and inhibits the native cathepsin proteases present in ES products of *F. hepatica* adult worms

The ppFhCL3 ability of inhibiting native cathepsins was tested using ES extract from adult *F. hepatica* worms. Once the inhibitory effect of the ppFhCL3 was verified through the enzymatic assay (Fig 4A), a pull-down experiment was performed in order to isolate and identify those enzymes to which the ppFhCL3 binds and inhibits in the ES. The resulting reactions were resolved by Coomassie Blue- stained SDS-PAGE (Fig 4B). The LC-MS/MS analysis of the protein band pulled down by the ppFhCL3 revealed that ppFhCL3 is acting towards three different native *F. hepatica* cathepsin L's, FhCL1, FhCL2 and FhCL5 (Fig 4C), which is in line with the results we observed with enzymatic assays using recombinant FhCL2 (Fig 5).

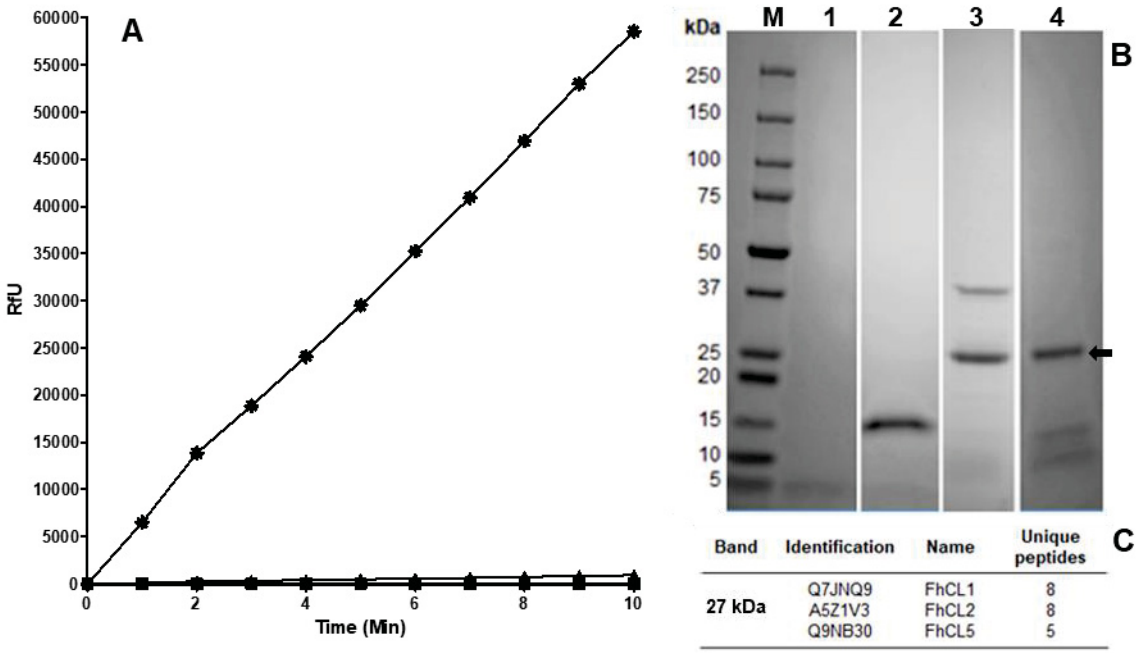


Figure 4. The ppFhCL3 binds and inhibits native *F. hepatica* cysteine proteases in the excretory-secretory (ES) products of adult worms. (A) Cysteine proteases activity (presented as relative fluorescent units, RfU) in adult *F. hepatica* ES was measured with the fluorogenic peptide substrate Z-Leu-Arg-NHMec in the absence of the inhibitors (asterisks), in the presence of recombinant propeptide ppFhCL3 (triangles) or in the presence of the cysteine protease inhibitor E64 (squares). (B) The ppFhCL3 was used in pull-down experiments to identify binding partners in adult *F. hepatica* ES and the results were analysed by SDS-PAGE as follows, Ni-NTA beads (lane 1), Ni-NTA beads and recombinant ppFhCL3 (lane 2), *F. hepatica* adult ES (lane 3), Ni-NTA beads, ppFhCL3 and *F. hepatica* adult ES (lane 4). (C) LC-MS/MS identification of proteins present in the ~27 kDa band (arrow).

4. The ppFhCL3 present high inhibitory activity towards *F. hepatica* collagenolytic enzymes, with optimum activity at pH 7.0

The activity of the recombinant ppFhCL3 as inhibitor was first tested with its cognate enzyme, revealing that the ppFhCL3 is a highly potent inhibitor of FhCL3. Following, the importance of the pH for the inhibitory activity was stablished by testing the hydrolytic activity of FhCL1, FhCL2, FhCL3, HsCL and HsCK in the presence or absence of the ppFhCL3 over a range of pH (4.5 to 7.0) (Fig 5). All enzymes showed a wide pH activity profile consistent with previous reports where cathepsins were shown to be active in various pH (Dalton et al., 2003; Corvo et al., 2009). Although 75% to 90% of the activity of the *F. hepatica* enzymes were inhibited at pH 5.5 and 6.5, the maximum inhibitory activity was obtained at pH 7.0. In contrast, the best inhibition towards HsCL and HsCK was obtained at pH 6.5 and 5.5, respectively.

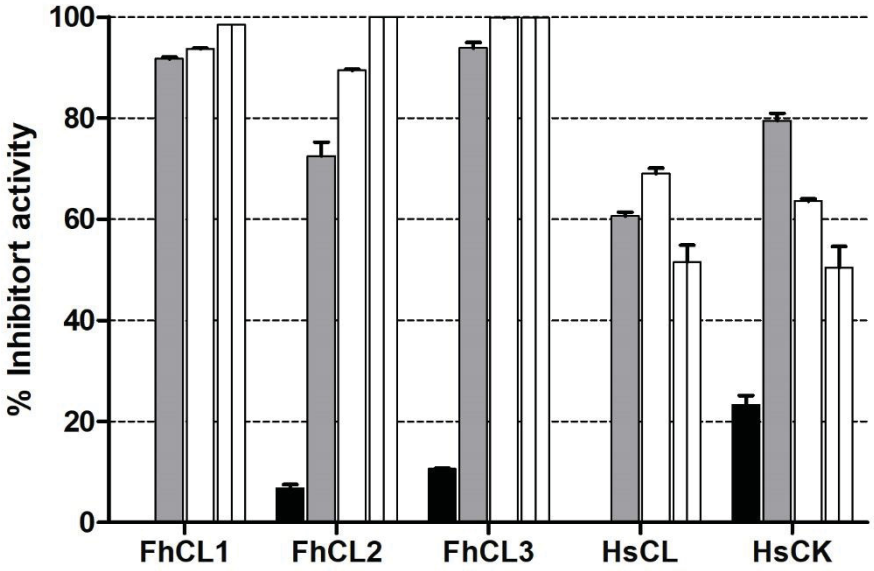


Figure 5. Inhibitory activity of ppFhCL3 against *F. hepatica* and humans cysteine proteases at different pH's. Inhibitory activity of ppFhCL3,10 nM, towards FhCL1, FhCL2, FhCL3, HsCL and HsCK at pH 4.5 (black bars), pH 5.5 (grey bars), pH 6.5 (white bars), pH 7.0 (striped bars). The activity is presented relative to the total activity of each enzyme in the absence of the ppFhCL3. Error bars indicate standard deviation of three separate experiments.

5. Structural studies of the ppFhCL3 interaction with *F. hepatica* and human cathepsins unveiled key residues involved in selectivity and inhibition of the propeptide

Using the previous resolved three-dimensional structure of FhCL1 enzyme as a model, the FhCL3 structure could be predicted (Fig 6). We generated a molecular model of the enzyme and ppFhCL3 and were able to exploit the interaction, and even compare the ppFhCL3 interaction with other mature cathepsins, including HsCL and HsCK. The *in silico* analysis provided insights into the interactions that could be essential for the correct binding of the pp to the enzyme, suggesting that pp residues p46, p47, p66 and p68 were of fundamental importance. The predicted interactions between p46, p47, p66 and p68 and amino acids of the mature enzymes is presented in the Table 2. Complementary, the alignment of the FhCL2, FhCL3, HsCL and HsCK is presented in the supplementary material (Sup Fig 1). The different predicted amino acid interactions between Y46 and K47 and mature FhCL3, HsCL or HsCK could explain the selectivity

observed of the ppFhCL3 towards *F. hepatica* enzymes, which was further experimentally determined.

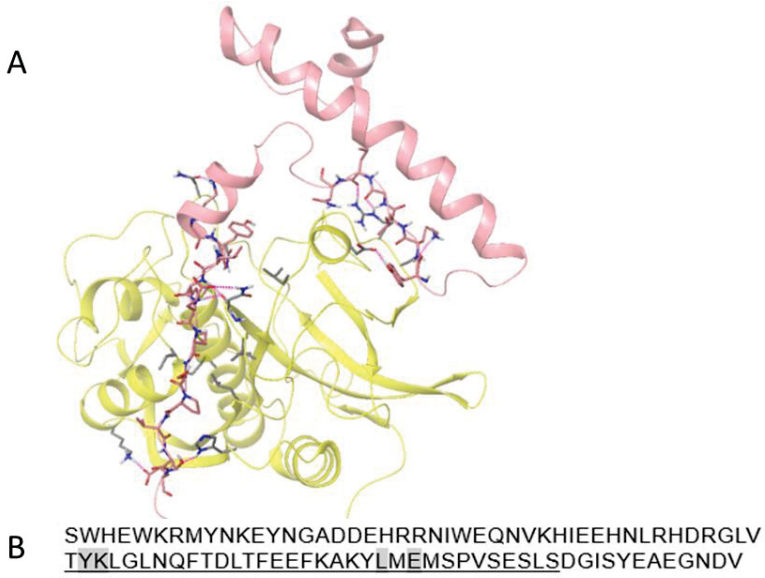


Figure 6. Structural representation of FhCL3 and its propeptide. (A) The analysis salients interactions of the ppFhCL3 within the active site and propeptide binding loop (PBL) of the mature protein. Dotted lines represent hydrogen bonds. (B) Primary sequence of the ppFhCL3. Residues highlighted were predicted to be involved in fundamental interactions with the mature domain. The sequence used to produce the synthetic peptide (33 residues) is underlined.

Table 2. The ppFhCL3 residues and the predicted amino acids residues they interact to in the mature domain of *F. hepatica* and human cysteine proteases.

ppFhCL3	**Mature domain**		
	FhCL3	HsCL	HsCK
Y46	D228	S255	S255
K47	S234 E233	E261 K260	K261 S260
L66	A225	G252	S252
E68	T248	D275	N275

6. The ppFhCL3 and variant ppFhCL3E^{68}/N^{68} bind and inhibit native and recombinant *F. hepatica* cathepsin proteases

The ability of the variant propeptides produced by amino acid substitutions inhibiting native cathepsins secreted by the invasive life stage of *F. hepatica* was assayed by testing the proteolytic activity of ES from NEJs 24 hr post-excystment in the presence and absence of the different propeptides. Our results shown that cathepsin activity in the ES extract is strongly inhibited by wild-type ppFhCL3 and ppFhCL3E^{68}/N^{68}, while the other two propeptides produced, ppFhCL3Y^{46}K^{47}/A^{46,47}, ppFhCL3L^{66}/G^{66}, were not able to inhibit cathepsins (Fig 7).

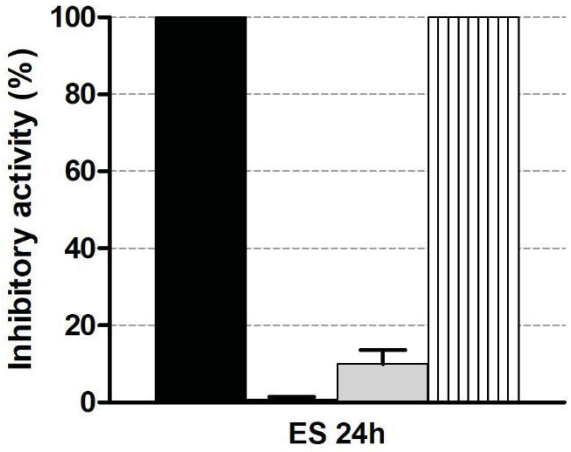


Figure 7. Inhibitory activity of the wild-type ppFhCL3, and variants ppFhCL3Y^{46}K^{47}/A^{46,47}, ppFhCL3L^{66}/G^{66}, ppFhCL3E^{68}/N^{68} against cysteine proteases in secreted by NEJ 24h post-excystment. The inhibitory activity of ppFhCL3 (black bars), ppFhCL3Y^{46}K^{47}/A^{46,47}(white bars), ppFhCL3L^{66}/G^{66} (grey bars), ppFhCL3E^{68}/R^{68} (striped bars) against ES product from NEJs 24h post-excystment. Inhibitory activity is presented relative to the total activity of each enzyme in the absence of the propeptides. Error bars indicate standard deviation of three separate experiments.

Having in consideration the high homology of the propeptides from *F. hepatica* cathepsins and the need of known the selectivity of ppFhCL3 as an inhibitor, its activity and the activity of all variants were also screened using a panel of serine and cysteine proteases (Fig 8 and Sup Fig 2). All propeptides were initially screened at 10 and 500 nM, resulting in a defined inhibitory profile (Fig 8A and B) that highlights the ability of ppFhCL3 specific and selectively inhibits *F. hepatica* cathepsin L's activity, since non-significant inhibition was verified towards *Fasciola* cathepsin B's or *S. mansoni* cathepsins (Sup Fig 2).

The inhibitory activity of the ppFhCL3 variants made by amino acids substitutions involving residues that interacting within the propeptide bind loop (PBL) of the mature enzyme, ppFhCL3$Y^{46}K^{47}/A^{46,47}$, or with the active site of the enzyme, ppFhCL3L^{66}/G^{66} and ppFhCL3E^{68}/K^{68} resulted in different profiles when compared to the wild type. The ppFhCL3$Y^{46}K^{47}/A^{46,47}$ and ppFhCL3L^{66}/G^{66} activity towards FhCL2 was completed abolished at 10 nM, while at 500 nM the ppFhCL3L^{66}/G^{66} showed only a poor activity. Similar results were observed with these same two variants towards FhCL3, which catalytic activity was only weakly inhibited by them at 500 nM (Fig 8B). In contrast, the substitution of the residue p68, E^{68}/K^{68}, interfere only slightly with the inhibitory activity of the pp, as demonstrated by the almost totally preserved inhibitory activity when used at 500 nM (Fig 8A, B).

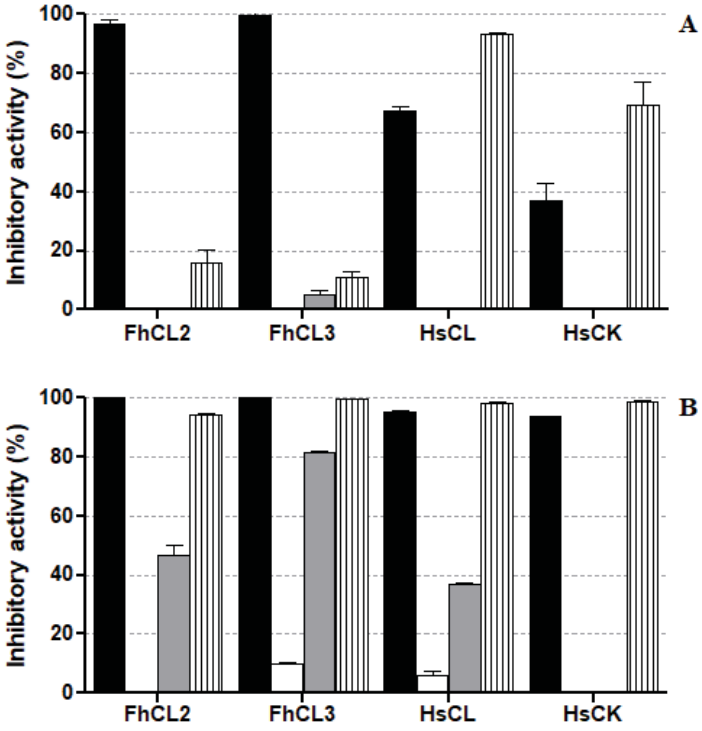


Figure 8. Inhibition profile of ppFhCL3 and variants against cysteine proteases. The inhibitory activity of ppFhCL3 (black bars), ppFhCL3_46,47 (white bars), ppFhCL3_66 (grey bars) and ppFhCL3-68 (striped bars), at 10 nM (A) and 500 nM (B) was screened using a panel of cysteine proteases, including *F. hepatica* cathepsin L2 (FhCL2), *F. hepatica* cathepsin L3 (FhCL3), Human cathepsin L (HsCL) and Human cathepsin K (HsCK). Inhibitory activity is presented relative to the total activity of each enzyme in the absence of inhibitors. Error bars indicate standard deviation of three separate experiments.

7. *Ki* values highlighting ppFhCL3 strong inhibitory activity against *F. hepatica* collagenolytic cathepsins, FhCL2 and FhCL3, a characteristic preserved in the variant ppFhCL3E^{68}/N^{68}

Table 3 presents the *Ki* values calculated for all relevant *F. hepatica* and human cathepsins (Examples of *Ki* curves are presented in Sup Fig 3). The low values obtained by the FhCL2 and FhCL3 in the presence of ppFhCL3, that were at least 500-fold lower than those values observed to human cathepsins, reaffirm the selectivity of the ppFhCL3 and also demonstrate its potency. The *Ki* obtained when the ppFhCL3 variants where assayed clearly reinforce the data verified in the screening tests, showing that the substitutions Y^{46}K^{47}/A^{46,47} and L^{66}/G^{66} have a strong effect in the inhibitory capacity of the ppFhCL3 towards all proteases tested. On the other hand, to replace the glutamic acid for arginine in the variant ppFhCL3E^{68}/R^{68} did not affect significantly its activity towards *F. hepatica* enzymes, although a slight improvement of the inhibition towards human cathepsins could be verified.

Table 3. *Ki* for inhibition of cysteine proteases by the ppFhCL3, ppFhCL3Y^{46}K^{47}/A^{46,47}, ppFhCL3L^{66}/G^{66}, ppFhCL3E^{68}/N^{68}

Enzyme concentration	Inhibition *Ki* (nM)			
	ppFhCL3	ppFhCL3 Y^{46}K^{47}/A^{46,47}	ppFhCL3 L^{66}/G^{66}	ppFhCL3 E^{68}/R^{68}
FhCL2	0.004 (±0.002)	>10 μM*	>10 μM	0.02 (±0.01)
FhCL3	<0.002*	>10 μM	8.00 (±2.3)	0.002 (±0.001)
HsCL	26.6 (±0.4)	>10 μM	>10 μM	6.04 (±0.44)
HsCK	2.0 (±0.5)	>10 μM	>10 μM	1.40 (±0.2)

* Values >10 μM and <0.002 could not be calculated accurately.

8. The 33 amino acids synthetic peptide did not preserve the inhibitory activity of the ppFhCL3

The chemically synthesised peptide (according the sequence highlighted in the Fig 9) included regions of the ppFhCL3 predicted to interact and stabilize the bind with the mature enzyme. The activity of the peptide was screened against relevant cysteine proteases, at 500 and 10 nM, and revealed a weak inhibitory activity (≤50% at 500 nM) when compared with the observed ppFhCL3 activity (Fig 9).

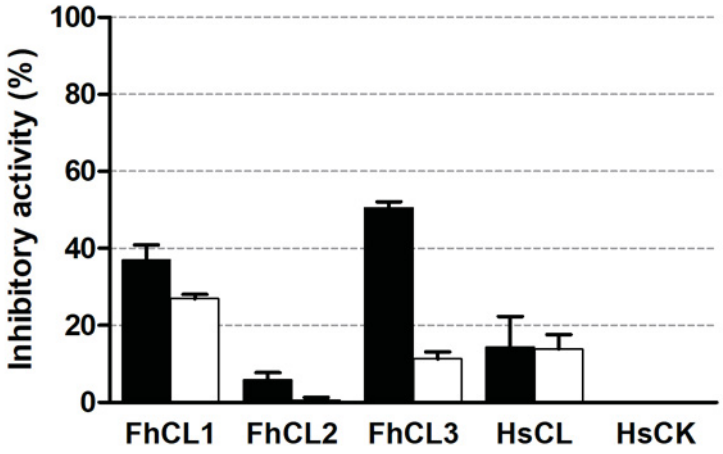


Figure 9. Inhibition profile of a 33-mer synthetic peptide derived from ppFhCL3 against *F. hepatica* and human cysteine proteases. The inhibitory activity of the 33-mer synthetic peptide was tested at 500 nM (black bars) and 10 nM (white bars) against the cysteine proteases FhCL1, FhCL2, FhCL3, HsCL and HsCK. Inhibitory activity is presented relative to the total activity of each enzyme in the absence of inhibitors. Error bars indicate standard deviation of three separate experiments.

DISCUSSION

Evidences of triclabendazole-resistant *F. hepatica* in livestock are associated with the growing consume of the drug and have been driven the search for alternative measures of control liver fluke disease (KELLEY et al., 2016). *F. hepatica* cathepsins (FhCs) are recognised as virulence factors and are associated with key processes that guarantee the success of the infection, including immune evasion and digestion of fibronectin, collagen and albumin, which place them as potential targets for the development of drugs and vaccines (DALTON et al., 1996; JAYARAJ et al., 2009; PIACENZA et al., 1998; WESOLOWSKA et al., 2018; ZAWISTOWSKA-DENIZIAK et al., 2013). By integrating transcriptomic and proteomic approaches, the analysis of molecules associated to NEJs

revealed the expression and secretion of two main cathepsin proteases, FhCL3 and FhCB (ROBINSON et al., 2009; ZAWISTOWSKA-DENIZIAK et al., 2013). Complementary studies using RNAi demonstrated that the FhCL3 present in the SE extract from NEJs is fundamental for excystment, penetration and migration of the larvae through the host intestinal wall (MCGONIGLE et al., 2008). Besides, FhCL3 cleaves collagen assuring parasite migration throughout the extracellular matrix (ECM) and connective tissue matrix of the host tissues (CORVO et al., 2009; ROBINSON et al., 2011). Since collagenolytic activity was observed for very few papain-like enzymes, including the FhCL2 secreted by *F. hepatica* adult worms, it surely is of extreme relevance for the parasite life-cycle and to inhibit its activity might block invasion and consequent tissue damage. Therefore, in this study we investigated the potential of the ppFhCL3 as inhibitor of FhCL3 and other cathepsins, its possible mechanism of inhibition, and the dynamic of FhCL3 activation.

The production and secretion of FhCs primarily involve the synthesis of a zymogen in specialised gastrodermal cells which lines the parasites gut, then their packaged in secretory vesicles and excretion into the parasite gut lumen, followed by release of them into host tissues (COLLINS et al., 2004; CORVO et al., 2009). In line with this and with the evidences that proFhCL3 progressively changes to mature enzyme during the first 48h of NEJs development (CANCELA et al., 2008; CORVO et al., 2009), our differential immunolocalization of the ppFhCL3 and proFhCL3 in NEJs 3 and 24h post-excystment (Fig 2) shows a stronger fluorescent signal in the gut of NEJs 3 hrs when compared to NEJs 24 hrs post-excystment. The temporal difference observed in our results strongly suggests that FhCL3 is being rapidly produced and secreted-excreted from the parasite gut, which is in accordance with the necessity that early stage larvae have of penetrating through the intestinal mucosa in the first 72 hrs (KENDALL, S; PARFIT, 1962). The cathepsin activity observed in the ES from NEJs 24h post-excystment also supports a mechanism of progressive FhCL3 activation. Complementary, the western blot analysis matched with previous data reported by Robinson et al. (2009), reaffirming the abundant presence of proFhCL3 in ES from NEJs. It is worth to note that even though in our western blots a band referent to the pp is absent in ES sample, the cathepsin activity verified in the 24h extract, inhibited when ppFhCL3 was added to the reaction, reinforce the presence of mature FhCL3 in the extract and that possibly our antibodies were not sensitive enough to detect it.

Our results indicate that both zymogen and mature FhCL3 are being excreted into host environment. The role of the prosegment in the infection or host-parasite interaction is yet to be better understood, but certainly it appears to produce a relevant immunological response, since rats immunized by natural infection with *F. hepatica* had a higher immunoreactivity to the proFhCL3 than to the mature enzyme enzyme (MILLIGEN; CORNELISSEN; BOKHOUT, 2000). Similar results were also found in other studies (CARMONA et al., 1996; HARMSEN et al., 2004). Indeed, the activity we verified in the NEJ's extract (Fig 4) needed no previous activation of the extracts meaning that at least part of FhCL3 is being pre-activated in the parasite gut, which is consistent with the previous described mechanism of autocatalytic activation of cathepsins. In addition, it has been shown that certain molecules present in the host tissues, such heparin and several other glycosoaminoglycans (GAGs) that compose the ECM, can increase the processing of zymogens to mature enzyme disrupting the interactions between the pp and mature cathepsin L and B (FAIRHEAD et al., 2012; KIHARA et al., 2002), a hypothesis yet to be tested for FhCL3 activation.

Although recently pp from human cathepsins have received a lot of attention, specially concerning to the development of specific inhibitors to HsCK, which is associated with reabsorption during osteoporosis disease (TABATABAEI-MALAZY et al., 2017), there are few studies exploring the importance of pp from parasite enzymes, including *F. hepatica*, regardless the central role of some of them, such FhCL3, in the infection. Certainly, there is a potential for application of cathepsins prosegments as candidates for novel immunoprophylactic and chemotherapeutic strategies, which is firstly supported by data showing the specificity and potency of the ppFhCL1 as inhibitor of FhCL1 enzyme, but not for FhCL2, two enzymes that are 90% similar in their amino acid sequences (ROCHE et al., 1999).

Enzymatic assays revealed that the ppFhCL3 we produced is a potent and selective inhibitor of both native and recombinant *F. hepatica* collagenolytic cathepsins, which was demonstrated by screening the inhibitory activity against cathepsins compound SE extracts from *F. hepatica* adult worms NEJs (Fig 4 and 7) and using a panel of recombinant serine and cysteine proteases derived from various organisms, including *F. hepatica*, *S. mansoni* and humans (Fig 5 and Sup Fig 2). Further, the ability of the ppFhCL3 to bind to the native enzymes, including FhCL2, was confirmed by pulling down proteins present in ES from adult worms followed by mass spectrometry analysis.

Unfortunately, due the availability of material the same experiment could not be performed with the ES from NEJs, but by putting together previous studies that elucidated the cathepsin composition of these extracts (ROBINSON et al., 2009) and our enzymatic assays in ES from NEJ 24h post-excystment we can establish that the ppFhCL3 is able to inhibit the cathepsin activity in ES from NEJs, mainly related to the presence of FhCL3. We also demonstrated that at pH 5.5-7.0 the ppFhCL3 strongly inhibits *F. hepatica* cathepsins, presenting higher potency at pH 7.0 (Fig 5), which is consistent with prior reports of parasite cathepsins being active at various pH and also with the described mechanism of activation of these enzymes involving acidic conditions (CORVO et al., 2009; DALTON et al., 2003). The maximum inhibitory activity of the ppFhCL3 at a neutral pH also corresponds to the environment of the host duodenum were NEJs are secreting-excreting the FhCL3, suggesting its possible application to prevent natural infections.

The low selectivity that ppFhCL3 demonstrates towards *F. hepatica* cathepsins from different clades, FhCL2 and FhCL3, *Ki* values of 0.004 nM (FhCL2) and <0.002 nM (FhCL3) (Table 3), could be explained by the high amino acid identity these proteases share, over 79% (Sup Fig 1). These enzymes also share a very similar active site that confers their collagenolytic activity and were observed to lay very close in a phylogenetic analysis (CORVO et al., 2009; ROBINSON et al., 2011). Guay et al. (2000), found similar results when human cathepsin L subfamily of papain-like cysteine proteases (cathepsins S and K) were screened against the pp from either enzyme. It is worth mentioning that ppFhCL3 shows minor inhibitory effects towards human cathepsins, HsCL and HsCK, to which the *Ki* obtained were at least 500-fold higher when compared to the values obtained with *F. hepatica* cathepsins.

The availability of the three-dimensional structure of FhCL1 (STACK et al., 2008) allows targeting the activity of FhCL3 as a strategy to control fascioliasis. The structure-based drug design of inhibitors against FhCs with unknown structures have provided structural insights into the interaction and affinity of FhCL3 with various peptide substrates ((ROBINSON; DALTON; DONNELLY, 2008b), providing a broader understanding of the role and biology of this enzyme, leading to the development and test of specific inhibitors. A computational study combined virtual screening, molecular dynamics simulations, and binding free energy (ΔG_{bind}) calculations to design compounds to predict selective inhibitors to FhCL3 (HERNÁNDEZ ALVAREZ et al., 2015).

Although an interesting approach, the compounds were never tested. Also using computational approach, Ferraro et al. (2016) *in vitro* screened for non-peptide inhibitors for the same enzyme and identified a chalcone (C34) as an active inhibitor that was further verified to reduce NEJ's ability to penetrate the host gut wall. Interesting, to date the ppFhCL3 was never exploited in this sense and little is known about the mechanism of its inhibition and interaction towards its cognate enzyme and other cathepsins.

The observed selectivity of the ppFhCL3 within the collagenolytic enzymes requires a detailed structural interpretation to elucidate the mechanism of interaction. In order to advance in the knowledge of the mechanisms of interaction and inhibition ppFhCL3, we built the 3-DE model of the FhCL3 based on the FhCL1 crystal structure and generated models of interaction between ppFhCL3 with FhCL3, HsCL and HsCK. The analysis provided the framework essential to identify areas and even residues of the prosegment that might contribute to the interaction (Table 2), which was further confirmed by generating different ppFhCL3 with specific amino acid substitutions and also by synthesising a small peptide based on ppFhCL3 sequence and areas predicted to contribute for interaction and inhibition of mature enzymes. By testing the inhibitory activity of the pp variants, we confirmed that three residues we substituted are extremely important to the inhibitory activity towards both native and recombinant *F. hepatica* cathepsins, when compared to the ppFhCL3 wide type (Table 3 and Fig 7 and 8). The double substitution made in ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47} and the change of the residue Leu66p in ppFhCL3Leu^{66}/Gly^{66} abrogated the inhibitory activity of the pp towards FhCL2 and FhCL3 enzymes.

Our results shown that binding and inhibition of the ppFhCL3 to mature FhCL2 and FhCL3 occur fast and the inhibition of both enzymes is total (Fig 5). The models of interaction of pp-cathepsins provide a reasonable explanation for the inhibition observed and also for the absence of this activity when the residues Tyr46p and Lys47p are simultaneously substituted by residues of alanine. Our analyses indicating the importance of hydrogen bonds formed between tyrosine and lysine and the polar residues of aspartic acid and serine, respectively, present in the BPL, leading to an interference within the unique way in which the ppFhCL3 sits in the enzyme PBL forcing its extended C-terminal part into the substrate binding cleft of the mature enzyme. The effect of the modification Tyr^{46}Lys^{47}/Ala^{46}Ala^{47} demonstrates for the first time the importance of specific residues in the N-terminal portion of the pp.

A similar effect was observed when Leu66p was replaced by a glycine. The leucine residue is supposed to interact to another hydrophobic residue, an alanine, present in the active site of the mature FhCL2 and FhCL3. In all, the lack of inhibitory activity of ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47} and ppFhCL3Leu^{66}/Gly^{66} indicates an even importance of interaction within PBL and substrate cleft in order obtain a correct bind and stabilization of the ppFhC3 to both *F. hepatica* collagenolytic cathepsins, which indicates a conserved mechanism to control the activity of these enzymes. Moreover, despite the apparent importance of the foretold charge interaction between Glu68p and amino acids of the active site of the enzymes, the substitution made in ppFhCL3Glu^{68}/Arg^{68} did not result in significant different inhibitory profile when compared to the ppFhCL3 wild type.

Studies with human cathepsins have shown that the BPL segment of the mature enzyme is not only involved in the binding of the pp, but the negatively charged side chains od residues present in PBL of cathepsin L permit interaction with residues containing positively charged side chains found in the major histocompatibility complex (MHC) class II-associated invariant chain, a known inhibitor of cathepsins L, V, K, and F (Mihelic et al., 2008). The effect we observed by changing residues within both pp portions was similar to those results obtained by Coulombe et al. (1996) and Guo et al. (2000) regarding the importance of pp C- and N-terminal. The C-terminal was demonstrated to exert greater influence on the selectivity of the inhibition, whilst the N-terminus contributes to stabilise the backbone of the prosegment during the binding, essential for its inhibitory activity. Groves and colleagues (1998), also provided data showing that the C-terminal apparently does not contribute substantially to the binding and inhibition of the cognate enzyme but provides compensating charges to those residues contacting the enzyme surface and therefore could contribute to the specificity. Even though none of the variants produced displayed a significant change in terms of selectivity, the residues Tyr46p, Lys47p and Leu66p are clearly fundamental for the proper pp bind and inhibition, suggesting an equal importance of the C- and N-terminal parts. Therefore, our results indicating that a ‘clamp’ mechanism is required for the correct interaction between pp and mature FhCL3 and FhCL2, and here we have identified two parts of the clamp, one that clips inside (66p) and other outside (46,47p) of the active site of the mature enzyme.

To exploit the pp structure to generate short peptides are thought to be a good strategy to produce specific inhibitors to cathepsins, once they could prevent substrate

processing by competitive inhibition. Indeed, previously this strategy was applied and few small peptides were tested as potential inhibitor (COULOMBE et al., 1996; GUAY et al., 2000). Motivated by these data and the urgent need of a selective, small and reversible inhibitor for *Fasciola* cathepsins, as well as by the strong inhibitory capacity verified for ppFhCL3, we produced and tested the activity of a synthetic small peptide based on ppFhCL3 residues from 45p to 78p, which contains all those key amino acids here confirmed to be important to pp activity (Fig 6). The inhibitory assays showed that the peptide produced did not display an equivalent inhibitory activity when compared to the wide type ppFhCL3 (Fig 9), which could be a consequence of the synthetic production, lack of correct folding and even its use as substrate by the enzyme, since it is not possible to predict how these molecules are going to interact with the mature enzyme.

CONCLUSION

In summary, our study revealed that the ppFhCL3 is an extremely potent and selective inhibitor of *F. hepatica* CL's. Additionally, we describe for the first time a clamp mechanism essential for the ppFhCL3 bind and inhibition to FhCL3 and FhCL2, two enzymes that have collagenolytic activity fundamental to *F. hepatica* invasion of host tissue. The clamp here identified involves residues of the ppFhCL3 that interact with the BPL (Tyr46p, Lys47p) and active site (Leu66p) of the mature enzyme, both essential for the inhibitory activity observed. Together our results providing remarkable new structural insights regarding to pp-cathepsin interaction and may contribute to design a useful strategy to develop novel inhibitors against other FhCL's, which might hinder host invasion and penetration processes.

EXPERIMENTAL PROCEDURES

1. Molecular modelling of ppFhCL3 and variants cysteine protease inhibition: The obtained homology model was first docked to the crystal structure of FhCL1 previously reported by us (PDB code: 2O6X) using the HEX 6.1 protein-protein docking program and subjected to molecular dynamics simulations using MacroModel 10.8. In docking, the shape and electrostatic-based assessment of binding was conducted with over 100 generated and analysed docking solutions. The points of interaction of ppFhCL3 and FhCL3 mature enzyme is pointed to the active site and surrounding of FhCL3 enzyme, was selected for molecular dynamics simulations. 10 ns of molecular dynamics

simulations and 5000 steps of followed minimization were conducted in implicit solvent at temperature 300K to obtain the final complex. OPLS_2005 force field was used in MacroModel calculations. Similar docking and simulation steps were used to study ppFhCL3 binding and interaction with (PDB code: 1YK7). The image with the molecular models was prepared with Maestro 10.2.

2. Gene and peptide production: The propeptides sequences of the *F. hepatica* cathepsin L3 (FhCL3) was designed based on the full gene sequence of the protein (ABW75768.2). The cDNA encoding the propeptide with a C-terminal His-tag was cloned into a pET-28a(+) (kanamycin resistant) by GenScript (Genscript Biotech, USA). Three variants of the ppFhCL3 gene were similarly synthesized with a C-terminal His-tag; these included the variant ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47} whereby the tyrosine residue at position 46p and a lysine at position 47p were substituted to alanine residues, ppFhCL3Leu^{66}/Gly^{66} whereby the leucine residue at position 66 was substituted to a glycine residue and ppFhCL3Glu^{68}/Arg^{68}, whereby the glutamic acid residue at position 68p was substituted to an arginine residue (Fig. 3C). Additionally, a 33 amino acids long peptide derived from ppFhCL3 was chemically synthesised, according the sequence provided and highlighted in Fig 3C (GL Biochem, Shanghai).

3. Differential immunolocalization of ppFhCL3 and FhCL3 antibody in Newly Excysted Juveniles by confocal microscopy: Polyclonal antibodies against ppFhCL3 and FhCL3 were obtained from rabbits immunized with each of the respective recombinant protein by Eurogentec, Liège, Belgium. NEJs (3 and 24h post-excystment) were fixed with 4% paraformaldehyde in 0.1 M PBS (Sigma-Aldrich) overnight at 4 °C, and then washed three times with antibody diluent (AbD: 0.1 M PBS containing 0.1 % [V/V] Triton X- 100, 0.1 % [W/V] bovine serum albumin and 0.1 % [W/V] sodium azide). NEJs were then incubated in 0.1 M PBS containing either anti-ppFhCL3 or anti-FhCL3 at 1:500 dilutions, overnight at 4 °C, followed by three washes in AbD. As a negative control, separate samples of NEJs 6 and 24 hrs post-excystment were incubated in 0.1 M PBS containing rabbit pre-immune antiserum at 1:500 dilution. All NEJs samples were then incubated with the secondary antibody, fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (Sigma-Aldrich), at 1:200 dilution, overnight at 4 °C. To counter-stain muscle tissues, NEJs were incubated in AbD containing 200 µg/ml phalloidintetramethylrhodamine isothiocyanate (TRITC) overnight at 4 °C. Following three final washes in AbD, NEJs were whole-mounted in a 9:1 glycerol solution

containing 0.1 M propyl gallate and viewed using confocal scanning laser microscopy (CSLM) (Leica TCS SP5) under the HCX PL APO CS 100x oil objective lens. Leica type F immersion oil was used in viewing and all images taken at RT.

For western blots analysis, FhCL3, ppFhCL3, ES from NEJs 3 hrs post-excystment and somatic antigens from NEJs 3 and 24 h post-excystiment (5 µg each) were resolved by 4-20% SDS-PAGE and then electrotransferred into nitrocellulose membranes. Membranes were blocked (5% milk in 1x PBS-Tween 0.05%) and then probed with either anti-ppFhCL3 or anti-FhCL3 as primary antibodies (diluted 1:7500 in 5% blocking solution), and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) (diluted 1:5000 in PBST 0.05%) as secondary antibody. After washes the western blots were developed by the addition of the chromogenic substrate SIGMA FAST™ BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Sigma-Aldrich) and imaged using a G:BOX Chemi XRQ imager (Syngene). A negative control probed with pre-immune rabbit serum was tested in the same conditions.

4. Inhibitory profile of propeptides and synthetic peptide: The inhibitory specificity of the propeptides ppFhCL3, ppFhCL1, ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47}, ppFhCL3Leu^{66}/Gly^{66}, ppFhCL3Glu^{68}/Arg^{68} as well as the peptide were screened using a panel of serine and cysteine proteases, to name: bovine trypsin (SigmaAldrich), bovine chymotrypsin (Sigma-Aldrich), bovine thrombin (SigmaAldrich), human cathepsin B (SigmaAldrich), human cathepsin L (SigmaAldrich), human cathepsin S (SigmaAldrich), human cathepsin K (Enzo Life Sciences), and purified *F. hepatica* cathepsin L1 (FhCL1), L2 (FhCL2), L3 (FhCL3), B1 (FhCB1), B2 (FhCB2), B3 (FhCB3), *Schistosoma mansoni* cathepsin B (SmCB) and *S. mansoni* cathepsin L3 (SmCL3), which were all produced as functionally-active recombinant forms in *Pichia pastoris* in our laboratory (School of Biological Sciences, Medical Biology Centre, Queen's University Belfast).

All inhibition assays were performed in reaction buffer, Sodium Acetate buffer pH 7.0, (100 mM sodium acetate, 1 mM EDTA, 1 mM DTT, brij L23 0.01%) at 37°C. Enzymes and substrates concentrations employed in each assay are presented in Table 1. The inhibitory activity of all propeptides were screened at 10 nM and 500 nM. The synthetic peptide was first resuspended in 1% Dimethylsulphoxide (DMSO) in Mill-Q water and the concentrations adjusted to 10 nM and 500 nM. The propeptides were first incubated in reaction buffer for 10 min at 37°C, at either concentration, then each protease was added in a 100 µL volume reaction for 10 min at 37 °C. The peptide was incubated

for 60 min at 37°C, at either concentration, prior each protease was added. At this point, both reactions were conducted with the same procedure, and reaction volumes were then brought to 200 µL with the addition of fluorogenic substrate and the proteolytic activity started to be measured immediately as relative fluorescent units (RfU) in a PolarStar Omega Spectrophotometer (BMG LabTech, UK). All assays were carried out in triplicate. Additionally, the pH dependency of the inhibitory activity of the ppFhCL3 towards the enzymes FhCL1, FhCL2, FhCL3, HsCL and HsCK was tested using Sodium Acetate buffer in pH's ranging from 4.5 to 7.0.

The activity of the ppFhCL3 was also screened against native cysteine proteases present in excretory-secretory extracts (ES) from adult *F. hepatica* and NEJs. Briefly, adult *F. hepatica* ES was produced as described in Smith et al. (2016), and *F. hepatica* metacercariae (Italian isolate; Ridgeway Research, UK) were excysted and the NEJs were cultured in RPMI 1640 medium (ThermoFisher Scientific) for 24 h at 37 °C. The extracts were then concentrated to 1 mg/mL by ultrafiltration and stored at -80 °C until use (Robinson et al., 2009; Cwiklinski et al., 2015). The enzymatic assays were performed as described above, with the ES products being adjusted to an approximately cathepsin protease concentration of 1.5 nM per well

5. Determination of inhibition constant: The dissociation constants (K_i) of inhibition of the ppFhCL3, ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47}, ppFhCL3Leu^{66}/Gly^{66}, ppFhCL3Glu^{68}/Arg^{68} against relevant cysteine proteases (FhCL2, FhCL3, HsCL and HsCK) were determined by decreasing concentrations (nM) of the propeptide through serial dilution in the reactions. K_i determinations were performed using non-lineal regression analysis in GraphPad Prism 5.0 Software (http://www.graphpad.com). Initial velocities were fitted to Morrison's equation (Equation 1) and the resulting apparent K_i (K_i *app*) fitted to Equation 2 to determine the K_i of cysteine protease in the presence of the inhibitors (Morrison, 1969). The reported values of K_i are the averages of three measurements. Where *vi* is the initial velocity of a reaction containing the inhibitor and *v0* is the initial velocity of a reaction without the inhibitor; [*E*] is the concentration of enzyme catalytic sites; [*I*] is the inhibitor concentration; [*S*] is the substrate concentration and K_m is the known Michaelis-Menten constant for a given substrate with a given enzyme.

Equation 1 $$\frac{vi}{vo} = 1 - \frac{([E]+[I]+Ki^{app} - \sqrt{([E]+[I]+ ki^{app})^2 - 4[E][I]}}{2[E]}$$

Equation 2 $$Ki^{app} = Ki\ (1 + \frac{Km}{[S]})$$

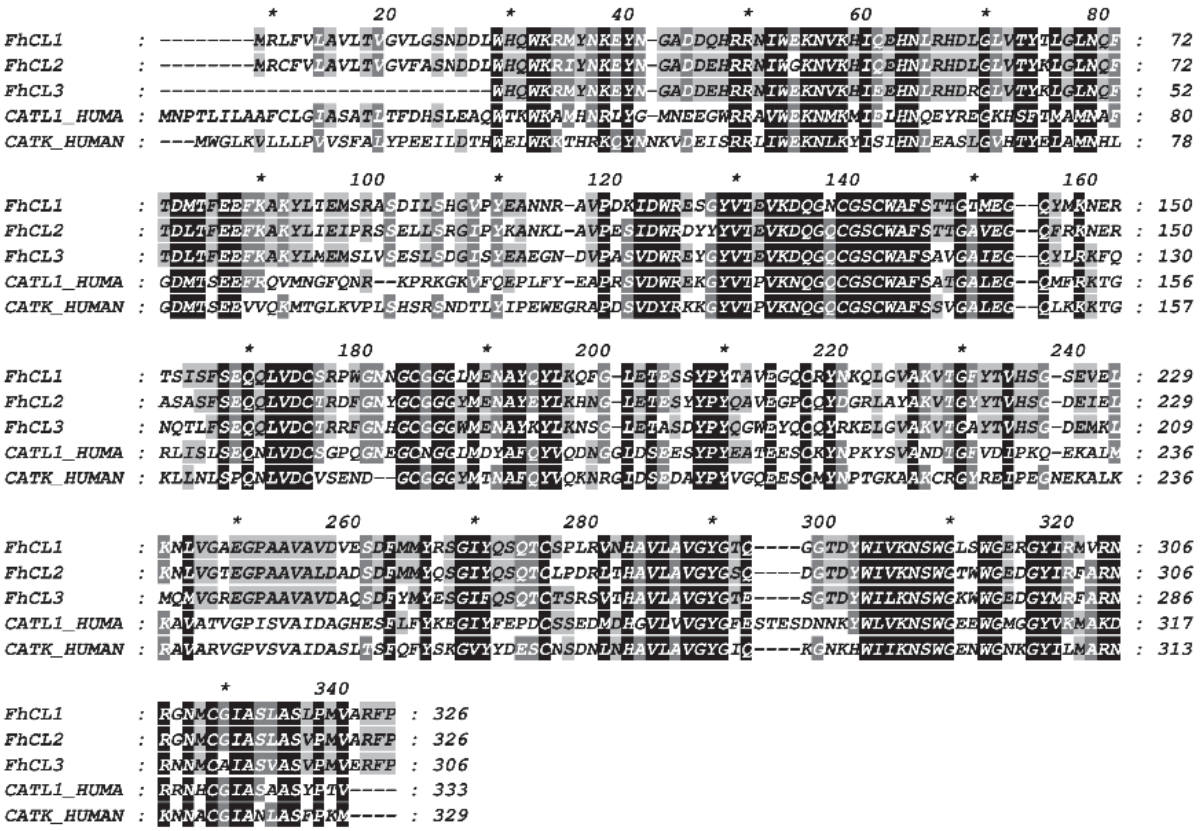
6. Pull-down of proteins from adult F. hepatica ES by ppFhCL3: The ppFhCL3, 1 µM, was incubated in 10 µL bed volume of pre-washed Ni-NTA beads for 1 hr at room temperature, then adult *F. hepatica* ES (40 µg) was added to the reaction and incubated for one hour at room temperature. The beads were washed three times (50 mM NaH_2PO_4, 300mM NaCl, 10mM $C_3\ H_4\ N_2$) before adding Elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM $C_3\ H_4\ N_2$). Eluted samples were resolved in 4-20% SDS-PAGE gel (Bio-rad), stained with Biosafe Coomassie (BioRad) and imaged using a G:BOX Chemi XRQ imager (Syngene). The composition of the protein band at ~27 kDa from the ES sample pulled down with ppFhCL3 was analysed by LC-MS/MS at the Fingerprints Proteomics Facility, University of Dundee, Scotland. The propeptide without the presence of ES was submitted to the same procedure as a control of the experiment.

Table 1. List of proteases and substrates used in enzymatic assays with ppFhCL3

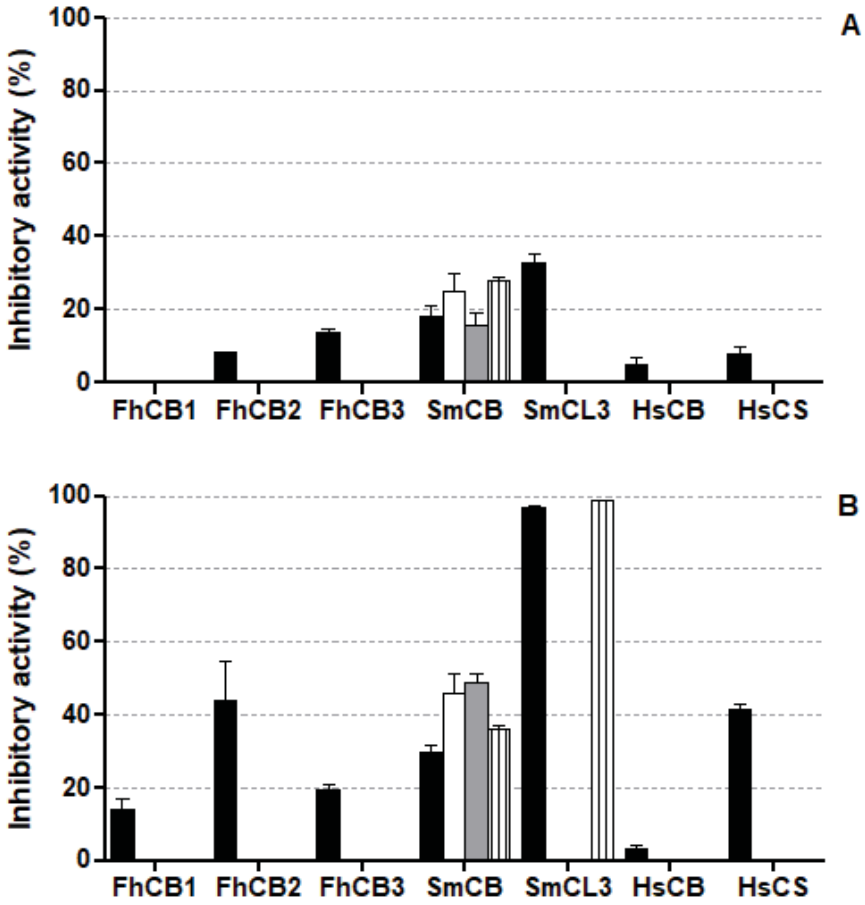
Enzyme	Substrate
F. hepatica cathepsin L1	Z-Leu-Arg-NHMec (20 μM)
F. hepatica cathepsin L2	Z-Leu-Arg-NHMec (20 μM)
F. hepatica cathepsin L3	Z-Gly-Pro-Arg-NHMec (20 μM)
F. hepatica cathepsin B1	Z-Phe-Arg-NHMec (20 μM)
F. hepatica cathepsin B2	Z-Val-Arg-NHMec (20 μM)
F. hepatica cathepsin B3	Z-Val-Arg-NHMec (20 μM)
Human cathepsin L	Z-Phe-Arg-NHMec (20 μM)
Human cathepsin K	Z-Phe-Arg-NHMec (20 μM)
Human cathepsin S	Z- Leu-Arg-NHMec (20 μM)
Human cathepsin B	Z-Phe-Arg-NHMec (20 μM)
Trypsin	Z-Leu-Arg-NHMec (20 μM)
Chymotrypsin	Suc-Ala-Ala-Pro-Phe-NHMec (20 μM)
Thrombin	Z-Gly-Pro-Arg-NHMec (2 μM)
Schistosoma mansoni cathepsin B	Z-Phe-Arg-NHMec (20 μM)
Schistosoma mansoni cathepsin L3	Z-Phe-Arg-NHMec (20 μM)

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

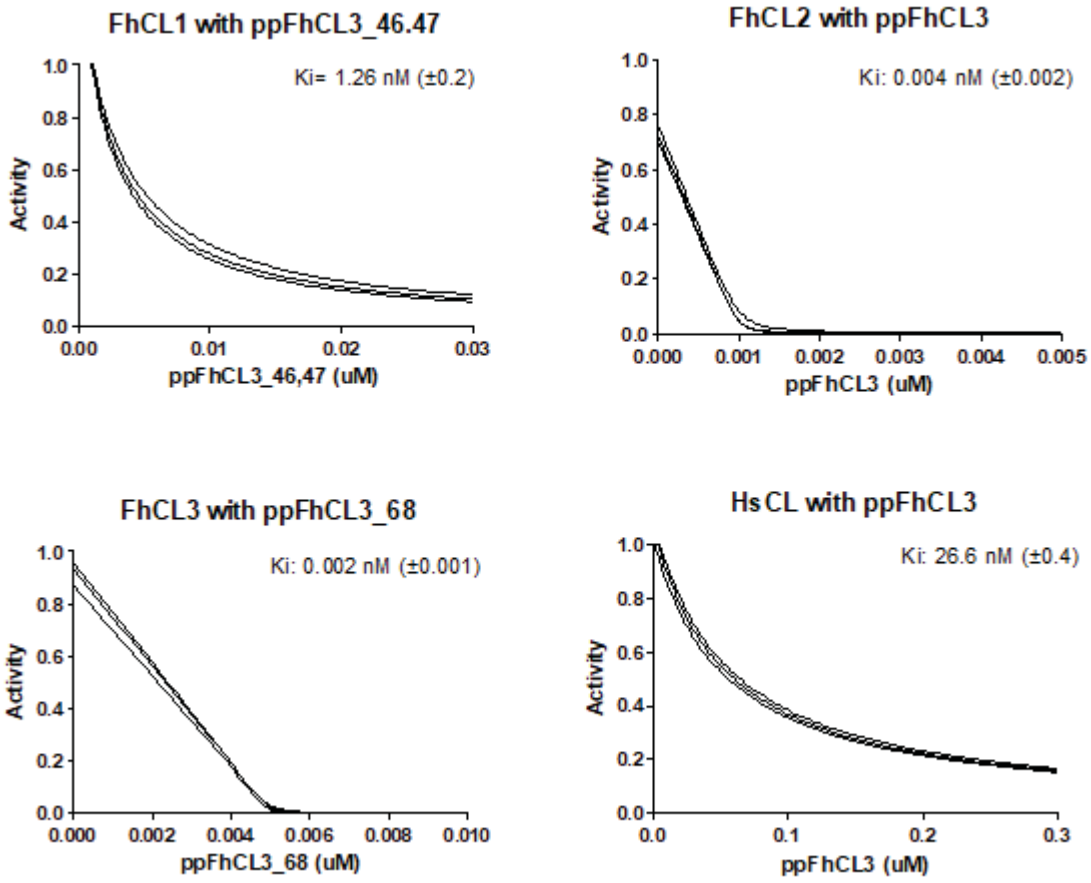
SUPPLEMENTARY MATERIAL



Sup 1. Sequence alignment of the main *F. hepatica* and *Homo sapiens* cathepsins L. FhCL1 (Q24940),-FhCL2 (A5Z1V3), FhCL3 (B3TM67), HsCL (NP_001903) and HsCK (P43235).



Sup 2. Inhibition profile of ppFhCL3 and variants against *F. hepatica*, *S. mansoni* and Human cysteine proteases. The inhibitory activity of ppFhCL3 (black bars), ppFhCL1_46,47 (white bars), ppFhCL1_66 (grey bars) and ppFhCL1_68 (striped bars), at 10 nM (A) and 500 nM (B) was screened using a panel of cysteine proteases, including *F. hepatica* cathepsin B1 (FhCB1), *F. hepatica* cathepsin B2 (FhCB2), *F. hepatica* cathepsin B3 (FhCB3), *S. mansoni* cathepsin B (SmCB), *S. mansoni* cathepsin L3 (SmCL3), Human cathepsin B (HsCB) and Human cathepsin S (HsCS). Inhibitory activity is presented relative to the total activity of each enzyme in the absence of inhibitors. Error bars indicate standard deviation of three separate experiments.



Sup 3. Relative inhibition constants of the ppFhCL3 and variants against *F. hepatica* and human cathepsin L's. *Ki*'s were calculated by decreasing concentrations (nM) of the propeptide through serial dilution in the reactions. Assays were carried out in triplicate and results are presented as average ± standard deviation.

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5. Final considerations

This work approached *Fasciola hepatica* infection in different hosts (humans, dairy cattle and buffaloes) and also, explored an innovative new alternative treatment for the disease. Even though several research groups around the world are working on fascioliasis control, eradication of this parasitic infection is rarely an option. Therefore, all the studies regarding to this disease are essential to broaden our knowledge and to reduce the effects caused by *F. hepatica*.

Within the concept of public health defined by the Brazilian Regulation Law No 8,080, 1990, health is a fundamental right of the all human being. For this reason, several programs were created to minimize damages caused by diseases that affect humans and animals. If we look from 1980 to today, it is possible to verify a decrease in the prevalence of parasitic diseases (i. e. intestinal parasitic helminths, intestinal protozoa) in the Brazilian population. However, thousands of people still suffer from the consequences of these parasitic diseases or are exposed in risky areas. As already mentioned in this work, human fascioliasis is a neglected disease in Brazil, and also, the diagnosis may be compromised by a diverse number of issues, such as the lack of health professionals to diagnose the disease adequately and the lack of specific diagnosis methods in routine laboratory procedures. We believe, from our reports (chapters one and two), that a new perspective about human fascioliasis will be adopted, both by the scientific community and by health professionals. Given the fact that Balneario Piçarras, from now on, is very much aware of the occurrence of the disease in the region, and doctors have already been advised on how to proceed about it - things will be improved and will change for better. We have provided information protocols (see addendum 4) for the doctors containing orientations about fascioliasis and we hope they will be adopted in their routine practice.

Epidemiological studies are undeniably important to determine the prevalence, intensity of infection, geographical distribution, risk areas and several factors concerning diseases. From these studies, intervention strategies can be recommended according to the local husbandry and particular climatic conditions, avoiding the dispersion of diseases to new areas and consequently new cases and their consequences. In terms of animal fascioliasis, each geographical affected area has its particularities, as well as each affected host, therefore local and regional studies are required. The prevalence of fascioliasis in dairy cattle in the state of Paraná and in buffaloes in the South of Brazil, including São Paulo were unknown to date. From these results, we expect that areas with a high

prevalence of the disease will receive more attention, and preventive control. Also, motoring strategies can be applied, changing the worrisome scenario that was forecasted by us.

It is worth to mention that buffaloes represent a significant portion of the livestock economy, they are an excellent alternative for areas where cattle breeding is not satisfactory. Besides that, buffalo's meat and products are well valued. However, by the fact that they are large animals, clinical symptoms can be neglected and the occurrence of diseases like fascioliasis may not (and was not) reported in buffaloes.

In relation to the impact of fascioliasis to dairy cattle, the association between the disease and economic losses are necessary to show to farmers and all the industry around, the importance of animal welfare, health control and the importance of diagnosis and treatment of parasitic diseases. It has been described in the literature that costs with anthelmintic treatment in dairy herds is lower than the impact caused by the disease, highlighting the need for veterinary advice regarding prophylactic measures to reduce economic losses. Even though the findings (milk parameters) that were reported in Chapter 3 were low (4.5% RDA Model), the relationship between the presence of antibodies in affected animals and milk quality parameters was not random. The data clearly showed that there was a negative impact of the disease on the animals, clinically affecting the animals. Thus, we consider that the disease can generate a significant economic loss, mainly if we consider that the monthly income of many families in the state of Paraná come from the milk industry. An appropriate approach to continue the investigations about the impact of the disease on dairy cattle, would be to work with positive and negative farms (in pairs) and study the animals individually. In this way, others parameters could be considered, such as milk yield, coprological test (to determine past and recent infections), animal management and contamination risk factors in a comparison model. We could even evaluate the value of treatment and the recovery of the animals' performance.

Neglected diseases, such as fascioliasis, face big problems resulting from the lack of investment for the development of new drugs. Pharmaceutical companies have less interest in developing more effective diagnostic methods and new drugs because neglected diseases are caused by infectious agents or parasites that are considered endemic in low-income populations and there would be no market to develop new products. Considering that the best option to treat *F. hepatica* infection is the use of

triclabendazole due to its unique efficacy against immature and adult flukes, the search for new alternative treatments is urgent. Wherein, resistance has been reported in several countries. Taking this into consideration, here we found promising results (Chapter 5) that will be continuously explored in the future to get a new target drug, hopefully to treat animals and humans.

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ADDENDUM 1. Document from the Sandwich Doctorate (June 2017 to February 2018) realized in the School of Biological Sciences under the supervision of Dr John Pius Dalton at Queen's University Belfast, Northern Ireland.

Inhibitory properties and mechanisms of *Fasciola hepatica* cathepsin L

By

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A chapter that will be submitted for the requirements for the degree of Doctor of Philosophy (PhD) in the Federal University of Paraná, Brazil

School of Biological Sciences

Queen's University Belfast

February 2018

DECLARATION

I declare that this chapter is my own work. It is based on research carried out in the School of Biological Sciences, Queen's University Belfast, during the months Jun 2017 – January 2018. All data reported in this chapter are property of the Queen's University Belfast.

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And, finally, thank you my Angels of Light. "*Assim seja*".

Abstract

The propeptide extention to the N-terminal of the cathepsin L zymogen is a well-known regulator of enzyme catalytic activity. Here we demonstrate that the propeptide from *F. hepatica* cathepsin L3 (ppFhCL3) is a potent inhibitor of recombinant and native parasite cathepsin L's. Differential immunolocalization of the FhCL3 zymogen and its propeptide in newly excysted juveniles (NEJ's), as well as the the verification of cathepsin L activity in excretory-secretory extracts from adult worms and NEJ's, suggest that the propeptide is releases from the FhCL3 zymogen in the parasite gut. Amino acids substitutions made in the ppFhCL3 sequence revealed that alterations of the residues p46 and p47 (ppFhCL3_Tyr^{46}Lys^{47}/Ala^{46}Ala^{47}) abrogates the inhibitory activity towards FhCL3 and FhCL2 enzymes, possibly by interfering with the unique way by which ppFhCL3 sits in the mature enzyme forcing its extended part into the substrate bind cleft. The substitution ppFhCL3_Leu^{66}/Gly^{66} also resulted in loss of inhibitory activity, whilst the variant ppFhCL3_Glu^{68}/Arg^{68} showed a mild variation in its activity when compared to the wild type. Unexpectedly, none of the variants lost the ability of inhibiting the FhCL1 enzyme, which may reflect key structural differences between these enzymes. Accordingly, we describe for the first time a 'clamp' mechanism essential for the binding of the propetide to FhCL3 and FhCL2, two enzymes that are known to have a collagenolytic activity fundamental during *F. hepatica* infection. The clamp involves residues of the propeptide that interact with the mature enzyme in regions outside (Tyr46p, Lys47p) and inside the active site (Leu66p). Together our results give remarkable insights regarding the propeptide-cathepsin interaction and open up the possibility of exploring these features in order to design new and selective inhibitors for *F. hepatica* cathepsins.

TABLE OF CONTENTS

1. Introduction

Proteases are enzymes that cleave or degrade peptides catalysing the hydrolysis of peptide bonds (Barrett & McDonald, 1986). According MEROPS, a peptidase database, these proteins are classified into families based on the nature of the catalytic residues in: Aspartic (A), Cysteine (C), Glutamic (G), Metallo (M), Asparagine (N), Mixed (P), Serine (S), Threonine (T) and Unknown (U). Among them, cysteine proteases are widely distributed in organisms, including vertebrates, invertebrates, viruses and plants, where they are involved in several biological process inside the lysosome, cytoplasm, nucleus and even in extracellular environment (Brömme, 2001; Chen *et al.,* 2017). The majority of cysteine proteases belong to the papain superfamily (Clan CA, family C1:CA1), which are generally referred as cathepsins. More than 20 cathepsins of different groups, A to Z, were already described in organisms, and special interest towards these enzymes has increased because of their importance in the development of pathologies such as cancer, atherosclerosis and osteoporosis (Chen *et al.,* 2017; Kramer *et al.,* 2017; Weiss-Sadan *et al.,* 2017).

Structurally, cysteine proteases consist of an amino terminal domain that is mostly α-helical and a carboxy-terminal domain featuring an antiparallel β-sheet, with a cysteine and histidine catalytic residues forming a thiolate-imidazolium dyad (Beveridge, 1996). To protect the cell from the consequences of uncontrolled degradative activity, these proteases are synthesized as inactive enzymes (Roche *et al.,* 1997). For this reason, cathepsin L proteases are synthesised as pre-pro-enzymes that consist of a pre-peptide, a pro-peptide and the mature (catalytic) enzyme. The inactive enzyme will be subsequently processed into a proenzyme (zymogen) during their passage through the endoplasmic reticulum and Golgi apparatus, where it loses the prepeptide (Roche *et al.,* 1997). Although, the catalytic power of the mature enzyme is already present at this stage it is nearly completely masked by the presence propeptide (Sivaraman *et al.,* 2000; Quraishi & Storer 2001).

The proenzyme is activated to the mature enzyme under specific conditions in the environment where it has to exert its function. Those that are lysosomal cathepsins will be activated once inside the organelle, where their propeptide is cleaved and dissociated, possibly due an autocatalytic reaction under acidic conditions, as revealed by *in vitro* experiments (Mason *et al.,* 1987; Stack *et al.,* 2008). Alternatively, the cathepsins can be activated in the cytoplasm or be secreted to the extracellular environment where its

activation will occur upon low pH conditions. Aisch & Biswas (2017) studies support the pH importance for enzyme activation, as they showed that the cathepsin L central residue, Arg31, is the key residue in this pH sensing network. Furthermore, apart from being potent inhibitors of the proteolytic activity of its cognate mature enzyme, the propeptide region of the cathepsins is also fundamental as it is involved in foldase activity, maintenance of stability, intracellular sorting and regulation of enzyme activity (Tao *et al.,* 1994; Roche *et al.*, 1997).

Specifically, the propeptide region is a N-terminal extension of the mature enzyme, also known as cathepsin prosegment (Capetta *et al.,* 2010). Structurally, propeptides are constituted by ~95 amino acid residues, ranging from 12-15 kDa. They consist of a N-terminal part (P-domain) formed by two crossed helices, α1p and α2p, that anchors in the corresponding propeptide binding loop (PBL) of the mature enzyme and forces the adjacent extended part, C-terminal, of the propeptide into the active site cleft (Coulombe *et al.*, 1996; LaLonde *et al.,* 1999; Cancela *et al.,* 2010). The C-terminal part is ~20 residues long and binds in the substrate-binding cleft in an opposite orientation to that of the substrate in order to avoid being degraded, thereby physically blocking the active site. Altogether, these features confer the propeptides with a unique ability to inhibit of their cognate cathepsin (Coulombe *et al.,* 1996; Sivaraman *et al.,* 2000). Complementary structural analysis of procathepsins L show that the prosegment folds and interacts with the enzyme in a similar way to that observed for procathepsin B, suggesting that the general mechanism of inhibition that the propeptide exerts on the mature enzyme is conserved across the papain superfamily (Coulombe *et al.*, 1996).

Within the structure of the prosegment, two regions, ERFIN and GNFD, are described as highly conserved motifs that contribute to specific interactions with the catalytic domain, and are also involved in inhibition and folding of the protease (Vernet *et al.,* 1995). The ERFIN motif comprising the residues 21p-77p (Glu27p-X_3-Arg-X_3-Phe X_2-Asn-X_3-Ile- X_3-Asn46p) and is found in cathepsins but not in cathepsins B (Karrer *et al.,* 1993). Although this motif is of obvious importance, amino acid mutations within it generally result in variants that display a high structural similarity to the wild type (Karrer et al. 1993). The importance of the GNFD motif, which comprising residues 59p-65p (Gly59p- X_1-Asn-X_1-Phe-X_1-Asp65p), was exploit by Capetta and collaborators (2002), which verified that a double point mutation (Asn70p to Ile and Phe72p to Ile) within the

motif led to significant changes in the propeptide conformation and a marked decrease in the affinity to the mature enzyme.

The ability of cathepsin proteases to degrade specific substrates is a characteristic defined by the structure of their substrate-binding subsites (S2, S1 and S1'), as well as of their comparatively broad binding areas (S4, S3, S2', S3'). The specificity of substrate binding is principally governed by the residues that make up the S2 subsite. This subsite forms a deep pocket capable of holding the P2 amino acid of the substrate and positioning the scissile bond into the S1 subsite for cleavage (Turk *et al.,* 2002; Cancela *et al.,* 2008). Taking this in consideration, Robinson and collaborator (2011), studied and compared the substrate specificity of different active recombinant *F. hepatica* cathepsins, FhCL1, FhCL2 and FhCL3. The study showed that the collagenase-like activity observed for FhCL3 and FhCL2 is due to the presence of the residues of Trp67 and Tyr67 in the S2 subsite of their active sites, respectively. Both, Trp and Try are able to accommodate either Gly or Pro residues at P2 in the substrate. On the other hand, in FhCL1 the presence of a Leu67 within the S2 subsite makes it impossible for the enzyme to readily accept Pro into its active site (Robinson *et al.,* 2011).

Although cathepsins from parasites perform key roles to establish infection in the host during their life cycles, human cathepsins have been fair more studied. Indeed, proteases are fundamental during host invasion as parasites secrete proteolytic enzymes that have the capacity to cleave a number of host proteins including collagen, haemoglobin and immunoglobulins (Zawistowska-Deniziak *et al.,* 2013). In addition, most of the parasites, including trematodes, express different proteases that were shown to be main players in host–parasite interactions (Robinson & Dalton 2011). These proteins are found in excretory-secretory products (ES), as well as in somatic antigens, derived from helminths and can be differentially expressed during the growth and maturation of the parasite within the host, which demonstrate the parasite's ability to change the host interface along the course of infection (Cancela *et al.,* 2008).

F. hepatica liver flukes, are trematode parasites with a complex life cycle that involves snails belonging to the Lymnaeidae family as intermediate host, and mammals, including humans as definitive host. The biological cycle begins when the infective metacercariae are ingested by the definitive host and excyst within the intestine as newly excysted juveniles (NEJ) that burrow through the duodenal wall and migrate through the host eroding the liver parenchyma while maturing. After approximately 6-8 weeks the

parasite finally reaches the biliary ducts to develop into fertile hermaphrodite adult worms that reproduce, laying around 25.000 eggs per fluke per day. These eggs leave the host through the faeces and reach the environment and continuing the cycle (Valero *et al.,* 2002). During all life stages of *F. hepatica* proteases are expressed and the different isoforms of some of them, such the cathepsins (Table 1), were proposed to play important roles in functional specialization of the parasite (Dalton *et al.,* 2006), as well as being responsible for the pathogenic effects of the infection (Brady *et al*., 1999; Corvo *et al.,* 2009).

Table 1. Differential expression of cathepsin proteases in *F. hepatica* development stages.

***F. hepatica* stages**	**Cathepsin B's**			**Cathepsin L's**				
	FhCB1	FhCB2	FhCB3	FhCL1	FhCL2	FhCL3	FhCL4	FhCL5
Metacercariae	X	X	X			X		
NEJ	X	X	X			X	X	
Immature	X	X	X	X	X	X		X
Adult	X			X	X			X

Data based on Meemon *et al.,* 2004; Robinson *et al.,* 2008; Robinson *et al.,* 2009; Zawistowska-Deniziak *et al.,* 2013.

Two main classes of cathepsin proteases are expresses and secreted by *F. hepatica*: cathepsin B (CB) and cathepsin L (CL) (Corvo *et al*., 2013). While all seven CB's identified belong to a single clade (CB1 to CB7), it was verified that *F. hepatica* express a total of 17 members of CL's that were classified in five clades (papain-like). A phylogenetic analysis of these CL's member is shown in Figure 1. In addition, while adult worms expressing CL's from clades 1 (CL1), CL2, and CL5, NEJs were shown only to express CL3 and CL4 (Cancela *et al.,* 2008; Robinson *et al.,* 2008; Corvo *et al.,* 2013; Cwiklinski *et al.,* 2015) (Table 1).

MetacercariaL excystment to NEJs that are capable of invading the gut and continuing the parasite life cycle is a fundamental step for *F. hepatica* infection and is highly associated with the secretion of specific proteases. According to transcriptomic

and proteomic analysis, the *F. hepatica* cathepsin CL3 (FhCL3) and FhCB account for over 80% of the total protease activity detectable in NEJs, including their excretory-secretory (ES) products (Robinson *et al.,* 2009). The abundance of FhCL3 could be related to its strong collagenase activity that might be critical to enable the NEJs penetrate the intestinal wall (Corvo *et al.,* 2009), while it was demonstrated that FhCB1, for instance, has important digestive functions in the gut of NEJ, they also might play a role in immune evasion through the cleavage of host antibodies to assist in parasite invasion and migration (Wilson *et al.,* 1998; Beckham *et al.,* 2009). As the fluke develops during migration through the host's liver, the secretion of FhCL3 and FhCBs is reduced to 2% and 3% of the total of secreted proteases, respectively. Concurrently, the secretion of other cathepsins start and increase, a trend that continues throughout parasite development and until they reach the host's bile duct as an adult worm, in which the profile of proteases found is FhCL1 (69%), FhCL2 (22%) and FhCL5 (9%) (Robinson *et al.,* 2009).

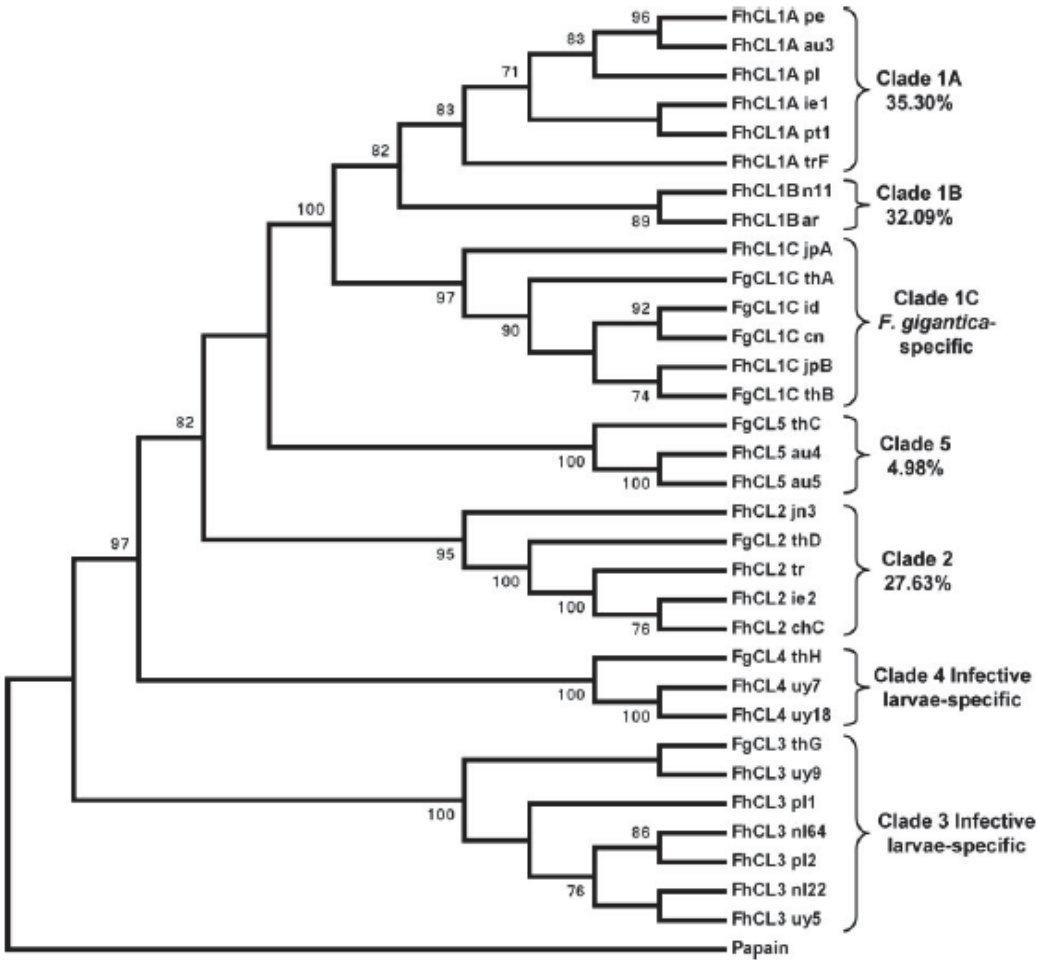


Figure 1. Phylogenetic relationships of the *Fasciola* cathepsin L gene family (Based on Robinson *et al.,* 2008).

For *F. hepatica* adult worms, the CL's are essential as they are pivotal to the parasite's ability to digest haemoglobin (Hb), their main source of nutrient. The worm obtains the Hb by making punctures with a muscular pharynx in the bile duct wall and uses it to supply the amino acids needed for egg production. The digestion of the Hb occurs within the lumen of the parasite gut, which is believed to be slightly acidic (around pH 5.5) and therefore necessary for cathepsin protease activation. The Hb digestion is a predominant role of FhCL1, and to a lesser extent to FhCL2 and FhCL5, respectively (Dalton *et al.,* 2006; Robinson *et al.,* 2008b). Of note, stability studies have shown that the FhCL-like proteases are active over a wide pH range (3.0–8.0) (Dowd *et al.,* 2000), which is indeed an important aspect of these proteases biology. Cathepsins excreted-secreted by *F. hepatica* were verified as being active in neutral physiological pH, which correlates with them being of fundamental importance in events such as host's tissues penetration, cleavage of host's immunoglobulins and suppression of immune cell proliferation (Dalton *et al.,* 2003; Lowther *et al.,* 2009).

The identification of major virulence factors is one of the primary goal in parasite research since they can eventually lead to the development of specific control measures, either through drugs or immunogens. When it comes to *F. hepatica* research, this is of special importance in light of the emerging drug resistance in several countries, especially considering that nowadays chemotherapy using benzimidazoles, in particular triclabendazole due its unique efficacy against immature and adult flukes, is the first option to treat and prevent cattle and sheep infections (Moll *et al.,* 2000; Brockwell *et al.,* 2014; Kelley *et al.,* 2016). In this context, cathepsins proteases stand out as good target candidates for new alternatives of treatment. Besides that, various studies have been demonstrating promising results using cathepsin L-like vaccine candidates (vaccine trials have usually been conducted using the whole cathepsin proteins, propeptide and mature enzyme). Dalton et al. (1996) tested FhCL1 and FhCL2 combined with Hb, both purified from ES products derived from adult worms, and obtained 53.7% and 51.9% of protection level in cattle, respectively. Later, Dalton and collaborators (2003) gathered data from vaccine trials carried out along eight years and showed that purified native FhCL1 and FhCL2 can induce protection, ranging from 33 to 79%, in vaccine trials with sheep and

cattle experimentally infected with *F. hepatica* metacercariae. A most recent study used recombinant FhCL1 in vaccine trials and showed that those vaccinated cattle exposed to fluke-contaminated pastures had fluke burden reduction of 48.2% (Golden *et al.,* 2010).

As highlighted above, a very important moiety of cathepsin proteases are their N-terminal extension, the propeptide, which previous studies have showed to have a high and selective inhibitory activity towards their cognate protease (Coulombe *et al*., 1996). The cathepsins FhCL1 and FhCL2, for example, are enzymes that share 90% similarity in their amino acids sequence, however the propeptide from FhCL1 enzyme (ppFhCL1) have shown higher potency as an inhibitor of FhCL1 than FhCL2 mature enzyme (Roche *et al.,* 1999). Moreover, the propeptide from FhCL1 had no inhibitory effect on human cathepsin L (Roche *et al.,* 1999). Ultimately, the apparent selectivity of these propeptides indicate specific roles in the parasite biology and in the host-parasite interface. Additionally, the observed specific and potent inhibitory activity of the ppFhCL1 towards mature FhCL1 are both desirable characteristics in proteases inhibitors, and indeed raise the possibility of exploring the prosegment to target and disrupt the function of *F. hepatica* cathepsins with the aim of developing alternatives for fascioliasis control.

Although FhCL1 and FhCL3 belonging to the same family they are expressed in distinct parasite life stages, suggesting diverse functions and mechanisms which possibly reflects the needs of the parasite in each condition and environment. Given these central roles that cathepsins play in different stages of *F. hepatica* infection, combined with the necessity of alternative methods for fascioliasis control, in the present study we investigate the differential specificity and potency of ppFhCL1 and ppFhCL3 as proteases inhibitors. Taking into consideration that specific inhibitors to FhCL3 might interfere and block *F. hepatica* infection in an early stage, variants of the ppFhCL3 with special substitutions based on 3-D structural analyses have been produced and tested to decipher the roles of specific residues in the inhibitory function of the propeptide. Here, we demonstrated that the ppFhCL3 is a highly potent and selective *F. hepatica* CL's inhibitor. The interaction between the ppFhCL3 and FhCL3 and FhCL2 it is described here for the first time as a 'clamp' mechanism involving residues of the propeptide that interacting with the mature enzyme in regions outside (Tyr46p, Lys47p) and inside the active site (Leu66p). In addition, changes in these residues interfere with the selectivity of the ppFhCL3. These results have opened up the possibility of designing new selective inhibitors for *Fasciola* cathepsins, a novel approach towards the treatment of fascioliasis.

2. Material and Methods

2.1 Gene and peptide production

The propeptides sequences of the *F. hepatica* cathepsin L1 (FhCL1) and cathepsin L3 (FhCL3) were designed based on the full genes sequences of each protein (ABW75768.2) (GenBank). The cDNA encoding the propeptides with a C-terminal His-tag were cloned into a pET-28a(+) (kanamycin resistant) by GenScript (Genscript Biotech, USA). Three variants of the ppFhCL3 gene were similarly synthesized with a C-terminal His-tag; these included the variant ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47} whereby the tyrosine residue at position 46p and a lysine at position 47p were substituted to alanine residues, ppFhCL3Leu^{66}/Gly^{66} whereby the leucine residue at position 66 was substituted to a glycine residue and ppFhCL3Glu^{68}/Arg^{68}, whereby the glutamic acid residue at position 68p was substituted to an arginine residue (Fig. 2). Additionally, a 33 amino acids long peptide derived from ppFhCL3 was chemically synthesised, according the sequence provided and highlighted in Fig 2 (GL Biochem, Shanghai).

2.2. Expression of the recombinant propeptides

2.2.1 Small-scale expression of propeptide:

the plasmids encoding the ppFhCL1, ppFhCL3, ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47}, ppFhCL3Leu^{66}/Gly^{66}, ppFhCL3Glu^{68}/Arg^{68} were transformed into *Escherichia coli* One Shot™ BL21 Star™ (DE3) (Thermo Fisher) cells by thermal shock and grow in Luria-Bertani (LB) agar (Sigma-Aldrich) plates for 24 hrs at 37℃. A single colony of *E. coli* transformed cells was transferred into 15 mL LB broth (Sigma-Aldrich) supplemented with kanamycin (1 mg/mL) at 37°C with shaking overnight. Then, 250 µL of the culture was transferred into 10 mL of fresh LB broth supplemented with kanamycin and incubated at 37℃ with shaking until the culture reached an A_{600} of 0.6 (culture in log phase). The protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the cultures were maintained at 37℃ for a further 3 hr. The culture was centrifuged at 12000 x *g* for10 min at 4 °C, the supernatant was discarded and the pellet suspended in 50 µL of distilled water and 50 µL of 2x Laemmli Sample Buffer (Bio-rad). After the addition 50 mM DTT, the sample was incubated at 95℃ for 10 min, and a 10 µL analysed by 4-20% SDS-PAGE (Bio-rad). The gel was stained with Biosafe Coomassie (BioRad) and imaged using a G:BOX Chemi XRQ imager (Syngene).

Sub-cultures were grown separately and once protein expression was confirmed glycerol stocks were prepared and stored at -80 °C.

2.2.2 Large-scale expression and purification:

Glycerol stocks were used to inoculate 50 mL of LB broth supplemented with kanamycin that were incubated overnight at 37°C with shkaing. The culture was then transferred into a 1l LB broth supplemented with kanamycin, incubated at 37°C on a shaker until reach the A_{600} of 0.6 and protein expression induced with 1 mM IPTG for 3 hr at 30°C. The pellets were resuspended in ST buffer (10 mM Tris, 150 mM NaCl, pH 8.0) and lysozyme (10 µg/mL), on ice, sonicated (6 x 10 sec), and centrifuged at 15000 x *g* for 30 min at 4 °C. The supernatant was diluted 1:4 with Lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, pH 8.0), and and passed over a Ni-NTA beads column (Qiagen) at a rate of 0.5 mL/min. After successive washes with Wash buffer (50 mM sodium phosphate buffer, 20 mM imidazole, pH 8), the recombinant protein was eluted from the column using five mL Elution buffer (50 mM sodium phosphate buffer, 250 mM imidazole, pH 7) and buffer exchanged by dialysis into 1x Phosphate buffer (PBS) overnight at 4 °C. The protein yield was determined by Bradford assay with bovine serum albumin (BSA) as a standard, and the purity of the protein expressed was verified by 4-20% SDS-PAGE (Bio-rad), stained with Biosafe Coomassie (BioRad) and imaged using a G:BOX Chemi XRQ imager (Syngene).

2.3. Synthetic peptide

The synthetic peptide was resuspended in 1% Dimethylsulphoxide (DMSO) in Mill-Q water and the concentrations adjusted to 10, 5.0, 2.5 and 1.0 nM to perform inhibition assays.

2.4. Inhibitory profile of the propeptides and synthetic peptide

The inhibitory specificity of the propeptides ppFhCL3, ppFhCL1, ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47}, ppFhCL3Leu^{66}/Gly^{66}, ppFhCL3Glu^{68}/Arg^{68}, as well of the peptide were screened using a panel of serine and cysteine proteases, including: bovine trypsin (SigmaAldrich), bovine chymotrypsin (Sigma-Aldrich), bovine thrombin (SigmaAldrich), human cathepsin B (SigmaAldrich), human cathepsin L (SigmaAldrich), human cathepsin S (SigmaAldrich), human cathepsin K (Enzo Life Sciences). Purified *F.*

hepatica cathepsin L1 (FhCL1), L2 (FhCL2), L3 (FhCL3), B1 (FhCB1), B2 (FhCB2), B3 (FhCB3), *Schistosoma mansoni* cathepsin B (SmCB) and *S. mansoni* cathepsin L3 (SmCL3), which were all produced as functionally-active recombinant forms in *Pichia pastoris* in our laboratory (School of Biological Sciences, Medical Biology Centre, Queen's University Belfast).

All inhibition assays were performed in reaction buffer, sodium acetate buffer pH 7 (100 mM sodium acetate, 1 mM EDTA, 1 mM DTT, brij L23 0.01%) at 37°C. Enzymes and substrates concentrations employed in each assay are presented in Table 2. The inhibitory activity of all propeptides were screened at 10 and 500 nM and for the peptide 10 to 1.0 nM. The peptide was first incubated in reaction buffer for 60 min at 37°C, at either concentration, then each protease was added in a 100 µL volume reaction for 10 min at 37 °C. For the propeptides reaction, they were incubated with each protease in a 100 µL volume reaction for 10 min at 37 °C. At this point, both reactions were conducted with the same procedure. Reaction volumes were then brought to 200 µL with the addition of the appropriate fluorogenic substrate, and the proteolytic activity measured over 60 min as relative fluorescent units (RfU) in a PolarStar Omega Spectrophotometer (BMG LabTech, UK). All assays were carried out in triplicate.

The activity of the ppFhCL3 and ppFhCL1 were also screened against native cysteine proteases present in excretory-secretory extracts (ES) from adult *F. hepatica* and NEJs. Briefly, adult *F. hepatica* ES was produced as described in Smith et al. (2016), and *F. hepatica* metacercariae (Italian isolate; Ridgeway Research, UK) were excysted and the NEJs were cultured in RPMI 1640 medium (ThermoFisher Scientific) for 3 and 24 hrs at 37 °C. The extracts were then concentrated to 1 mg/mL by ultrafiltration and stored at -80 °C until use (Robinson *et al.,* 2009; Cwiklinski *et al.,* 2015). The enzymatic assays were performed as described above, with the ES products being adjusted to an approximately cathepsin protease concentration of 1.5 nM per well.

2.5. pH dependency of ppFhCL1 and ppFhCL3 inhibitory activity

The pH dependency of the inhibitory activity of the ppFhCL3 and ppFhCL1 towards the enzymes FhCL1, FhCL2, FhCL3 and HsCL was tested using reaction buffer (100 mM sodium acetate, 1 mM EDTA, 1 mM DTT, brij L23 0.01%) in different pH's, ranging from 4.5 to 7.0.

2.6. Determination of inhibition constant (*Ki*)

The dissociation constants (K_i) of inhibition of the ppFhCL3, ppFhCL1, ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47}, ppFhCL3Leu^{66}/Gly^{66}, ppFhCL3Glu^{68}/Arg^{68} against relevant cysteine proteases (FhCL1, FhCL2, FhCL3, HsCL and HsCK) were determined by decreasing concentrations (nM) of the propeptide through serial dilution in the reactions. K_i determinations were performed using non-lineal regression analysis in GraphPad Prism 5.0 Software (http://www.graphpad.com). Initial velocities were fitted to Morrison's equation (Equation 1) and the resulting apparent K_i (K_i *app*) fitted to Equation 2 to determine the K_i of cysteine protease in the presence of the inhibitors (Morrison, 1969). The reported values of K_i are the averages of three measurements. Where *vi* is the initial velocity of a reaction containing the inhibitor and *v0* is the initial velocity of a reaction without the inhibitor; [*E*] is the concentration of enzyme catalytic sites; [*I*] is the inhibitor concentration; [*S*] is the substrate concentration and K_m is the known Michaelis-Menten constant for a given substrate with a given enzyme.

Equation 1 $$\frac{vi}{vo} = 1 - \frac{([E]+[I]+Ki^{app} - \sqrt{([E]+[I]+ ki^{app})^2 - 4[E][I]}}{2[E]}$$

Equation 2 $$Ki^{app} = Ki\ (1 + \frac{Km}{[S]})$$

2.7. Pull-down of proteins from adult *F. hepatica* ES by ppFhCL3 and ppFhCL1

The ppFhCL3 and ppFhCL1, 2 µM and 2 µM, respectively, were incubated in 10 µL bed volume of pre-washed Ni-NTA beads for 1 hr at room temperature. Then adult *F. hepatica* ES (40 µg) was added to each propeptide and incubated for an hour at room temperature. The beads were washed twice (50 mM NaH_2PO_4, 300mM NaCl, 10mM $C_3H_4N_2$) before adding elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM $C_3H_4N_2$) and eluted samples were analysed by 4-20% SDS-PAGE (Bio-rad) stained with Biosafe Coomassie (BioRad) and imaged using a G:BOX Chemi XRQ imager (Syngene). The composition of the protein bands at ~27 kDa from the ES sample pulled down with each propeptide were analysed by LC-MS/MS at the Fingerprints Proteomics Facility, University of Dundee, Scotland. The propeptides without the presence of ES were submitted to the same procedure as a control of the experiment.

2.8. Differential immune recognition of cysteine proteases and its propeptides in ES from *F. hepatica* adult worms and NEJs 3 and 24 hrs post-excystment

Polyclonal antibodies against ppFhCL3 and FhCL3 were obtained from rabbits immunized with each of the respective recombinant protein by Eurogentec, Liège, Belgium. Mature and pro-enzymes FhCL1, FhCL2 and FhCL3, and ES from adult *F. hepatica* and NEJs 3 and 24 hrs post-excystment, as well as the ppFhCL3 and ppFhCL1 (5 µg each) were resolved by 4-20% SDS-PAGE and then electrotransferred into nitrocellulose membranes. Membranes were blocked (5% milk in 1x PBS-Tween 0.05%) and then probed with either anti-appFhCL3 or anti-FhCL3 as primary antibodies (diluted 1:7500 in 5% blocking solution). After three washes with PBST 0.05%, the membranes were incubated with the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) (diluted 1:5000 in PBST 0.05%). After three washes the western blots were developed by the addition of the chromogenic substrate SIGMA FAST™ BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Sigma-Aldrich) and imaged using a G:BOX Chemi XRQ imager (Syngene). A negative control probed with pre-immune rabbit serum was tested in the same conditions.

2.9. Differential immunolocalization of ppFhCL3 and FhCL3 in NEJs

NEJs (6 and 24 hrs post-excystment) were fixed with 4% paraformaldehyde in 0.1 M PBS (Sigma-Aldrich) overnight at 4 °C, and then washed three times with antibody diluent (AbD: 0.1 M PBS containing 0.1 % [V/V] Triton X- 100, 0.1 % [W/V] bovine serum albumin and 0.1 % [W/V] sodium azide). NEJs were then incubated in 0.1 M PBS containing either anti-ppFhCL3 or anti-FhCL3 at 1:500 dilutions, overnight at 4 °C, followed by three washes in AbD. As a negative control, separate samples of NEJs 6 and 24 hrs post-excystment were incubated in 0.1 M PBS containing rabbit pre-immune antiserum at 1:500 dilution. All NEJ samples were then incubated with the secondary antibody, fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (Sigma-Aldrich), at 1:200 dilution, overnight at 4 °C. To counter-stain muscle tissues, NEJs were incubated in AbD containing 200 µg/ml phalloidintetramethylrhodamine isothiocyanate (TRITC) overnight at 4 °C. Following three final washes in AbD, NEJs were whole-mounted in a 9:1 glycerol solution containing 0.1 M propyl gallate and viewed using confocal scanning laser microscopy (CSLM) (Leica TCS SP5) under the HCX PL APO

CS 100x oil objective lens. Leica type F immersion oil was used in viewing and all images taken at RT.

2.10. Molecular modelling of ppFhCL3 and variants cysteine protease inhibition

The obtained homology model was first docked to the crystal structure of FhCL1 previously reported by us (PDB code: 2O6X) using the HEX 6.1 protein-protein docking program and subjected to molecular dynamics simulations using MacroModel 10.8. In docking, the shape and electrostatic-based assessment of binding was conducted with over 100 generated and analysed docking solutions. The points of interaction of ppFhCL3 and FhCL3 mature enzyme is pointed to the active site and surrounding of FhCL3 enzyme, was selected for molecular dynamics simulations. 10 ns of molecular dynamics simulations and 5000 steps of followed minimization were conducted in implicit solvent at temperature 300K to obtain the final complex. OPLS_2005 force field was used in MacroModel calculations. Similar docking and simulation steps were used to study ppFhCL3 binding and interaction with (PDB code: 1YK7). The image with the molecular models was prepared with Maestro 10.2.

3. Results

3.1. Screening of the recombinant expression of the propeptides in BL21 (DE3) *E. coli* cells

In order to illustrate the structural differences between the ppFhCL3 and ppFhCL1, Fig 2 presents an alignment of their primary sequences. The propeptide from human cathepsin L is also presented in order to highlight features that have been further studied in this species and compare with those presented by *F. hepatica* propeptides. The residues that we substituted in the variants and the sequence of the synthetic peptide chemically produced are highlighted as well.

```
                    *        20         *        40
ppFhCL3   : ------SWHEWKRMYNKEYNGADDEHRRNIWEQNVKHIEEHNLRHDRGLV
ppFhCL1   : --SNDDLWHQWKRMYNKEYNGADDQHRRNIWEKNVKHIQEHNLRHDLGLV
ppHsCL    : DHSLEAQWTKWKAMHNRLYGMNEEGWRRAVWEKNMKMIELHNQEYREGKH

             *        60         *        80         *       100
ppFhCL3   : TYKLGLNQFTDLTFEEFKAKYLMEMSP---VSESLSDGISYEAEGNDVHHHHHH--- : 95
ppFhCL1   : TYTLGLNQFTDMTFEEFKAKYLTEMSR---ASDILSHGVPYEANNRAVHHHHHH--- : 99
ppHsCL    : SFTMAMNAFGDMTSEEFRQVMNGLQNRKPRKGKVFQEPLFYEAPRSVDWREKGYVTP :107
```

Figure 2. Expression and purification of ppFhCL1, ppFhCL3, ppFhCL3$Y^{46}K^{47}/A^{46,47}$, ppFhCL3L^{66}/G^{66} and ppFhCL3E^{68}/K^{68} from BL21 (DE3) *E. coli* cells. The sequence of ppFhCL3 is aligned with ppFhLC1 and ppHsCL. Amino acids mutated in ppFhCL3 were $Y^{46}K^{47}/A^{46,47}$, L^{66}/G^{66}, E^{68}/K^{68}, and are shown highlighted in grey. The 33 amino acids sequence used to synthetize the peptide is underlined. Those residues predicted to interact within the active site of the mature HsCL enzyme are highlighted in black.

As evidenced by the presence of a large band at ~ 12 kDa, all the propeptides were successfully expressed as soluble proteins in *E. coli* DE3 cells (Fig 3 A-D) after induction using IPTG. Because a more prominant band appears after 3 hrs of expression this was the time chosen for the large expression. The yield of each protein after purification using the Ni-NTA beads column and dialysis was determined by Bradford assay as follows: 2.84 mg/mL for ppFhCL1, 10.6 mg/mL for ppFhCL3, 3.44 mg/mL for ppFhCL3$Y^{46}K^{47}/A^{46,47}$, 4.78 mg/mL for ppFhCL3L^{66}/G^{66} and 5.25 mg/mL for ppFhCL3E^{68}/K^{68} (Fig. 4).

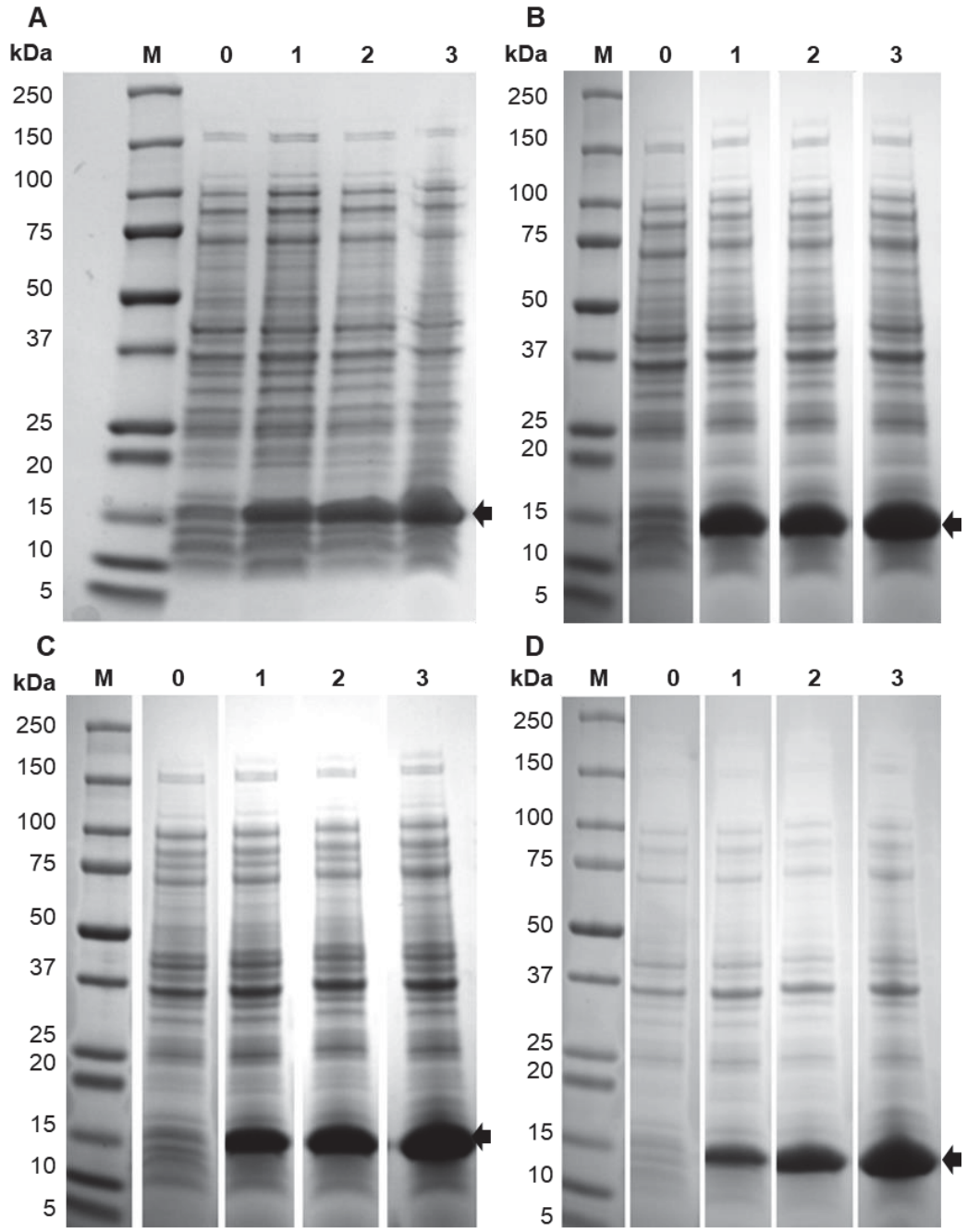


Figure 3. Small-scale eexpression of the ppFhCL3 (A), ppFhCL3Y^{46}K^{47}/A^{46,47} (B), ppFhCL3L^{66}/G^{66} (C) and ppFhCL3E^{68}/K^{68} (D) in BL21 (DE3) *E. coli* cells. The proteins present in the pellet before induction of expression (lane 0), and after 1 h (lane 1), 2 hrs (lane 2) and 3 hrs (lane 3) were resolved in 4-12% SDS-PAGE, stained with Biosafe Coomassie (BioRad). Molecular weight marker in kDa (M).

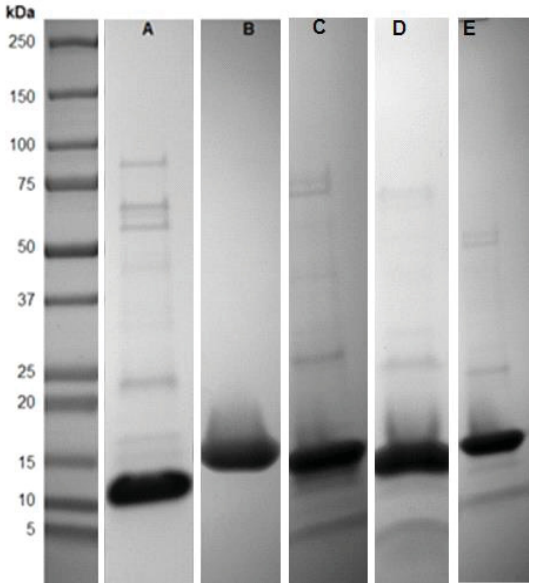


Figure 4. Large-scale expression of the recombinant forms of ppFhCL1 (*lane A*), ppFhCL3 (*lane B*) ppFhCL3Y^{46}K^{47}/A^{46,47} (*lane C*), ppFhCL3L^{66}/G^{66} (*lane D*) and ppFhCL3E^{68}/K^{68} (*lane E*) expressed in *E. coli* BL21 cells, after purification using the Ni-NTA beads column and dialysis against 1X PBS. The yield estimated of the propeptides varied from 2.8 – 10.6 mg/mL. Proteins resolved by 4-12% SDS-PAGE (BioRad), stained with Biosafe Coomassie (BioRad).

3.2. The ppFhCL3 and ppFhCL1 present high selective inhibitory activity against *F. hepatica* cathepsins L

The activity of the recombinant ppFhCL3 and ppFhCL1 were screened using a panel of proteases that included serine and cysteine proteases. Both propeptides were initially screened at 10 and 500 nM which demonstrated a defined inhibitory profile and revealed the selectivity of the ppFhCL3 and ppFhCL1 by *F. hepatica* cathepsins L as the inhibition towards the *F. hepatica* CB's was minimal at 10 nM. Moreover, both propeptides showed poor activity against cathepsins from *S. mansoni*, and a high inhibitory activity against the HsCL and HsCK was only obtained at 500 nM (Fig 5).

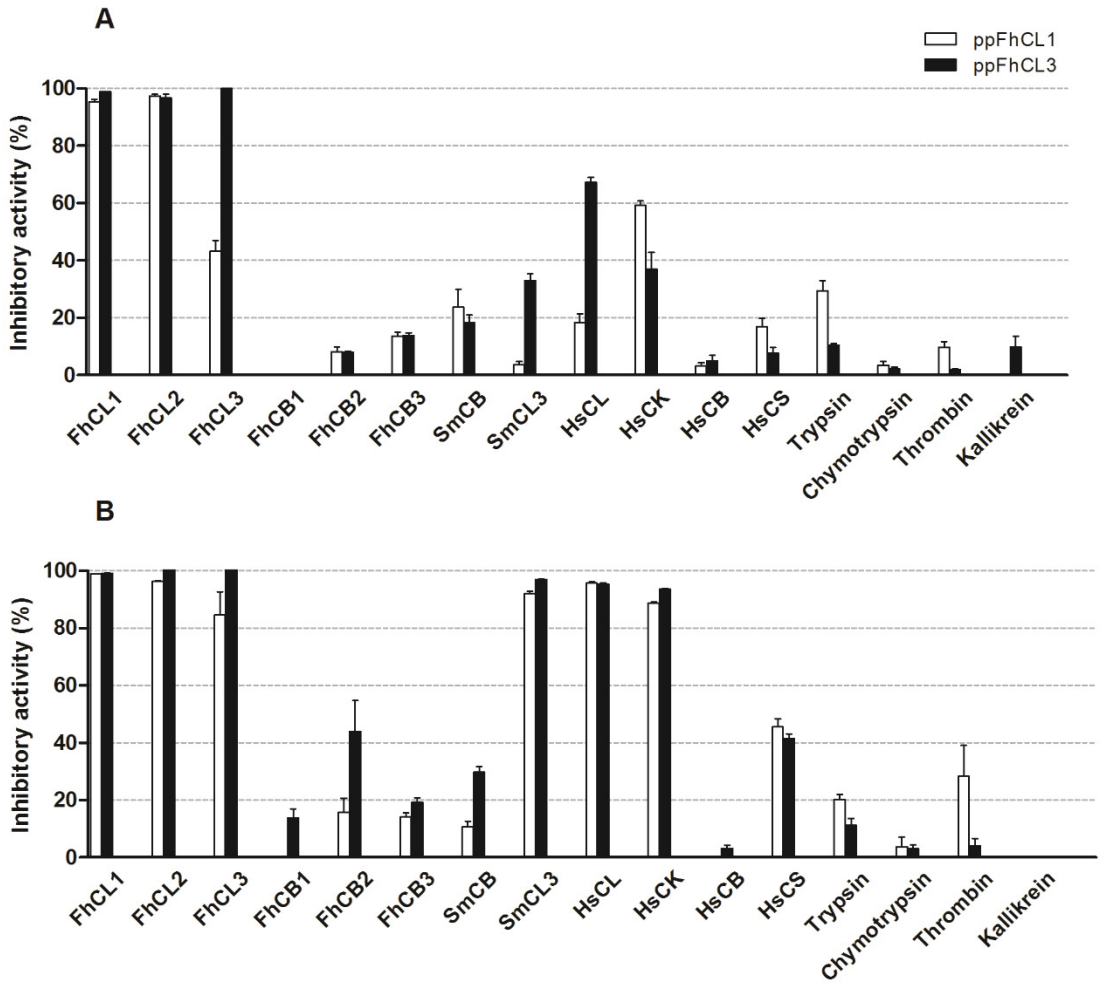


Figure 5. Inhibition profile of ppFhCL3 and ppFhCL1 against cysteine and serine proteases. The inhibitory activity of ppFhCL3 (black bars) and ppFhCL1 (white bars), at 10 nM (A) and 500 nM (B) was screened using a panel of cysteine proteases, including *F. hepatica* cathepsin L1 (FhCL1), *F. hepatica* cathepsin L2 (FhCL2), *F. hepatica* cathepsin L3 (FhCL3), *F. hepatica* cathepsin B1 (FhCB1), *F. hepatica* cathepsin B2 (FhCB2), *F. hepatica* cathepsin B3 (FhCB3), *Schistosoma mansoni* cathepsin B (SmCB), *S. mansoni* cathepsin L3 (SmCL3), Human cathepsin L (HsCL), Human cathepsin K (HsCK), Human cathepsin B (HsCB), Human cathepsin S (HsCS). Serine proteases screened including trypsin, chymotrypsin, thrombin and kallikrein. Inhibitory activity is presented relative to the total activity of each enzyme in the absence of inhibitors. Error bars indicate standard deviation of three separate experiments.

3.3. The inhibitory activity of the propeptides is pH dependent

The effect that pH exerts on the ability of the ppFhCL3 and ppFhCL1 to bind and inhibit different cathepsins L from *F. hepatica* and human, FhCL1, FhCL2, FhCL3 and HsCL, was verified by varying the pH of the running buffer, from 4.5 to 7.0. Fig 6 shows that the activity of the enzymes in the presence of the one or other propeptide decreases as pH rises, where at pH 7.0 the activity of the enzymes FhCL1, FhCL2 and FhCL3 are completely abrogated by the propeptide.

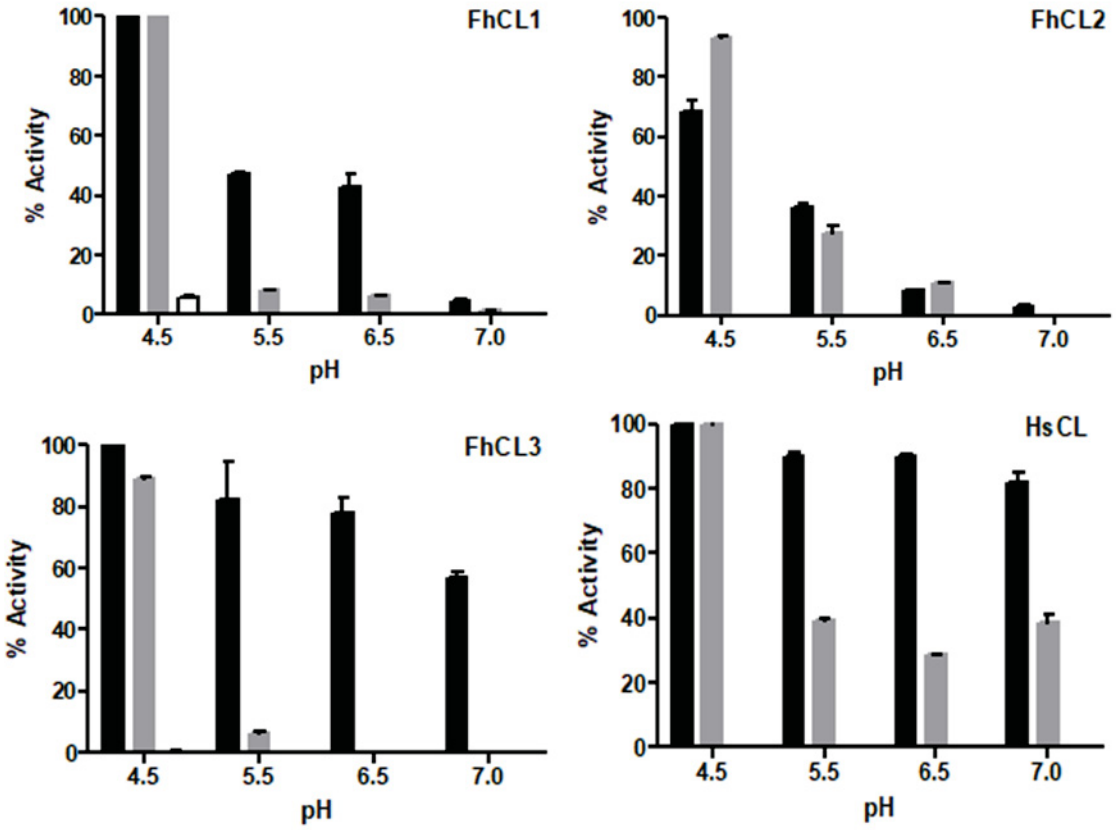


Figure 6. Effect of pH on the inhibitory activity of the ppFhCL3 and ppFhCL1. Activity of FhCL1, FhCL2, FhCL3 and HsCL in the presence of 10 nM ppFhCL3 (grey bars), ppFhCL1 (black bars) or E64 (white bars) at different pH. All assays were performed using sodium acetate buffer at different pH's. The activity is presented relative to the total activity of each enzyme in the absence of the propeptides in the same pH. Error bars indicate standard deviation of three separate experiments.

3.4. Structural studies of the interaction of the ppFhCL3 with *F. hepatica* and human cathepsin L's shown key residues interaction that could explain the selectivity and mechanism of inhibition of the ppFhCL3

Using the previous resolved three-dimensional structure of FhCL1 enzyme as a model the FhCL3 structure could be predicted, showing high homology with FhCL1 and providing insights into the interactions responsible for the binding of the propeptide to the mature enzyme. The role of the residues p46, p47, p66 and p68 were considered of fundamental importance; the participation of these amino acids in the propeptides-enzymes interactions is shown in the Fig. 7A, B and C. In the Table 2 we summarize the comparison of the residues in ppFhCL1 and HsCL participating of the same interaction, as well as those amino acids residues that are predict interact with them in the mature *F. hepatica* and humans cathepsin L's. Complementary, the complete alignment of the FhCL1, FhCL2 FhCL3 and HsCL is presented in the figure 8.

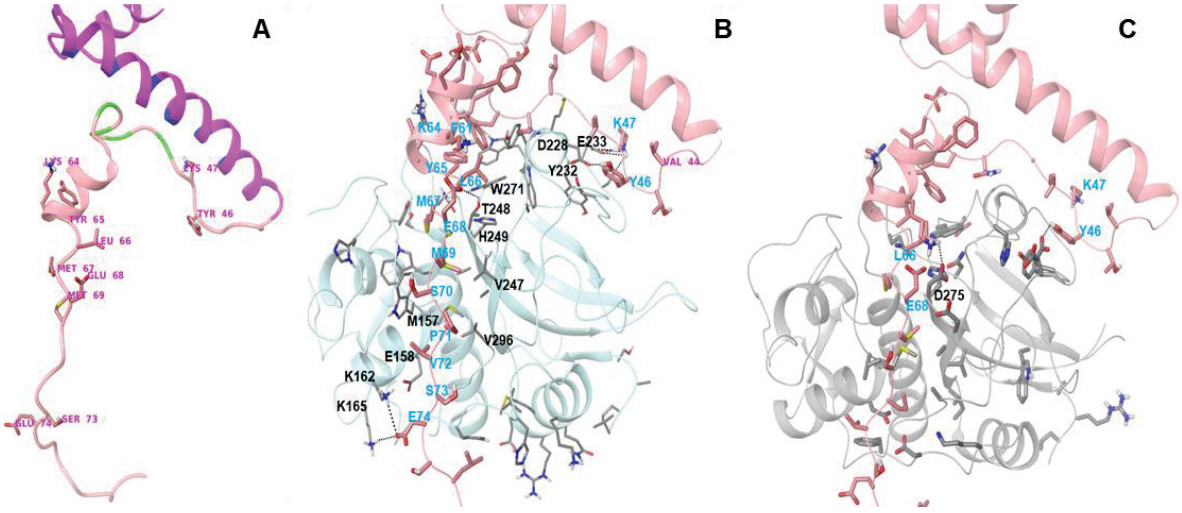


Figure 7. Structural representation of ppFhCL3 and its interactions with FhCL3 and HsCL, with points of mutation highlighted. Homology model of FhCL3 built displaying the main points of interactions between the propeptide and mature proteins. Hydrogen bond in dotted line. Those residues modified in the active site and in the surrounding are also shown.

Table 2. Comparison of the propeptides residues interaction with the active site and surrounding region residues of cysteine proteases from *F. hepatica* and *Homo sapiens*.

ppFhCL3	ppFhCL1	ppHsCL	FhCL1	FhCL2	FhCL3	HsCL
Y46 -----	Y50	F52	D248	D248	D228	S255
K47-----	T51	T53	S254	S254	S234	E261
L66	L70	N72	V245	A245	A225	G252
E68 -----	E72	L74	N268	T268	T248	D275

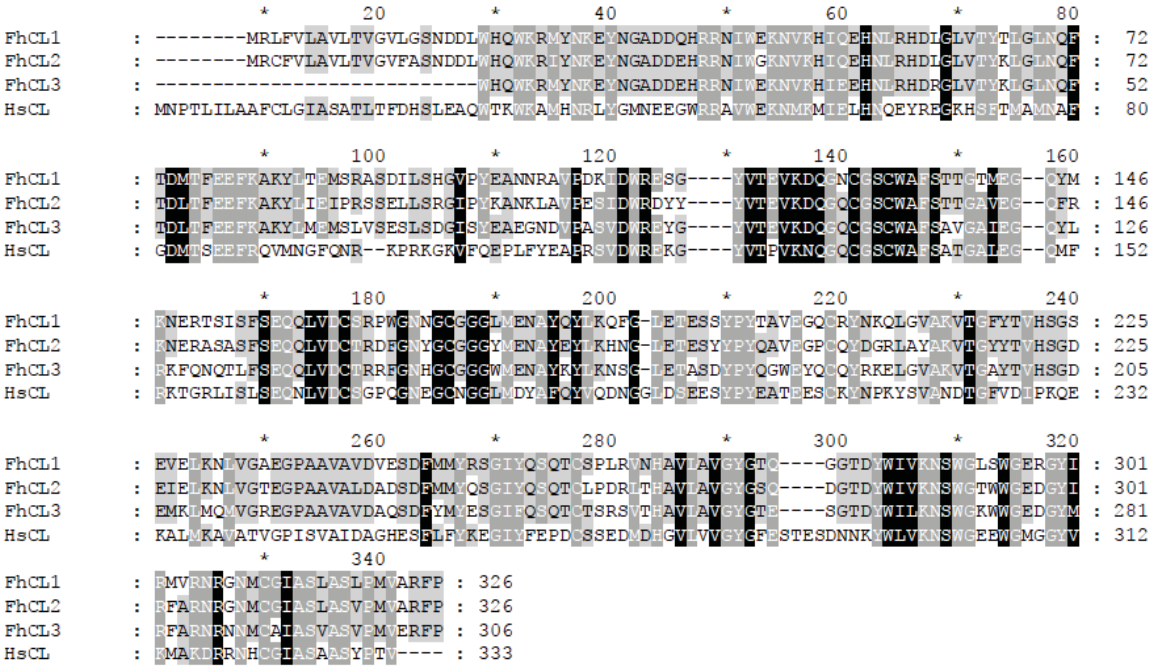


Figure 8. Sequence alignment of the main *F. hepatica* and *Homo sapiens* cathepsins L. FhCL1 (Q24940), FhCL2 (A5Z1V3), FhCL3 (B3TM67) and HsCL (NP_001903).

3.5. The variants ppFhCL3Y^{46}K^{47}/A^{46,47} and ppFhCL3L^{66}/G^{66} completely lost their inhibitory activity towards FhCL2 and FhCL3, but not FhCL1 enzyme

Considering the wider spectrum of inhibition that the ppFhCL3 showed, when compared to the ppFhCL1, a structural analysis of this propeptide was performed with the aim to identify those residues that could be responsible for the selective inhibitory activity that ppFhCL3 presents towards *F. hepatica* CLs. From these analyses three

precise substitutions were chosen, one involving residues that do not interact within the active site of the mature enzyme, ppFhCL3Y^{46}K^{47}/A^{46,47}, and two involving alterations of residues that were predict to interact with the substrate binding cleft, ppFhCL3L^{66}/G^{66} and ppFhCL3E^{68}/K^{68}. After recombinant expression the activity of the variants were tested in identical conditions previously used to the ppFhCL3. The results of the screening showed that the inhibitory activity of ppFhCL3Y^{46}K^{47}/A^{46,47} and ppFhCL3L^{66}/G^{66} towards FhCL2 and FhCL3 enzymes was completely abrogated. Unexpectedly, however, both variants still exhibited potent inhibitory activity against FhCL1. In contrast, the substitution of the residue p68, E^{68}/K^{68}, did not interfere with the inhibitory activity of the propeptide, as shown by the preserved inhibitory activity when compared to the ppFhCL3 (Fig 9).

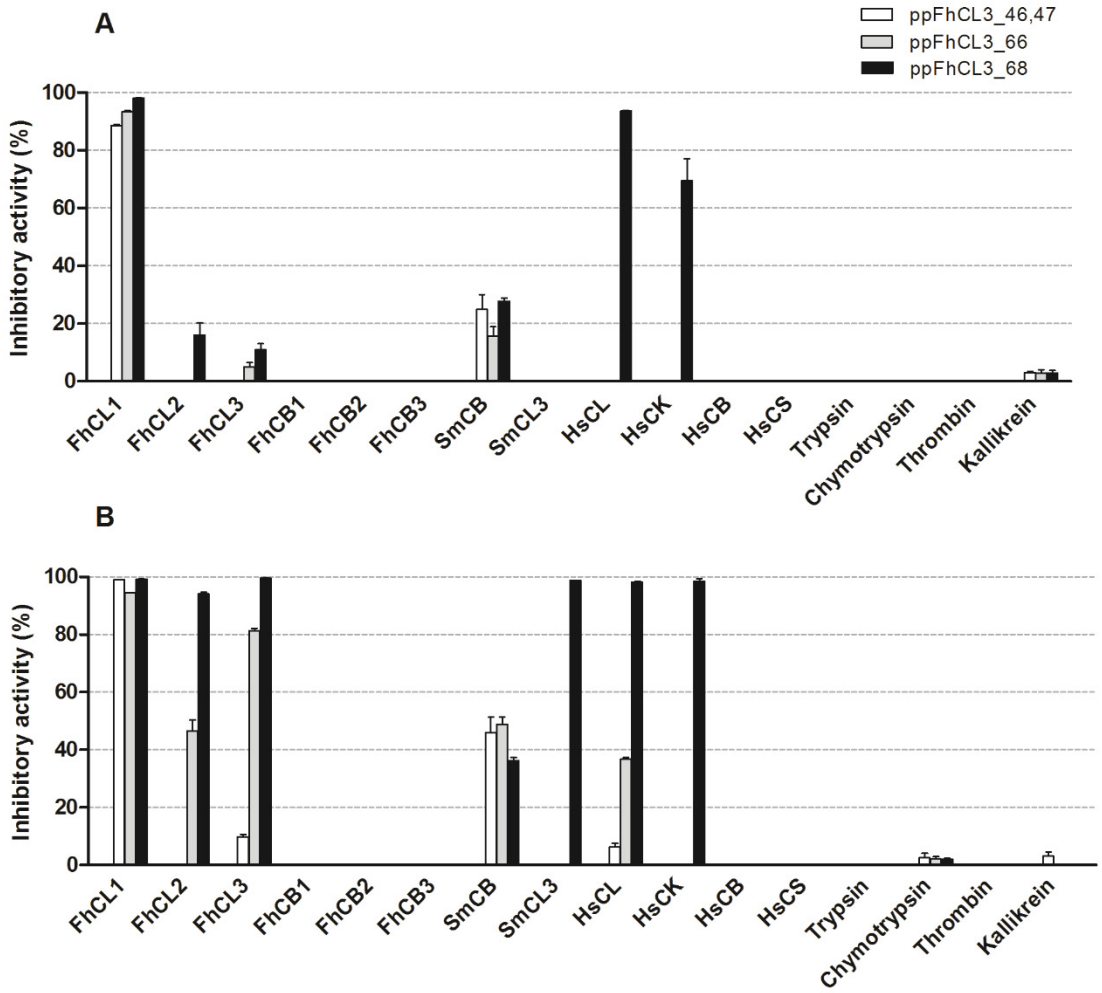


Figure 9. Inhibition profile of ppFhCL3Y^{46}K^{47}/A^{46,47}, ppFhCL3L^{66}/G^{66} and ppFhCL3E^{68}/K^{68} against cysteine and serine proteases. The inhibitory activity of

ppFhCL3Y^{46}K^{47}/A^{46,47} (white bars), ppFhCL3L^{66}/G^{66} (grey bars), ppFhCL3E^{68}/K^{68} (black bars) at 10 nM (A) and 500 nM (B) was screened using a panel of cysteine proteases, including *F. hepatica* cathepsin L1 (FhCL1), *F. hepatica* cathepsin L2 (FhCL2), *F. hepatica* cathepsin L3 (FhCL3), *F. hepatica* cathepsin B1 (FhCB1), *F. hepatica* cathepsin B2 (FhCB2), *F. hepatica* cathepsin B3 (FhCB3), *S. mansoni* cathepsin B (SmCB), *S. mansoni* cathepsin L3 (SmCL3), Human cathepsin L (HsCL), Human cathepsin K (HsCK), Human cathepsin B (HsCB), Human cathepsin S (HsCS). Serine proteases screened including trypsin, chymotrypsin, thrombin and kallikrein. Inhibitory activity is presented relative to the total activity of each enzyme in the absence of inhibitors. Error bars indicate standard deviation of three separate experiments.

3.6. The synthetic peptide lost significantly the inhibitory activity towards FhCL1, FhCL2, FhCL3 and HsCL compared to the ppFhCL3

The activity of the synthetic peptide was screened against relevant cysteine proteases. The screening was performed in four different concentrations, 10 to 1.0 nM, resulting in a particular inhibitory profile that revealed that the highest inhibition is observed at 2.5 nM for all the screened proteases. A slight selectivity of the peptide is shown for the FhCL3 enzyme. However, we observed that the inhibitory potency decreased significantly when compared with the ppFhCL3 (Fig 10).

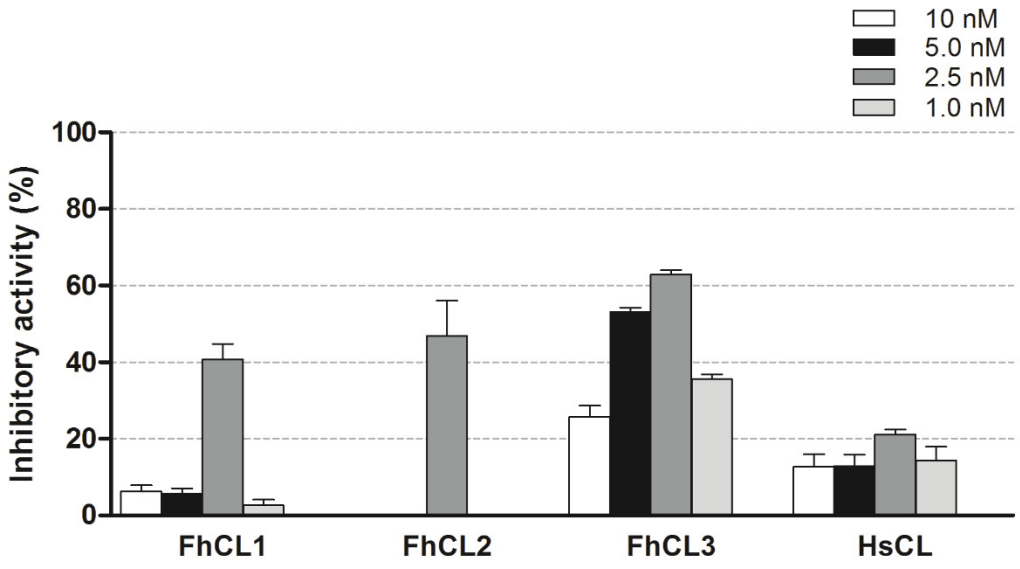


Figure 10. Inhibition profile of synthetic peptide against cysteine proteases in different concentrations. The inhibitory activity of synthetic peptide was screened in different concentrations, 10 nM (white bars), 5 nM (dark bars), 2.5 (grey bars) and 1.0 nM (lighter grey bars), against cysteine proteases, including *F. hepatica* cathepsin L1 (FhCL1), *F. hepatica* cathepsin L2 (FhCL2), *F. hepatica* cathepsin L3 (FhCL3) and human cathepsin L (HsCL). Inhibitory activity is presented relative to the total activity of each enzyme in the absence of inhibitors. Error bars indicate standard deviation of three separate experiments.

3.7. The *Ki*'s determination revealed that the ppFhCL3 is a highly potent inhibitor of FhCL1, FhCL2 and FhCL3 enzymes, a characteristic preserved only in the variant ppFhCL3E^{68}/N^{68}

The Table 3 presents the *Ki* values calculated for all relevant cathepsins from *F. hepatica* and humans (individual *Ki* curves can be visualized in the Fig 11.1 and 11.2). The low values obtained by the FhCL1, FhCL2 and FhCL3 in the presence of ppFhCL3, that were 650 and 50-fold lower than those values observed to human cathepsins, HsCL and HsCK, respectively, reaffirm the selectivity of the ppFhCL3 and ppFhCL1, and also demonstrate the potency of these inhibitors. Moreover, the *Ki*'s obtained when the ppFhCL3 variants where used to inhibit FhCL1 shown that all variants are still highly potent against this enzyme. The same preserved inhibitory activity is also verified to the variant ppFhCL3E^{68}/N^{68} against all enzymes tested

Table 3. *Ki* for inhibition of cysteine proteases by the ppFhCL1, ppFhCL3, ppFhCL3Y^{46}K^{47}/A^{46,47}, ppFhCL3L^{66}/G^{66}, ppFhCL3E^{68}/N^{68}

Enzyme concentration	**Inhibition *Ki* (nM)**				
	ppFhCL1	**ppFhCL3**	**ppFhCL3 Y^{46}K^{47}/A^{46,47}**	**ppFhCL3 L^{66}/G^{66}**	**ppFhCL3 E^{68}/N^{68}**
FhCL1 (2.7 nM)	0.90 (±0.07)	0.04 (±0.006)	1.26 (±0.2)	0.05 (±0.004)	0.3 (±0.001)
FhCL2 (5 nM)	1.11 (±0.28)	0.004 (±0.002)	>1 μM*	>1 μM*	0.02 (±0.01)
FhCL3 (5 nM)	2.47 (±0.41)	<0.002	>1 μM*	8.00 (±2.3)	0.002 (±0.001)
HsCL (0.2 nM)	48.33 (±4.06)	26.6 (±0.4)	>1 μM*	>1 μM*	6.04 (±0.44)
HsCK (2 nM)	356.23 (±57.88)	2.0 (±0.5)	>1 μM*	>1 μM*	1.40 (±0.2)

* values >1 μM could not be calculated accurately.

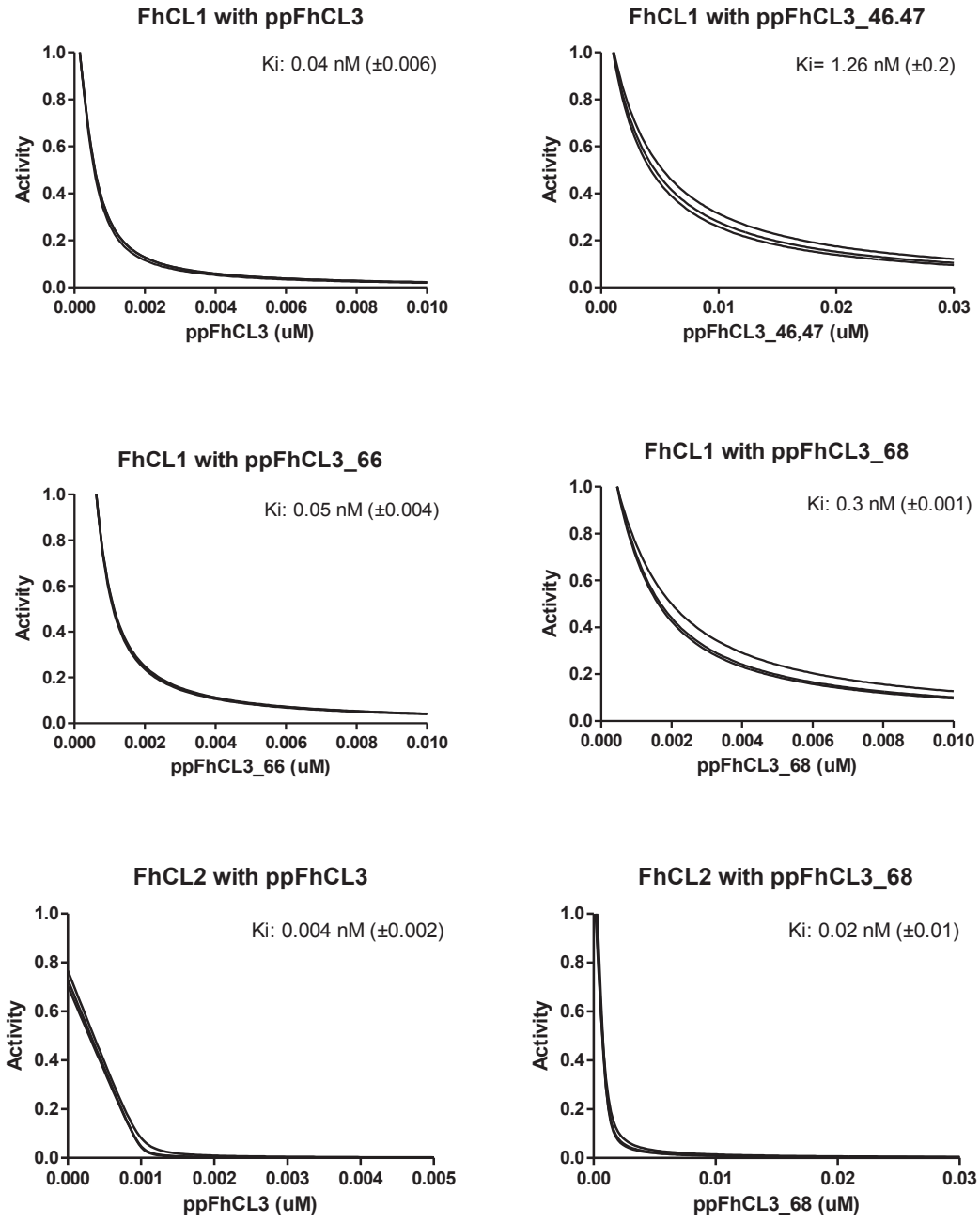


Figure 11.1. Relative inhibition constants of the ppFhCL1, ppFhCL3, ppFhCL3Y^{46}K^{47}/A^{46,47}, ppFhCL3L^{66}/G^{66}, ppFhCL3E^{68}/N^{68} against FhCL1 and FhCL2 enzymes.

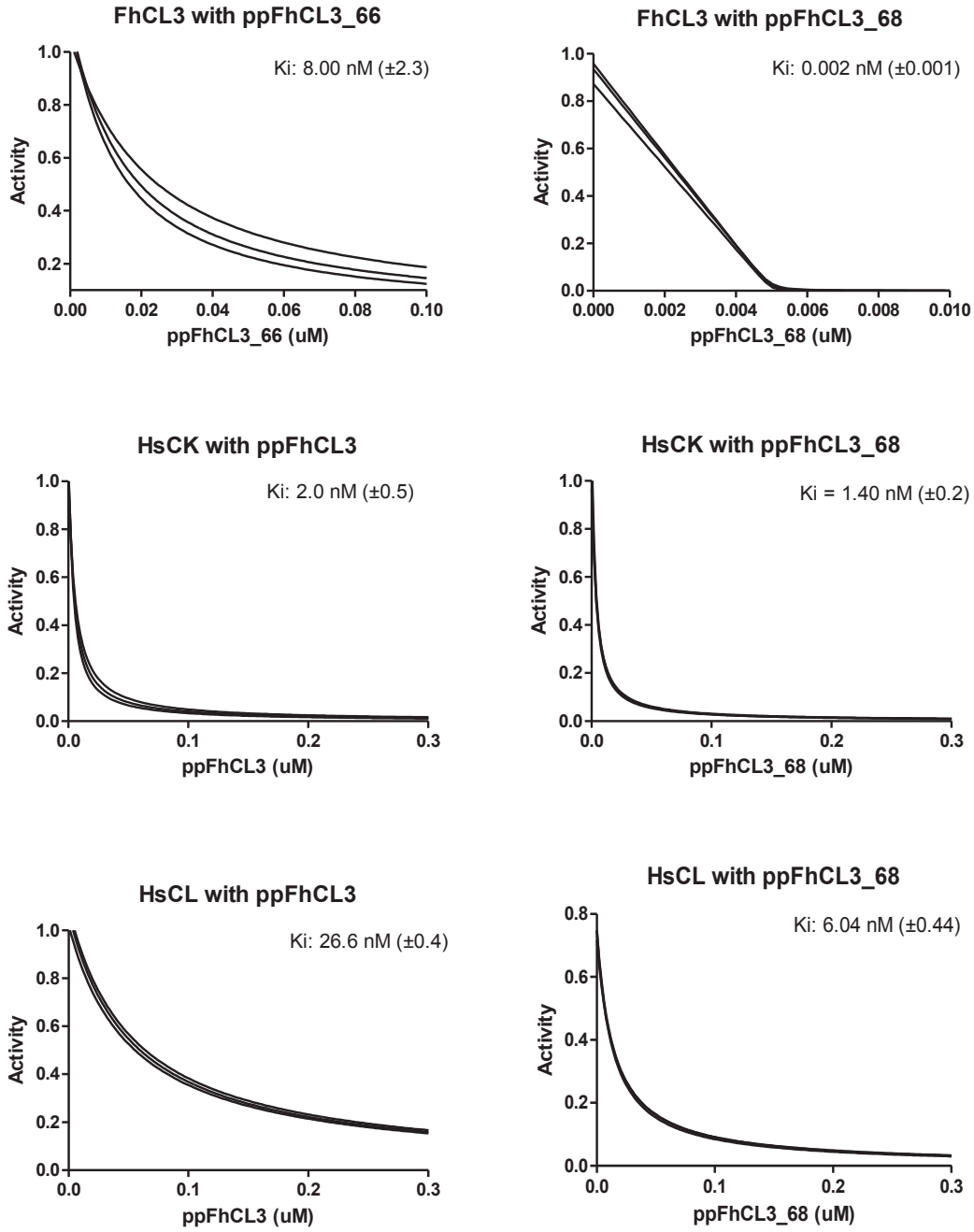
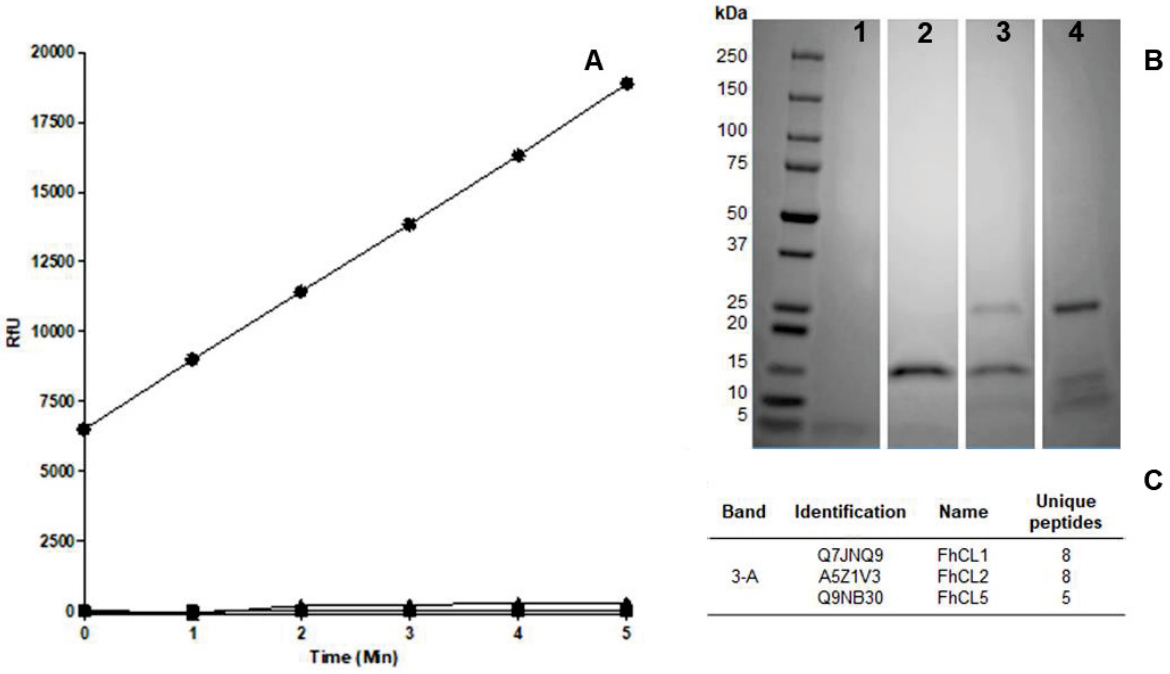


Figure 11.2. Relative inhibition constants of the ppFhCL1, ppFhCL3, ppFhCL3Y^{46}K^{47}/A^{46,47}, ppFhCL3L^{66}/G^{66}, ppFhCL3E^{68}/N^{68} against FhCL3, HsCK and HsCL enzymes.

3.8. The recombinant propeptides bind and inhibit the native cathepsin proteases present in ES products of *F. hepatica* adult worms

In order to verify the ability of the recombinant ppFhCL3 to effectively inhibit native cathepsins, we used the ES extract from adult *F. hepatica* worms. Once the inhibitory effect of the ppFhCL3 was verified through the enzymatic assay (Fig 12A), a pull-down experiment was performed in order to isolate and identify those enzymes in which the ppFhCL3 is binding, and therefore inhibiting, in the ES (Fig 12B). The LC-MS/MS analysis of the proteins pulled down by the ppFhCL3 revealed that the propeptide is acting towards three different native *F. hepatica* cathepsins, FhCL1, FhCL2 and FhCL5 (Fig 12C).



Band	Identification	Name	Unique peptides
3-A	Q7JNQ9	FhCL1	8
	A5Z1V3	FhCL2	8
	Q9NB30	FhCL5	5

Figure 12. The ppFhCL3 and ppFhCL1 bind and inhibit native *F. hepatica* present in the excretory-secretory extract (ES) from adult worms. (A) Cysteine proteases activity (presented as relative fluorescent units, RfU) in ES from adult *F. hepatica*, measured with the fluorogenic peptide substrate Z-Leu-Arg-NHMec in the absence of the inhibitors (circles), in the presence of ppFhCL1 (triangles), in the presence of ppFhCL3 (squares), and in the presence of E64 (asterisks). (B) SDS-PAGE gel stained with Biosafe Coomassie (BioRad) of the pull-down experiment from proteins present in ES from adult worms of *F. hepatica*. Ni-NTA beads (Lane 1), ppFhCL1 and Ni-NTA beads (Lane 2), ppFhCL3 and Ni-NTA beads (Lane 3), ppFhCL1, ES and Ni-NTA beads (Lane 4); ppFhCL3, ES and Ni-NTA beads (Lane 5) and ES alone (Lane 6). (C) Identification of

the proteins present in the ~27 kDa band from the lanes 4 and 5 by LC-MS/MS analysis revealed sequences consistent with FhCL1, FhCL2 and FhCL5.

3.9. The ppFhCL3 and variants ppFhCL3L^{66}/G^{66}, ppFhCL3E^{68}/N^{68} bind and inhibit native cathepsins proteases in the ES extract of NEJ

The ES from NEJ obtained at 3 and 24 hrs post-excystment were used in an enzymatic assay to verify the proteolytic activity in the presence and absence of the ppFhCL3 and variants, in order to verify the ability of these propeptides to inhibit native cathepsins that are being secreted by the infective life stage of *F. hepatica.* The assay clearly demonstrates that the ppFhCL3 inhibits the activity of cathepsins in the ES extract at different intensities, that could be attributed to the presence of different subclasses of cathepsins in the extract (Fig 12).

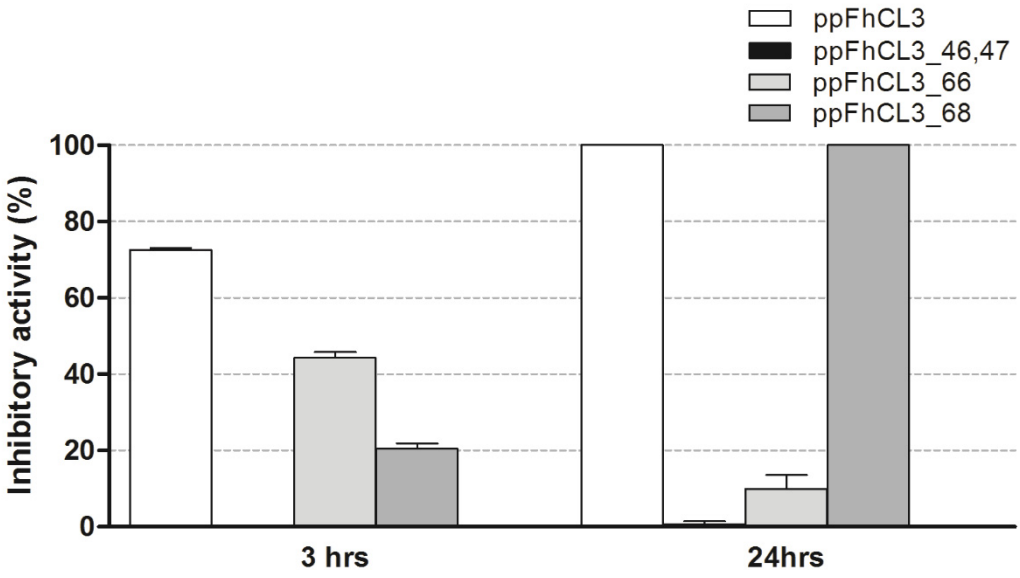


Figure 13. Inhibitory activity of the ppFhCL3, ppFhCL3Y^{46}K^{47}/A^{46,47}, ppFhCL3L^{66}/G^{66}, ppFhCL3E^{68}/N^{68} against ES from NEJ, 3 and 24 h post-excystment. The inhibitory activity of ppFhCL3 (white bars), ppFhCL3Y^{46}K^{47}/A^{46,47}(dark bars), ppFhCL3L^{66}/G^{66} (lighter grey bars), ppFhCL3E^{68}/N^{68} (grey bars) against ES from NEJ, 3 and 24 h post-excystment. Inhibitory activity is presented relative to the total activity of each enzyme in the absence of inhibitors. Error bars indicate standard deviation of three separate experiments.

3.10. Antibodies to FhCL3 and ppFhCL3 detect the *F. hepatica* cathepsins in the NEJ somatic extracts and secretions.

The presence of the FhCL3 in the NEJ ES and somatic antigens was demonstrated by probing these extracts with anti-FhCL3 (Fig 14B, lanes 8, 9 and 10). While the anti-ppFhCL3 cross-recognizes all *F. hepatica* cathepsins (Fig 14A, lanes 2, 3 and 4) the anti-FhCL3 demonstrate to be a more specific antibody, able to distinguish the cathepsins (Fig 14B, lanes 2, 3 and 4).

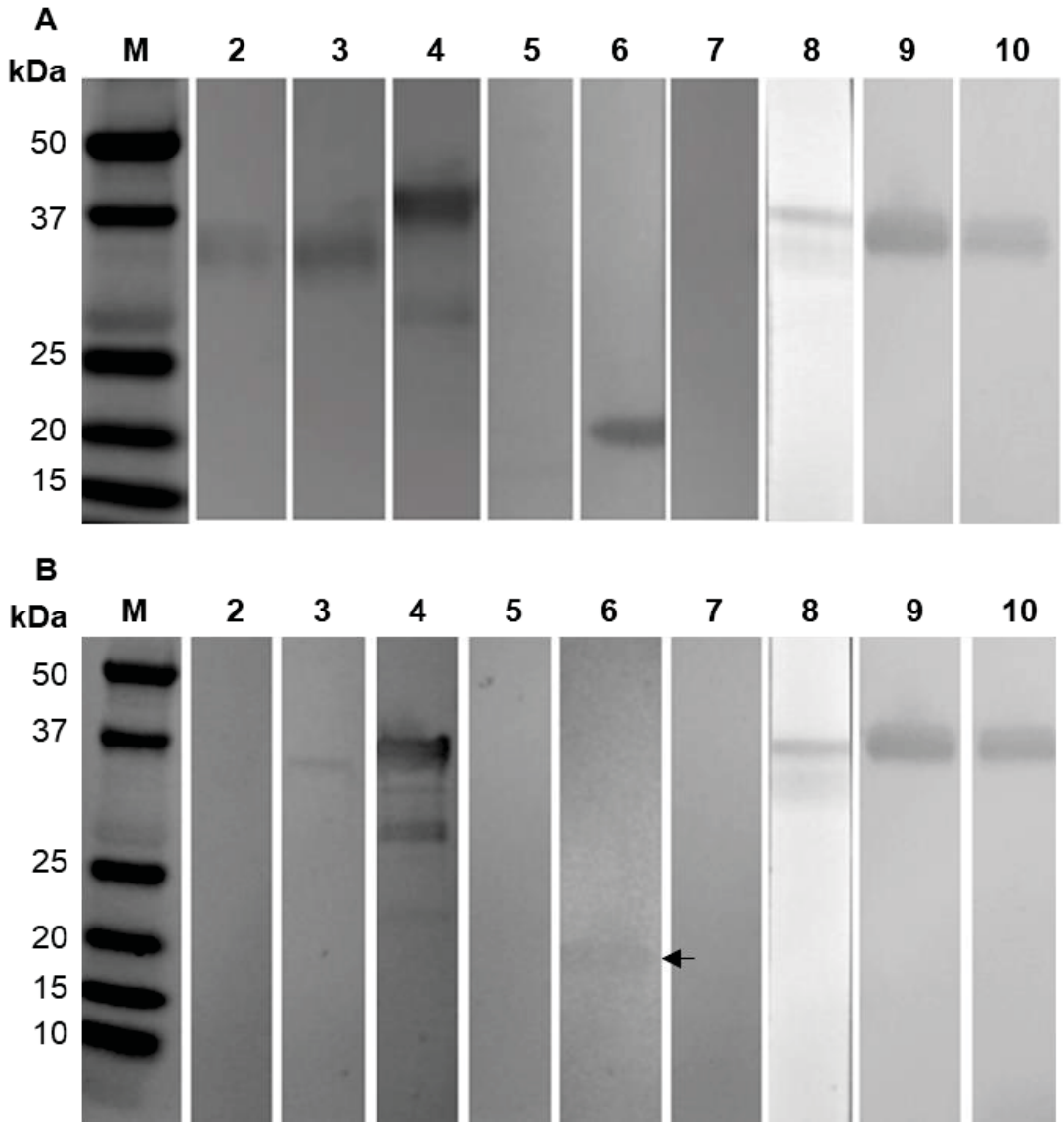


Figure 14. **Recognition of *F. hepatica* cysteine proteases by antibodies anti-FhCL3 and anti-ppFhCL3. A:** Western blot probed with anti-ppFhCL3 (dilution 1:7500) as primary antibody. Molecular weight marker in kDa (M), FhCL1 (Lane2), FhCL2 (Lane 3), FhCL3 (Lane 4), ppFhCL1 (Lane 5), ppFhCL3 (Lane 6), excretory-secretory extract

from adult *F. hepatica* (Lane 7), excretory-secretory extract from NEJs 3 hrs post-excystment (Lane 8), somatic extract from NEJ 3 hrs post-excystment (Lane 9), somatic extract from NEJ 24 hrs post-excystment (Lane 10). **B:** Western blot probed with anti-FhCL3 (dilution 1:7500) as primary antibody. Molecular weight marker in kDa (M), FhCL1 (Lane2), FhCL2 (Lane 3), FhCL3 (Lane 4), ppFhCL1 (Lane 5), ppFhCL3 (Lane 6), excretory-secretory extract from adult *F. hepatica* (Lane 7), excretory-secretory extract from NEJs 3 hrs post-excystment (Lane 8), somatic extract from NEJ 3 hrs post-excystment (Lane 9), somatic extract from NEJ 24 hrs post-excystment (Lane 10). WB probed with secondary antibody anti-IgG

3.11. Immunolocalization of ppFhCL3 and FhCL3 in NEJs indicates a fast production and secretion-excretion of FhCL3 by the NEJ

To localize ppFhCL3 and FhCL3 expression within the NEJ, confocal microscopy was carried out on 3 and 24 h post-excystment NEJs. These were probed with polyclonal anti-ppFhCL3 and anti-FhCL3 antibodies and counter-stained with TRITC-labeled phalloidin to provide NEJ structural visualization of muscle tissue under fluorescence microscopy. Higher fluorescence intensity was observed in NEJs 3 h post-excystment using anti-ppFhCL3 and anti-FhCL3 (Fig. 15, panels 1B and 1C) when compared in NEJs 24 h post-excystment (Fig. 15, panels 2B and 2C). The expression observed by fluorescence indicates that the presence of the propeptide in the gut is more evident in the first hours after excystment. This pattern is consistent with the fact that the enzyme is activated within the parasite gut and it is released in a processed and active mature form. Low and diffuse background fluorescence was observed in negative control NEJs (3 and 24 h post-excystment) probed with pre-immune sera but was not considered relevant (Fig. 15, panels 1A and 2A).

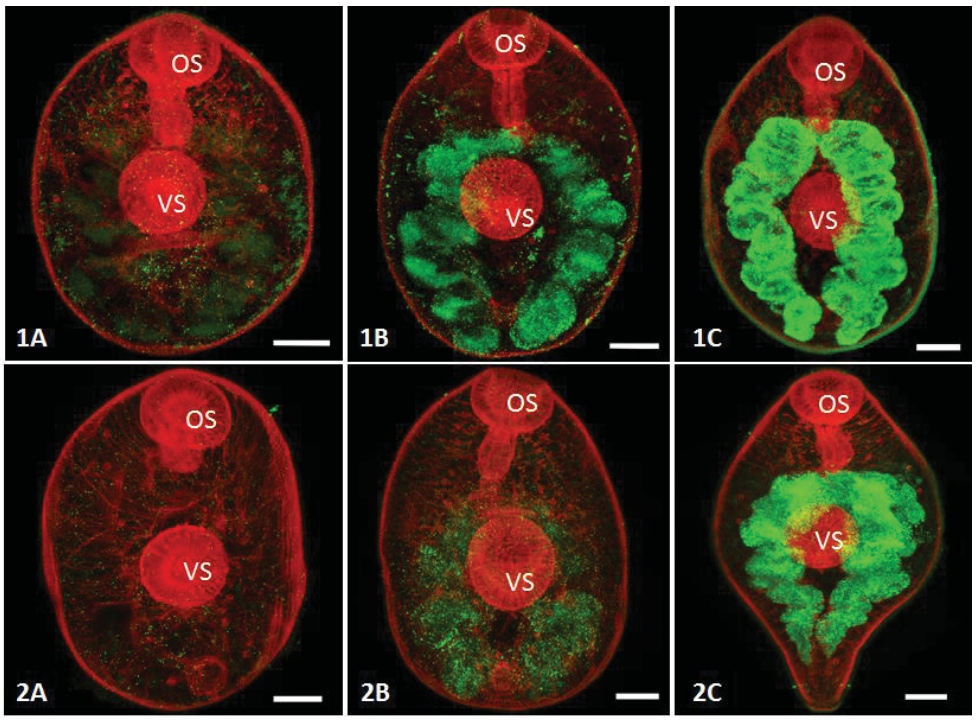


Figure 15. **Immunolocalization of ppFhCL3 and FhCL3 in NEJs 6 and 24 h post-excystment by confocal scanning laser microscopy.** Fixed NEJ's were probed with either rabbit pre-immune antiserum (1A, 2A), anti-ppFhCL3 (1B, 2B) and anti-FhCL3 polyclonal antibodies raised in rabbit (1C, 2C) followed by secondary antibody fluorescein isothiocvanate (FITC)-labelled goat anti-rabbit IgG. The expression of ppFhCL3 and FhCL3 were compered in different time points post-excystment, 6 h (1A, 1B and 1C) and 24 h (2A, 2B and 2C). All specimens were counter-stained with phalloidin-tetramethylrhodamine isothiocyanate (TRITC) to stain muscle tissue (red fluorescence) and provide structure. *OS*, oral sucker. *VS*, ventral sucker. *Scale bars*, 20 μM.

4. Discussion

The cathepsin L proteases are among the major molecules secreted during the various stages of the *F. hepatica* life cycle (Robinson et al., 2008), and these enzymes have been implicated in several processes, including invasion, migration, establishment of infection and parasite survival (Dalton et al., 2006; Corvo *et al.,* 2009). The strict temporal expression of different cathepsins throughout *Fasciola* development suggests the specialization of these proteases, reflected in the differential ability of NEJs and adult worms to penetrate host tissues, evade host immune response and degrade haemoglobin, for instance. Accordingly, while FhCL1 is highly expressed by adult worms, FhCL3 is

only found at NEJ stage (Robinson *et al.,* 2009), and due to the pivotal function attributed to these enzymes during fascioliasis, it is reasonable that specific inhibitors able to disturb their activity might lead to the development of an novel means of parasite control.

Although recently propeptides from human cathepsins have received a lot of attention, specially concerning to the development of specific inhibitors for HsCK, an enzyme associated with bone reabsorption during osteoporosis disease (Tabatabaei-Malazy et al. 2017), there are few studies exploring the importance of propeptides from parasites, including *F. hepatica* cathepsins. Certainly, previous studies have shown the potential application of these molecules as candidates for novel immunoprophylatic and chemotherapeutic strategies. Roche et al. (1999), for instance, demonstrated the specificity and potency of the propeptide from FhCL1 as an inhibitor of FhCL1 enzyme, but not for FhCL2, two enzymes that share 90% similarity in their amino acid sequence. Here, we demonstrated that the ppFhCL3 is a highly potent and selective *F. hepatica* CLs inhibitor, an activity clearly dependent on the pH of the environment (Fig 6).

The ppFhCL3 is an equipotent inhibitor of FhCL1, FhCL2 and FhCL3, a characteristic demonstrated by the *Ki* values that we obtained for the inhibition of FhCL1 (0.04 nM), FhCL2 (0.004 nM), and FhCL3 (<0.002 nM) (Table 3). Similar results were also verified by Guay and colleagues (2000), which showed the low selectivity of propeptides when human cathepsin L subfamily of papain-like cysteine proteases (cathepsins S and K) were screened against the propeptides from each enzyme. It is worth mentioning that the ppFhCL3 shows minor inhibitory effects towards human cathepsins, HsCL and HsCK, to which the *Ki* obtained were respectively 665 and 50-fold higher when compared to the value observed for FhCL1.

Here, the selectivity of the ppFhCL3 was analysed by screening of its inhibitory activity against a panel of serine and cysteine proteases derived from various organisms, including *F. hepatica*, *S. mansoni* and humans. The evidence of little or no inhibition of *F. hepatica* CBs, as well as *S. mansoni* CLs and CBs, HsCL, HsCK, and serine proteases, highlight the ppFhCL3 selectivity, which is likely be related to the way the propeptides bind to the mature enzymes. This propeptide-enzyme association is known to involve non-covalent bonds in a mechanism and structural basis that has been extensively investigated because their elucidation could lead to the development of a selective, small and reversible inhibitor (Fox *et al.,* 1995; Carmona *et al.,* 1996). In this context, our study

is the first to explore and compare the inhibitory properties of the ppFhCL3 against cathepsins from different organisms.

The observed selectivity of the ppFhCL3 within a subfamily requires a detailed structural interpretation to elucidate the mechanism of interaction. In this regard, the three-dimensional analyses of the propeptide interaction with different cathepsins provided the framework essential for the identification of areas and even residues in the prosegment that might contribute to the interactions. The 3-D model of the FhCL3 was built based on the FhCL1 crystal structure that was resolved by our laboratory, enabling us to study the ppFhCL3-FhCL interaction and compare those amino acids that are possibly participating in this binding with FhCL1, FhCL2, FhCL3, HsCL and HsCK (Table 2). Ultimately, this analysis led us to choose precise variation in the propeptide structure that could be used to explain its mechanism of inhibition. Three variants, involving the alteration of four residues, were generated, including a double mutation applied in the portion of the propeptide that was predicted to interact with the PBL in the mature enzyme, whilst the other two variants involved changes in amino acids predicted to interact directly with the active site of the proteases (Table 2).

Two of the propeptides generated clearly displayed a different inhibitory activity compared to the wild type (Table 3). The double amino acid mutation, ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47}, results in an abrogated inhibitory activity of the propeptide towards FhCL2 and FhCL3 enzymes, possibly by interfering with the unique way in which it sits in the mature enzyme forcing its extended part into the substrate binding cleft. This is the first time that the importance of specific residues located within the N-terminal portion of propeptide are shown. Coulombe et al. (1996) and Guo et al. (2000) have demonstrated that in the cathepsin L-like subfamily, when the C- and the N-terminal part of the propeptide are considered, the latter exerts a greater influence on the selectivity of the inhibition. The extreme N-terminus of these region contains a crossed helices that contributes to stabilising the backbone of the prosegment during the binding.

Similar importance to the residue p66 was found in our results, since the variant ppFhCL3Leu^{66}/Gly^{66} lost its ability to inhibit FhCL2 and FhCL3 enzymes. When compared to the results obtained with the ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47}, this finding suggests that the substitution of the Leu66p located within the region of the ppFhCL3 that interacts with the active site has an major importance to the propeptide inhibitory activity. Potentially the effect of this mutation is related to the inability of the glycine, an small

hydrophilic amino acid, to interact properly with hydrophobic residues present in the active site of FhCL2 and FhCL3. This is the first time that this change has been made and hence this is a novel result.

Groves and colleagues (1998) suggested that the differential charge distribution observed in different mature enzymes should be considerable in the propeptide-enzyme specific recognition. In their work the C-terminal extended portion of the propeptide apparently does not contribute substantially to the binding and inhibition of the cognate enzyme, but provides compensating charges to those residues contacting the enzyme surface and therefore could contribute to the specificity. Indeed, with regard to the third variant created, ppFhCL3Glu^{68}/Arg^{68}, the molecular modelling analysis demonstrated that the E68p, a negative amino acid, would interact with amino acid D275 in the mature HsCL, which would result in an effect of repulsion that could explain the low inhibitory activity of the ppFhCL3 towards the human enzyme. In order to verify this effect, we changed this residue for an Arginine, which is positively charged. In contrast of what was expected, this variant did not result in a significant change in the inhibitory activity, since the *Ki* value demonstrated only a 5-fold less inhibition towards the HsCL when compared to the ppFhCL3.

Together, the results of our substitutions were able to demonstrate the key importance of the residues Tyr46p, Lys47p and Leu66p, in the binding and inhibitory activity of the propeptide towards FhCL2 and FhCL3 enzymes, since that to replace 46p, 47p or 66p residues equally annul the inhibition. Therefore, for the first time we demonstrate that a 'clamp' mechanism is required for the interaction between propeptide and mature cathepsins, having identified two parts of the clamp, one that clips inside (66p) and other outside (46,47p) of the active site of the mature enzyme. Our findings indicate that the clamp is required for the correct siting and orientation of the prosegment over the enzyme. Previously, Coulombe et al. (1996) generated truncated HsCL propeptides by removing sequential residues of different regions of it. The variant 21p-95p showed a *Ki* of 11.5 nM and the variant 52p-95p, 2900 nM, demonstrating that the presence of the α-2 and α-3 helices are required for the proper propeptide activity as inhibitor. However, because in their truncated versions still have the equivalent residues 46p, 47p and 66p they could be contributing to the residual activity observed in their variants.

It is worth noting, that all three variants here studied retained their inhibitory activity against the FhCL1 enzyme (Table 3). Even though this is an intriguing result, it

could indicate that the residues 46p, 47p and 66p have central importance to the selectivity of the ppFhCL3 towards different cathepsins L. The different inhibitory profile of the variants could be explained by structural features of the FhCL1, FhCL2 and FhCL3. Indeed, previously it was demonstrated that FhCL1 and FhCL3 enzymes are very different, sharing only 50% of the cleavage sites (Corvo *et al.,* 2013). Moreover, phylogenetic analyses previously reported by Robinson et al. (2008) raises the hypothesis that the similar inhibition of the FhCL2 and FhCL3 by the ppFhCL3 may be explained by the clades distribution of these enzymes in the phylogenetic tree. These enzymes are closely related in the analysis indicating a higher degree of identity between them, than between FhCL1 and FhCL3, for instance (Fig 2).

According to crystal structures of the HsCK, Guay et al. (2000), considered that the majority of the interactions between the prosegments and human cathepsins involve residues located between Thr55p and Leu78p, and indicated that these amino acids are responsible for the selective inhibition. In contrast, our studies indicate that the residues Tyr46p and Lys47p seems to stronger contribute to the selectivity towards FhCL1 enzyme, while Glu68p, for instance, doesn't significantly modify the inhibition of the ppFhCL3.

In order to verify if the region comprising between 45p and 78p of the ppFhCL3 could itself bind and inhibit cathepsins as the whole propeptide does we chemically synthesized and tested a peptide 33 amino acids long (45p-78p) (Fig 1A). The results of the inhibitory assays shown that the peptide is still able to exert some inhibitory activity towards FhCL1, FhCL2 and FhCL3, at a considerable low concentration (2.5 nM), although it is not comparable to the potency presented by the ppFhCL3. Short peptides derived of cathepsins have been tested as potential inhibitor with no success (Guay *et al.,* 2000; Coulombe *et al.,* 1996), which could be a consequence of the synthetic production, lack of correct folding and even their use as substrate by the enzyme, since it is not possible to predict how these molecules are going to interact with the mature enzyme.

The invasive stage of the parasite, NEJ, expresses and secretes cathepsins that were previously associated with its ability to penetrate and migrate through the host tissues, and are therefore considered potential vaccine targets due the possibility to block *F. hepatica* infection prior the liver damage (Zawistowska-Deniziak *et al.,* 2013). Taking into consideration that FhCL3 is the main cathepsin secreted by NEJ, and that the secretion may variate according the time post-excystment of the NEJ, here we also

analysed the inhibitory activity of the ppFhCL3 and variants against the ES extract from NEJ 3 and 24 hrs post-excystment. It is important to highlight that no previous activation of the extracts was required for the enzymatic assays. Our results revealed strong cathepsin activity in the NEJ extracts analysed, which were almost completely abrogated when ppFhCL3 was added to the reaction (Fig 13). A less evident inhibition was observed by adding the variants ppFhCL3Leu^{66}/Gly^{66} and ppFhCL3Glu^{68}/Arg^{68}, while the ppFhCL3Tyr^{46}Lys^{47}/Ala^{46}Ala^{47} was not able to inhibit the activity of the extracts, supporting the data obtained with the recombinant enzymes. Moreover, it is important to notice that the residual activity verified in the extracts could be due the presence of cathepsin B's, which the ppFhCL3 are not able to inhibit according our analysis.

Enzymatic and pulldown experiments using ES extract from adult worms confirmed that the ppFhCL3 is also able to bind and inhibit native FhCL1, FhCL2, and even FhCL5 enzymes, consistent with the complete abrogated enzymatic activity in the extract when the propeptide was added. Furthermore, the identity of the enzymes that the propeptide binds to in the ES extract were confirmed by mass spectrometry; this approach identified three proteins, FhCL1, FhCL2 and FhCL5, that were pulled down from the extract by the ppFhCL3 (Fig 12A, B and C). These data are in agreement with the previous analysis of this ES extract from adult *F. hepatica* (Robinson *et al.,* 2009).

Furthermore, in order to understand how the FhCL3 is being produced and secreted-excreted by the NEJs we performed experiments of differential immunolocalization in NEJ 6 and 24 hrs post-excystment, using antibodies prepared against FhCL3 and ppFhCL3 (Fig 15). The presence of a stronger fluorescent signal in the gut of NEJ 6 hrs post-excystment than in NEJ 24 hrs, for those probed with anti-FhCL3 and ani-ppFhCL3, strongly suggests that FhCL3 is being rapidly produced and secreted-excreted from the parasite gut. Indeed, immunolocalization studies have been used to investigate the functions and localization of related *F. hepatica* cathepsins. For instance, FhCB2 was identified in the caecal epithelium of metacercariae, NEJ and 2 week-old juveniles suggesting their involvement in digestion of host connective tissues and evasion of the host immune responses during the parasite penetration and migration (Chantree *et al.,* 2012). Another study showed that the CLs are present in the cytoplasm and lamellae of the caecal-epithelial cells, when stimulated by the presence of food, for nutrient digestion in adult worms (Meemon *et al.,* 2010).

Our immunolocalization results, together with the evidence that the cathepsins present in ES extracts from NEJ are already active, suggest that the NEJ's are secreting the FhCL3 already processed from the zymogen to the active mature form into the host environment, which is line with the need of these parasite stages to penetrate through the intestinal mucosa in up to 72 hrs (Kendall and Parfitt, 1962). Considering the already described collagenolytic activity of FhCL3 (Robinson *et al.,* 2011), our data reaffirm the central involvement of this enzyme in the NEJ migration towards the parenchyma of the host.

In summary, our study revealed that the ppFhCL3 is a highly potent and selective *F. hepatica* CL's inhibitor. Additionally, here we describe for the first time a clamp mechanism essential for the ppFhCL3 and FhCL3 and FhCL2 interaction, two enzymes that are known to have a collagenolytic activity that is fundamental during *F. hepatica* invasion of host tissue. The clamp involves residues of the propeptide that interact with the mature enzyme in regions outside (Tyr46p, Lys47p) and inside the active site (Leu66p), and are both essential for the propeptide as an inhibitor and regulator of cathepsin L activity. In addition, change to these residues interferes with the selectivity of the ppFhCL3. Together our results give remarkable new insights regarding to propeptide-cathepsin interaction and open up the possibility of exploring these features to design new and selective inhibitors for *F. hepatica* cathepsins.

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APPENDIX A

Buffer and medium Recipes

Luria-Bertani (LB) agar

1% Lb

0,75% agar

LB

1% LB

ST buffer

150 mM NaCl

10 mM Tris-HCl

Solution adjusted to pH 8.0 with NaOH

Lysis buffer

50 mM NaH_2PO_4

300 mM NaCl

5mM imidazole

Solution adjusted to pH 8.0 with HCl

Wash buffer

50 mM NaH_2PO_4

300 mM NaCl

10 mM imidazole

Solution adjusted to pH 8.0 with HCl

Elution buffer

50 mM NaH_2PO_4

300 mM NaCl

250 mM imidazole

Solution adjusted to pH 7.0 with HCl

Sodium acetate buffer

100 mM sodium acetate

1 mM DTT

1 mM EDTA

0.01% Brij L23

Solution adjusted to pH 7.0 with HCl to complete 100 mL

Activation CLs buffer

100 mM sodium acetate

500 mM DTT

500 mM EDTA

0.01% Brij L23

Solution adjusted to pH 4.5 with HCl to complete 100 mL

Activation CBs buffer

100 mM sodium acetate

10 mM DTT

1 mM EDTA

200 mM NaCl

50 μg/mL dextran sulphate

Solution adjusted to pH 4.5 with HCl to complete 100 mL

ADDENDUM 2. Academic production.

1. Published papers:

- Santos C., Campestrini L.H., **Pritsch I.**, Yamassaki F.T., Zawadzki-Baggio S.F., Maurer J.B., Molento M.B. (2018). Chemical characterization of the *Opuntia ficus-indica (L.) Mill.* hydroalcoholic extract and its efficiency against gastrointestinal nematodes of sheep. Veterinary Sciences, 5 (3), 1-13.
- **Pritsch, I**. and Molento, M.B. (2018). Recount of reported cases of human fascioliasis in brazil over the last 60 years. Revista de Patologia Tropical, 47(2), 75-86.
- Molento, M. B., Bennema, S., Bertot, J., **Pritsch, I. C.**, & Arenal, A. (2018). Bovine fascioliasis in Brazil: Economic impact and forecasting. Veterinary Parasitology: Regional Studies and Reports, 12, 1-3.
- Bennema, S. C., Molento, M. B., Scholte, R. G., Carvalho, O. S., & **Pritsch, I**. (2017). Modelling the spatial distribution of *Fasciola hepatica* in bovines using decision tree, logistic regression and GIS query approaches for Brazil. Parasitology, 144(13), 1677-1685.
- Trevisan, L., **Pritsch, I. C.**, & Molento, M. B. (2017). Parasitological analysis of cress (*Nasturtium officinale, Barbarea verna* e *Lepidium sativum*) samples from Curitiba Area, Brazil. Archives of Veterinary Science, 22(3), 50-56.

2. Published technical papers:

- Marcelo Beltrão Molento e **Izanara Cristine Pritsch**. *Fasciola hepatica* em bovinos no Brasil: uma doença negligenciada. Jornal Agroin Comunicação. 2018. http://www.agroin.com.br/noticias/11960/*Fasciola-hepatica*-em-bovinos-no-brasil-uma-doenca-negligenciada
- Marcelo Beltrão Molento, **Izanara Cristine Pritsch** e Laura Trevisan. *Fasciola hepatica* e qualidade do leite em bovinos no estado do Paraná. Artigos científicos, Associação Paranáense de Criadores de Bovinos da Raça Holandesa do Paraná. 2017. https://www.apcbrh.com.br/artigo/artigos-cientificos

3. Accepted manuscripts

- Krystyna Cwiklinski, Sheila Donnellya, Orla Drysdale, Heather Jewhurst, David Smith, Carolina De Marco Verissimo, **Izanara C. Pritsch**, Sandra O'Neill, John P. Dalton, Mark W. Robinson. The cathepsin-like cysteine peptidases of trematodes of the genus *Fasciola*. Review. Advances in Parasitology.

4. Submitted manuscripts

- **Izanara Cristine Pritsch**, Emanoelli Cristini Augustinhak Stanula, Alan dos Anjos, Marcelo Beltrão Molento. Fascioliasis in Water Buffaloes: A 5-year forecast analysis of the disease based on a 15-year National survey in Brazil. Original Paper. Revista Brasileira de Parasitologia Veterinária.
- **Izanara Cristine Pritsch**, Ricardo Reis Schwendler, Magda Rejane Bordin Buttendorf, Raquel Garcia, Marcelo Beltrão Molento. Human fascioliasis by *Fasciola hepatica*: the first case report in Santa Catarina, Brazil. Case Report. Revista da Sociedade Brasileira de Medicina Tropical.
- Ana Isabella Iura Schafaschek, Thales Baggio, Anibal Moraes, Alexandre Fillus, Lucas Bochnia Bueno, André Guaraldo, **Izanara Cristine Pritsch**, Marcelo Beltrão Molento. Occurrence of *Haematobia irritans* in cattle in different livestock production systems. Original Paper. Veterinary Parasitology: regional studies and reports.

5. Manuscripts in preparation

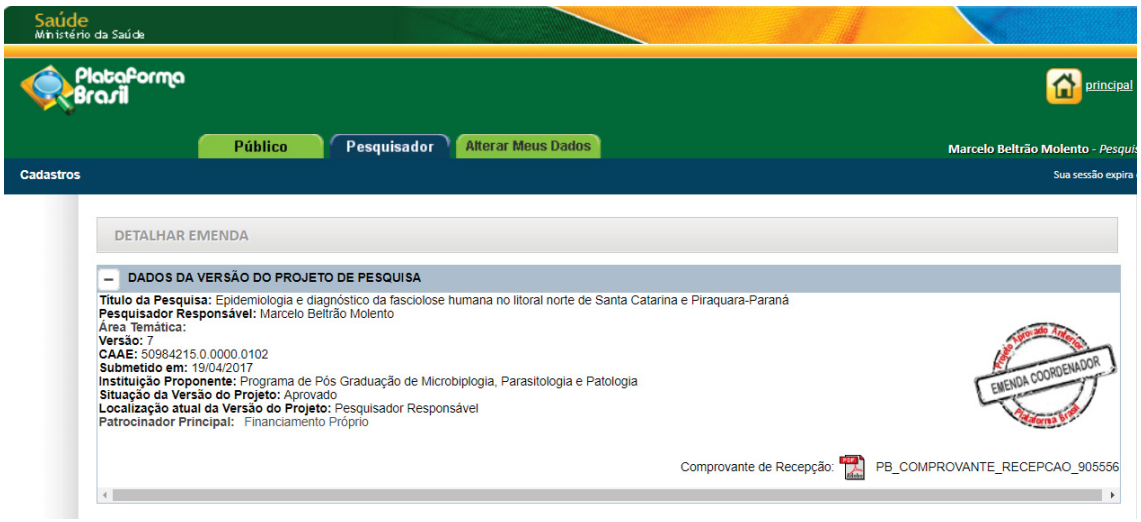
- **Izanara Cristine Pritsch**, Laura Trevisan, André Guaraldo, Grace Mulcahy, John Dalton, Rodrigo Almeida, Marcelo Beltrão Molento. Association between anti-*Fasciola hepatica* antibody levels in bulk tank milk and production parameters in dairy cows in Brazil. Original Paper. Parasitology.
- **Izanara C. Pritsch**, Irina G. Tikhonova, Heather L. Jewhurst, Orla Drysdale, Krystina Cwiklinski, Marcelo Beltrão Molento, John P. Dalton, Carolina De M. Verissimo. A propeptide 'clamp' mechanism is required for inhibition of *Fasciola hepatica* collagenolytic cathepsin L3. Original Paper. Journal of Biological Chemistry, 2019.
- M. B. Molento, L. H. Dutra, **I. C. Pritsch**, A. G. Pereira, A. Gavião, A. Pereira, R. S. de Sousa, J. G. A. Viana. Histopathological and quantitative findings in the liver and economic impact of *Fasciola hepatica* infection in cattle. Original Paper.

- Lucas Bochnia Bueno, Andreia Buzatti, Bruno Batista Bortoluzzi, Maria Chistine Rizzon Cintra, **Izanara Cristine Pritsch,** Lisando Pacheco-Lugo, Wanderson Duarte DaRocha, Marcelo Beltrão Molento. Silencing of the genes glc-5, DIM-1, GAPDH, GDH and β-tubulin in *Haemonchus contortus*. Original Paper.

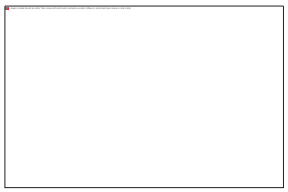
6. Published abstracts

- **Pritsch, I.C**.; Stanula, E.C.A.; Dolenga, C.J.R.; Molento, M.B. Prevalência de fasciolose em búfalos abatidos no brasil nos últimos 15 anos. XX Congresso Brasileiro de Parasitologia Veterinária. Londrina, Paraná. 2018.
- **Izanara C. Pritsch**, Irina G. Tikhonova, Heather L. Jewhurst, Orla Drysdale, Krystina Cwiklinski, Marcelo Beltrão Molento, John P. Dalton, Carolina de M. Verissimo. A propeptide 'clamp' mechanism is required for inhibition of *Fasciola hepatica* collagenolytic cathepsin L3. The British Society For Parasitology. Wales, United Kindom, 2018.
- **Izanara Cristine Pritsch**, Laura Trevisan, Grace Mulcahy, John Dalton, Marcelo Beltrão Molento. Prevalence of *Fasciola hepatica* in dairy cattle in the state of Paraná, Brazil. International Conference of the World Association for the Advancement of Veterinary Parasitology. Malaysia. 2017.
- **Izanara Cristine Pritsch**, Laura Trevisan, Cristina Mayumi, Andreas Lazaros Chryssafidis, John Dalton, Grace Mulcahy, Marcelo Beltrão Molento. Padronização e validação da técnica de ELISA para detecção de anticorpos anti-*Fasciola hepatica* em soro de sangue e leite bovino. XIX Congresso Brasileiro de Parasitologia Veterinária. Belém, Pará. 2016.

ADDENDUM 3. Research Project at Plataforma Brasil website - Ethics Committee.



ADDENDUM 4. Protocol for diagnosis/suspected human fascioliasis.



Ministério da Educação
Universidade Federal do Paraná
Setor de Ciências Agrárias
Departamento de Medicina Veterinária
Laboratório de Doenças Parasitárias

Protocolo para diagnóstico/suspeita de fasciolose

Exames laboratoriais auxiliares:

- Hemograma: eosinofilia
- TGO/TGP: TGP aumentado
- Gama GT: alterado
- Bilirrubina total: alterada

Outros exames:
*Presença do parasito no fígado e/ou ductos biliares

- Ultrassom abdominal: suspeito/sugestivo
- Tomografia computadorizada com contraste: suspeito

Sintomas que podem ser evidenciados:

- Dor abdominal
- Anemia e icterícia
- Hepatomegalia

Fase crônica da doença (após 12 semanas da infecção):

- Colestase
- Colangite esclerosante secundária

Observações:
A ausência de quaisquer alterações acima citadas não indica que o paciente não esteja com a doença, pois as manifestações clínicas variam muito entre os indivíduos. No entanto, os mesmos podem levar a suspeita quando associado a anamnese do paciente em relação ao consumo de vegetais crus (principalmente agrião/rúcula), que são a principal forma de transmissão da fasciolose.
Em caso de dúvidas contate o Laboratório de Doenças Parasitárias da UFPR. Após a suspeita clínica o paciente deverá ser encaminhado para o Laboratório Municipal de Piçarras para a coleta de sangue a assinatura do termo de consentimento livre e esclarecido (TCLE). A amostra será encaminhada para diagnóstico confirmatório pela técnica de ELISA.

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