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Journal and vol/article ref: jhl JHL1600056

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Q1 The distinction between surnames can be ambiguous, therefore to ensure accurate tagging for indexing purposes online (eg for PubMed entries), please check that the highlighted surnames have been correctly identified, that all names are in the correct order and spelt correctly.

Q2 1985 changed to 1995 to agree with the article title in the Reference list. OK?

Q3 A similar study in 2005 showed that almost 25% of cattle livers were condemned due to liver fluke in Peruvian abattoirs, with values up to 80% in certain regions. Should this be followed by a reference?

Q4 'outmost' changed to 'great' – OK?

Q5 'While it is clear…life cycle' Are the suggested changes here OK?

Q6 'There are reports of infection of the European deer (*Cervus elaphus*) in southern Argentina (Larroza & Olaechea, 2010) and the wild Pampas deer (*Ozotoceros bezoarticus*) in Uruguay (Hernandez & Gonzalez, 2011)' Are the suggested changes here OK?

Q7 'at houses' changed to 'in homes' – OK?

Q8 *Viscacia* here; *viscaccia* in the Reference list. Which is correct?

Q9 Should 'fractionary' be 'fragmented'?

Q10 'sensible' changed to 'sensitive' – OK?

Q11 Please give DILAVE in full.

Q12 'high drug pressure' changes to 'high drug selection pressure'. OK?

Q13 'to contrast this view' changed to 'to be opposed to this view' – OK?

Q14 parasite.wormbase.org – please check that this url is correct.

Q15 Has RNAi been explained correctly?

Q16 'might provide answers to these needs' changed to 'might provide some answers' Is this OK?

Q17 GST – please confirm that this is glutathione S-transferase (explained above).

Q18 'parenchyma' changed to 'liver parenchyma' – OK?

Q19 'analysis' changed to 'mass spectrometry' – OK?

Q20 'Interestingly *Fh*LAP and its orthologues from other while the M17 phylogenetic analysis demonstrates that all metazoan M17 LAPs fall into three well-defined clusters.' Changed to 'The M17 phylogenetic analysis demonstrates that all metazoan M17 LAPs fall into three well-defined clusters.' OK?

Q21 'possibilities' changed to 'potential' – OK?

Q22 'before being introduced' Can this be changed to 'which are then introduced'?

Q23 GSH is now explained at first mention. OK?

Q24 (TR) added here. Is this correct?

Q25 Please write 'NEJs' in full.

Q26 'lead target' – perhaps this should be 'prime target'?

Q27 'using' changed to 'following the use of' – OK?

Q28 Please check that the suggested changes to the Conclusions are acceptable.

Q29 Please provide details of financial support, together with grant numbers, if appropriate. If not, please state: This research received no specific grant from any funding agency, commercial or bnot-for-profit sectors.

Q30 Please declare any conflict of interest. If there are no interests to declare, please state 'None.'.

Q31 Alvarez Rojas, C.A., Jex, A.R., Gasser, R.B. & Scheerlinck, J.P.Y. (2014) Techniques for the diagnosis of Fasciola infections in animals. Room for improvement. 1st edn. Elsevier. Please give the place of publication.

Q32 Brennan, G.P., Fairweather, I., Trudgett, A., Hoey, E., McCoy, McConville, M., Meaney, M., Robinson, M., McFerran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G. & Brophy, P.M. There are no initials for McCoy. Please advise.

Q33 Canevari, J., Ceballos, L., Sanabria, R., Romero, J., Olaechea, F., Ortiz, P., Cabrera, M., Gayo, V., Fairweather, I., Lanusse, C. & Alvarez, L. (2014) Testing albendazole resistance in Fasciola hepatica: validation of an egg hatch test with isolates from South America and the United Kingdom. Journal of Helminthology 88, 286–92 This reference has been updated according to information in PubMed. The text citations have been changed from 2013 to 2014. OK?

Q34 Bol. Acad. C. Fís., Mat. y Nat. Please give this journal title in full.

Q35 Rev Inv Vet Peru Please give this journal title in full.

Q36 Ann Fac. Vet. (Uruguay) Please give this journal title in full.

Q37 Hodgkinson, J., Cwiklinski, K., Beesley, N.J., Paterson, S. & Williams, D.J.L. (2013) Identification of putative markers of triclabendazole resistance by a genome-wide analysis of genetically recombinant Fasciola hepatica. Parasitology 140, 1523–1533 Volume and page range added. Are these OK?

Q38 INIA-CIID – please give in full. Please give the place of publication.

Q39 collected in Huayllapampa, San Jerónimo, Cusco, Peru Galba. Should 'Galba' be deleted here?

Q40 Rev Vet Please give journal title in full.

Q41 13/17 changed to 13–17. OK?

Q42 Ortiz, P., Scarcella, S., Cerna, C., Rosales, C., Cabrera, M., Guzmán, M., Lamenza, P. & Solana, H. (2013) Resistance of Fasciola hepatica against triclabendazole in cattle in Cajamarca (Peru): a clinical trial and an in vivo efficacy test in sheep. Veterinary Parasitology 195, 118–121 Volume and page range added. Are these OK?

Q43 Rojas, J. de D. (2012) Resistance of Fasciola hepatica to triclabendazole in cattle of the Cajamarca countryside. Revista Veterinaria Argentina 1–6. Please give volume and check the page range.

Q44 Rev. Ibero-Latinoam.Parasitol Please give this journal title in full.

Q45 Rev.Brasil.Parasitol.Vet. Please give this journal title in full.

Q46 Spithill, T.W., Carmona, C., Piedrafita, D. & Smooker, P.M. (2012) Prospects for immunoprophylaxis against Fasciola hepatica (Liver Fluke). pp. 465–484 in Caffrey, C.R. (Ed.) Parasitic helminths: Targets, screens, drugs and vaccines. Weinheim, Germany, Wiley. The editor, publisher and place of publication have been added. Please check that the changes are OK.

Q47 Teofanova, D., Hristov, P., Yoveva, A. & Radoslavov, G. (2012) Issues associated with genetic diversity studies of the liver fluke, Fasciola heptica (Platyhelminthes, Digenea, Fasciolidae). pp. 251–274 in Caliskan, M. (Ed.) Genetic diversity in microorganisms. InTech Please give the place of publication.

Journal of Helminthology (2016) **0**, 1–11
doi:10.1017/S0022149X16000560

Fasciolosis in South America: epidemiology and control challenges

Q1 **C. Carmona[1] and J.F. Tort[2]***

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(Received 23 April 2016; Accepted 29 July 2016)

Abstract

Fasciolosis caused by *Fasciola hepatica* severely affects the efficiency of livestock production systems worldwide. In addition to the economic impact inflicted on livestock farmers, fasciolosis is an emergent zoonosis. This review emphasizes different aspects of the disease in South America. Available data on epidemiology in bovines and ovines in different countries, as well as a growing body of information on other domestic and wildlife definitive hosts, are summarized. The issue of drug resistance that compromises the long-term sustainability of current pharmacological strategies is examined from a regional perspective. Finally, efforts to develop a single-antigen recombinant vaccine in ruminants are reviewed, focusing on the cases of leucine aminopeptidase or thioredoxin glutathione reductase.

Fasciolosis as a zoonotic disease in South America

Fasciolosis is the parasitic infection caused by the two related but different liver-fluke species *Fasciola hepatica* and *Fasciola gigantica*. Both are responsible for massive economic losses affecting cattle and sheep farmers, estimated globally to be US$3.2 billion (Spithill *et al*., 1999). This negative impact is related to impaired energy conversion and anaemia in chronically infected animals, leading to a reduction in meat, milk and wool output, as well as fertility. Infected ruminants also suffer from impaired 'draft power' that impacts on production of crops, particularly rice (Kaplan, 2001; Charlier *et al*., 2014b).

Of the two species involved, *F. hepatica*, is widely distributed in all continents, while *F. gigantica* is found in tropical climates, with a more focal distribution in Africa, the Middle East, and South and East Asia. It has been calculated that there are more than 700 million animals at risk of infection (Spithill *et al*., 1999). Moreover, fasciolosis caused by *F. hepatica* is currently recognized by WHO as an emerging zoonosis in 51 countries, with 2.4 million estimated human cases and 180 million persons at risk of infection, mostly in South America and Africa. In South America the disease is endemic in Bolivia, Peru and Ecuador; sporadic cases are reported in the remaining countries (Mas-Coma *et al*., 2005; World Health Organization, 2007). A high prevalence (15–66%) of human liver-fluke infection has been described in Bolivia and Peru (Mas-Coma *et al*., 1999), with highest levels of human fasciolosis hepatica found amongst the indigenous Aymaran people in the Lake Titicaca Basin, particularly in children (Parkinson *et al*., 2007).

In the present review we examine different aspects of the epidemiology and control of fasciolosis in South American livestock. Advances in the diagnosis of *F. hepatica* infection in ruminants have not been included, since excellent reviews covering this issue have been published recently (Alvarez Rojas *et al*., 2014; Charlier *et al*., 2014a). In the region, serological and coprological approaches are being applied in human cases, but most of the data on prevalence in livestock rely on traditional egg-count methods and/or liver condemnation. Very recently,

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polymerase chain reaction (PCR) detection of liver-fluke DNA in faeces has been tested successfully (Carnevale *et al.*, 2015), while novel 'field friendly' loop-mediated isothermal amplification (LAMP) approaches (Martínez-Valladares & Rojo-Vázquez, 2016) have not yet been tested in the region.

Fasciolosis is endemic in areas dedicated to breeding cattle and sheep in most of the South American countries. Prevalence studies either using coprology or data from slaughterhouses have focused mainly on bovines. In northern Argentina an age-related analysis found prevalences ranging from 4.8% in animals aged from 12 to 18 months up to 77.0% in animals older than 5 years (Moriena *et al.*, 2004). Very high prevalences in cattle were registered in the northern Bolivian altiplano around La Paz, an area characterized by the highest levels of human infection ever recorded (Mas-Coma *et al.*, 1999). A retrospective study of liver condemnation at Chilean abattoirs between 1989 and 1995 found that 30.1% of bovine and 2.1% of sheep livers were positive for *F. hepatica* (Morales *et al.*, 2000), and human cases are emerging (Gil *et al.*, 2014). A similar study in 2005 showed that almost 25% of cattle livers were condemned due to liver fluke in Peruvian abattoirs, with values up to 80% in certain regions. High endemic foci of human fasciolosis are also found in the Andean valleys, particularly in Cajamarca, an area characterized by over 60% incidence in dairy cattle (Espinoza *et al.*, 2010; Ticona *et al.*, 2010). Uruguay, an agriculturally based country, has a population of 11.4 million cattle (the highest number of cattle per habitant) and 8.2 million sheep. In addition, meat and sheep farming occupy 60% of the land. Not surprisingly, fasciolosis is one of the most relevant parasitic infections in livestock, present in most of the territory. A recent serological study in the Salto Department showed 67% of positive animals, with the highest percentages in Angus cattle and those younger than 2 years (Sanchís *et al.*, 2011). Georeferenced prevalence data of *F. hepatica* in bovines were collected and mapped for the Brazilian territory during the period 2002–2011. The highest prevalence of fasciolosis was observed in the southern states, with disease clusters along the coast of Paraná and Santa Catarina and in Rio Grande do Sul (Bennema *et al.*, 2014).

A similar approach, using geographical information systems in Antioquia, Colombia, and prevalence data for the region (21%), was used to generate a national-scale climate-based risk model to forecast major transmission periods, with considerable annual differences (Valencia-López *et al.*, 2012). Clearly, these approaches could provide farmers and governmental agencies with valuable epidemiological information, with the aim of improving control strategies (Aleixo *et al.*, 2015). Altogether these data reflect the great economic importance of ruminant fasciolosis in South America.

South American natural reservoirs and the expansion of host range

It is generally assumed that the parasite arrived in the Americas with the European conquest, within the sheep, goats and/or cattle brought by the first colonizers, in the early 16th century (Mas-Coma *et al.*, 2009). Liver-fluke disease is now widespread in livestock in the continent, and can be mapped across the whole of Latin America.

While it is clear that the parasite could have travelled within the definitive host, its successful dispersion in the new lands would have depended on finding and adapting to novel snails in order to complete its life cycle (Mas-Coma *et al.*, 2005)[. Several members of the Lymnaeidae have been described as hosts, including *Lymnaea viatrix* (Nari *et al.*, 1986), *L. columella* (Pereira De Souza & Magalhães, 2000), *L.* (*Fossaria*) *cubensis* (Vignoles *et al.*, 2014), *Galba truncatula* (Iturbe & Muñiz, 2012) and *L. neotropica* (Mera y Sierra *et al.*, 2009). A recent molecular phylogeny of the Lymnaeidae showed the existence of three clades, representing their geographical origins from America, Eurasia and the Indo-Pacific region. Interestingly, while species involved in *F. gigantica* transmission are more restricted to African and Australasian species (following the general trend of trematodes for marked specificity for their intermediate host), *F. hepatica* has been reported to infect species of the three main clades (Correa *et al.*, 2010). This is a relevant difference that might underlie the success of *F. hepatica* dissemination, and should be taken into account in epidemiological control programmes, which should cover a broad spectrum of possible hosts rather than focusing on a single snail species.

Besides infecting cattle, sheep and goats, in the 500 years since its introduction the parasite has been confronted by different native species, and has been particularly efficient in gaining new hosts among native species. The South American camelids – llamas, alpacas and guanacos – the natural livestock of the Andean region, might have represented the first to be conquered, since these species would have been grazing with the introduced species. Domestic camelids are highly susceptible to liver-fluke infection, with reports of almost 60% prevalence in Bolivian alpacas (Ueno *et al.*, 1975), close to 50% in llamas and more than 70% in alpacas in the Peruvian Jauja region (Flores *et al.*, 2014), and even reaching 80% in llamas in the north of Argentina (Cafrune *et al.*, 1996). Reports of infection in wild camelids (Issia *et al.*, 2009; Larroza & Olaechea, 2010; Fugassa, 2015), despite being much lower than in farmed animals, indicate that they might be considered as reservoirs.

While camelids host liver flukes in the Andean and Patagonian regions, other wild ungulates that usually graze together with livestock, such as deer, can act as hosts to *F. hepatica* in the grasslands. There are reports of infection of the European deer (*Cervus elaphus*) in southern Argentina (Larroza & Olaechea, 2010) and the wild Pampas deer (*Ozotoceros bezoarticus*) in Uruguay (Hernandez & Gonzalez, 2011), but the extent and relevance of these species as reservoirs is still unknown. The small Pudu deer (*Pudu puda*) was also found occasionally to be infected in Chile (Bravo Antilef, 2015).

The host range has also extended to rodents, with reports of infection of capybaras (*Hydrochoerus hydrochaeris*) in Venezuela, Argentina, Brazil and Uruguay (Freyre *et al.*, 1979; Santarem *et al.*, 2006; El-Kouba *et al.*, 2008; Alvarez *et al.*, 2009; Cañizales & Guerrero, 2013; Fugassa, 2015), but the status of this species is still largely unknown. A more consistent role as reservoir could be assigned to the coypu (*Myocastor coypus*) (Silva-Santos *et al.*, 1992; Ménard *et al.*, 2001; Issia *et al.*, 2009; Gayo *et al.*, 2011;

Fugassa, 2015). This species has been introduced into Europe and it has been reported that almost 40% of the animals from an area where *F. hepatica* exists in livestock are infected and produce infective eggs (Ménard *et al.*, 2001). While the initial reports from Brazil showed lower incidences (Silva-Santos *et al.*, 1992), a more recent study in a Natural Reserve of Argentina showed that all specimens were infected (Issia *et al.*, 2009). The semi-aquatic habits of these herbivorous species, shared with those of the intermediate hosts, increase the probability of released liver-fluke eggs encountering suitable snails to complete the cycle.

The guinea pig (*Cavia porcellus*) is another rodent that might play a relevant role in dissemination of fasciolosis. In Peru 'cuyes' are traditionally valued for their meat, and are usually bred in homes and small family businesses. A report from the National Institute of Agriculture of Peru established *F. hepatica* as one of the parasitic infections found in this species, with a reported prevalence of 5% in farmed animals (INIA-CIID, 1991), and a similar value of 4.2% prevalence was found in wild animals (Dittmar, 2002). Vizcachas (*Lagidium viscacia*) are also known to harbour *F. hepatica* infection (Led *et al.*, 1979).

Other farm species brought to the continent by the Europeans, such as horses, pigs and mules, could have contributed to the dispersion, or acted as secondary hosts, as well as other introduced species, such as rabbits and hares (Mas-Coma *et al.*, 1997; Cuervo *et al.*, 2015).

The variety of mammals that can be hosts to *F. hepatica* highlights the enormous adaptability of the parasite. A notable extension to this was the first report of liver flukes in Aves, with the description of two cases in Australian farmed emus (*Dromaius novaehollandiae*) (Vaughan *et al.*, 1997). However, in that study only one small adult was found, and abnormal eggs were recovered, suggestive of an incomplete adaptation to birds as hosts. Two more recent reports of the liver fluke in farmed and wild populations of ñandues (*Rhea americana*) provide evidence that a notable host-range extension to Aves has indeed occurred in South America (Soares *et al.*, 2007; Martinez-Diaz *et al.*, 2013). The first of these studies describes the finding of normal adult worms and eggs in condemned livers of farmed ñandues from an endemic area of cattle and sheep fasciolosis in southern Brazilian. Furthermore, eggs were found in 4 out of 17 wild ñandues that grazed together with cattle and sheep. These eggs matured and produced swimming miracidia but their infectivity to snails was not tested (Soares *et al.*, 2007). A coprological study of ñandues across Argentina found *F. hepatica*-like eggs in the common ñandu (*R. americana*) from two farms and one wild bird, and also in Darwin's rheas (*R. pennata*) from one Patagonian farm. The latter came from a farm where two adult birds died before the sampling and, according to the owner, presented liver lesions, but unfortunately were not kept for further analysis (Martinez-Diaz *et al.*, 2013). The common ñandu usually grazes together with cattle, sheep and horses (and occasionally deer) in southern Brazil, Uruguay and the Argentinian pampas, while the lesser ñandu (*R. pennata*) is adapted to the Patagonia and altiplano regions, usually coinciding with sheep and guanacos.

This information supports the idea that when introduced to South America *F. hepatica* was able to adapt to a diversity of autochthonous grazing mammals that share ecological niches with sheep and cattle. In this sense, camelids are now probably one of the most relevant hosts to consider in the Andean region, while the role of rodents, such as guinea pigs and coypus, as reservoirs is strongly suggested. Despite the fractionary and anecdotal nature of several reports of liver flukes in South American wildlife, is evident that diverse species can host the parasite, and eventually act as reservoirs. The presence of egg-producing parasites in ñandues, raises the question whether other bird species, for example herbivorous waterfowl (chajas (screamers), swans, geese, ducks), living in endemic areas are also eventual hosts to liver flukes. Considering the migratory nature of some of these species, they might eventually contribute to the spread of the parasite. Systematic studies in this direction are clearly needed.

Control approaches

Current methods to control fasciolosis include the eradication of snails with molluscicides, grazing management, improving drainage systems to limit the habitat of the intermediate host and, most commonly, the use of anthelminthic drugs. Nevertheless, the emergence of drug resistance, the increasing concern by consumers for xenobiotic residues in the food chain and environment, and trade barriers have stimulated the search for novel control methods (Statham, 2015; Kelley *et al.*, 2016).

Emergence of drug resistance

While several drugs can be effective against adult flukes, triclabendazole (TCBZ) is also effective against immature flukes, and for that reason it is the drug of choice for the control of fasciolosis (Fairweather & Boray, 1999; Brennan *et al.*, 2007). The drug was introduced in the 1980s and the first report of resistance emerged in 1995 in Australia (Overend & Bowen, 1995), followed by reports in Europe (reviewed in Kelley *et al.*, 2016).

The first report of possible drug resistance in the Americas appeared in a sheep and goat farm in Parana State, Brazil. A liver-fluke outbreak causing animal deaths was treated with abamectin plus TCBZ, with reduced efficiency (66% in sheep and 57% in goats). The authors mention the abusive use of anthelmintics as a possible selecting force; however, TCBZ had not been administered in the past in the farm (Oliveira *et al.*, 2008).

Albendazole (ABZ) resistance was demonstrated experimentally in two flocks from La Paz, Bolivia, confirmed by sheep necropsy after treatment. While TCBZ was effective in one of the flocks, the other showed a reduced efficacy of TCBZ, with 36.6% reduction in worm burden (Mamani & Condori, 2009). A similar pattern of complete resistance to ABZ and reduced efficacy of TCBZ (with a fecal egg count reduction of close to 35% after 4 weeks) was observed in dairy cattle from the Junín region in Peru, an endemic area with a prevalence of 41% (Chávez *et al.*, 2012).

Reports of resistance to TCBZ on a cattle farm in Neuquén, Argentina were confirmed experimentally in a controlled trial (Olaechea *et al.*, 2011). A second case of

resistance was reported on a cattle and sheep farm from Entre Rios province, Argentina, where 4–5 annual treatments with different drugs were performed (mainly directed at gastrointestinal nematodes and not specifically for liver fluke). A clinical efficacy experiment in sheep showed that this isolate was resistant to ABZ but sensitive to TCBZ (Sanabria *et al.*, 2013). A sheep isolate from nearby Salto, Uruguay, maintained at DILAVE, was also resistant to ABZ and sensitive to TCBZ (Canevari *et al.*, 2014).

A more relevant focus of drug resistance has emerged in the Cajamarca region in Peru, an endemic area for cattle fasciolosis with reported prevalence up to 75% and, consequently, high drug selection pressure (Espinoza *et al.*, 2010). Confirmation of TCBZ resistance in three dairy farms by fecal egg count reduction (FECRT) following treatment was published locally (Rojas, 2012). Snails were infected with the resistant isolate, and the metacercariae obtained were used in an *in vivo* efficacy test in sheep, corroborating the resistant status (Ortiz *et al.*, 2013).

An egg-hatch assay was used to test the resistant status of several of these isolates, confirming the ABZ resistance status in the Entre Rios and the Uruguayan isolates, and indicating that the TCBZ-R Cajamarca (Peru) isolate is also resistant to ABZs, while the TCBZ-R INTA isolate from Neuquén is sensitive to ABZ (Canevari *et al.*, 2014).

Unfortunately, drug resistance has not been limited to farmed animals, but it has extended to humans, with the report of four cases in Chile (Gil *et al.*, 2014) and seven cases in the Cuzco region of Peru that did not respond to treatment with TCBZ (Cabada *et al.*, 2016). The implications of this spread are of serious concern, and this clearly emphasizes the zoonotic nature of the disease.

Genetic variation and omics approaches

Drug selection pressure might be the driving force to generate resistant parasite populations, but the molecular targets affected in each population might not be the same. A thorough isolation and characterization of the resistant strains found in the continent is warranted (Fairweather, 2011), and efforts in this direction have already started. Despite serval studies, the mechanism of action of TCBZ is still not clear (Brennan *et al.*, 2007; Kotze *et al.*, 2014). Studies of morphological and metabolic differences between susceptible and resistant strains has been reported, based on comparison of the first available well-characterized isolates of European origin (Mottier *et al.*, 2006; Solana *et al.*, 2009; Ceballos *et al.*, 2010; Hanna *et al.*, 2010; Scarcella *et al.*, 2011, 2012; reviewed in Kelley *et al.*, 2016). The search for mutations in putative target (tubulin) or effector (P-glycoprotein (PGP), glutathione S-transferase (GST)) genes has been based on European isolates (Ryan *et al.*, 2008; Wilkinson *et al.*, 2012; Fernández *et al.*, 2015), but confirmation in other isolates is needed. In fact, the PGP point mutation proposed as being associated with resistant isolates was not found to be associated with Australian isolates (Elliott & Spithill, 2014), and studies under way on some of the South American isolates have not found the variant to be associated with resistance (Solana and Tort, unpublished).

Studies of genetic diversity in the liver fluke have started to emerge, and are relevant in following the dispersal of the species and identifying and characterizing the emergence of variants with particular properties, such as drug resistance (reviewed in Ai *et al.*, 2011; Teofanova *et al.*, 2012). The genetic characterization of defined TCBZ-R populations of European and Australian origin based on mitochondrial markers (*nad*-1 and *cox*-1) showed that these populations are genetically diverse, suggesting that no 'bottleneck' occurred due to selective pressure (Walker *et al.*, 2007; Elliott *et al.*, 2014). A single, very recently published report characterizing liver flukes from Peru seems to be opposed to this view (Ichikawa-Seki *et al.*, 2016). No significant differences by host were found in the haplotypes of the mitochondrial *nad*-1 gene from cattle, sheep and pigs form the Cajamarca region, and, in general, the genetic diversity of the Peruvian flukes was low. In any case, this study highlights the need to characterize the liver-fluke variants circulating in South America.

The advent of new sequencing technologies facilitated knowledge of the genomes and transcriptomes of trematodes; in particular, the initial efforts in liver flukes concentrated on the transcriptomics and proteomics of the juvenile and adult stages (Robinson *et al.*, 2009; Cancela *et al.*, 2010; Young *et al.*, 2010). The first assembly of the *F. hepatica* genome, recently published, was surprisingly big (one-third of the human genome and almost four times bigger than that of *Schistosoma*) (Cwiklinski *et al.*, 2015a). This assembly (based mainly on UK samples) and a second one (generated mainly from US liver flukes) are now publically available in a trematode-specific database (www.trematode.net) (Martin *et al.*, 2015) and a more general worm parasite database (parasite.wormbase.org). These resources provide an essential framework for the disclosure of genes and regulatory pathways associated with drug resistance. In this sense, a genome-wide approach to map TCBZ resistance based on identifying single nucleotide polymorphisms (SNPs) in the progeny of genetic crosses between TCBZ-S and TCBZ-R strains is under way (Hodgkinson *et al.*, 2013).

The detailed analysis of the resources now available can detect distinct metabolic steps that might differ between host and parasite, and/or novel chokepoints that consequently result as relevant targets for anti-parasitic drug design and vaccines. However, as in other helminth genomes, most of the putative proteins predicted in the *F. hepatica* genome encode for proteins of unknown function. For this reason the development of experimental tools that can unravel the function of liver-fluke genes is necessary to evaluate and validate the relevance of the putative drug or vaccine candidates that emerge from the *in silico* analysis. So far, five studies from two groups demonstrate the viability and utility of RNA interference (RNAi) as a tool that might provide some answers (McGonigle *et al.*, 2008; Rinaldi *et al.*, 2008; Dell'Oca *et al.*, 2014; McVeigh *et al.*, 2014; McCammick *et al.*, 2016). Our group has reported the efficiency of this silencing methodology, and advanced it by optimizing several experimental parameters, using the vaccine candidate leucine aminopeptidase as one of the targets (Rinaldi *et al.*, 2008; Dell'Oca *et al.*, 2014). Adult cysteine proteases involved as vaccine targets have also been tested by RNAi (McGonigle *et al.*,

2008) and the evaluation of novel vaccine candidates, such as juvenile cathepsin CL3 (Corvo *et al.*, 2009), is under way.

Vaccine development

Immune control through the development of vaccines has emerged as a promising alternative control strategy, as it has been shown that ruminants can acquire resistance against metacercarial challenge after vaccination with irradiated metacercariae (Nansen, 1975), parasite extracts (Guasconi *et al.*, 2012) or individual antigens (Spithill *et al.*, 2012). However, vaccines have to reach an appropriate level of efficacy to make this control technology commercially viable within the framework of lack of adequate funding of this 'neglected' parasitic disease.

During the past 25 years single molecules have been used in experimental trials against *F. hepatica*, either as native or recombinant proteins: cathepsin L and cathepsin B peptidases, fatty acid binding proteins (FABP), paramyosin, leucine aminopeptidase, and the anti-oxidant enzymes peroxiredoxin and thioredoxin glutathione reductase (reviewed in Spithill *et al.*, 2012). Native FABP gave from 22 to 55% protection in natural hosts, while the recombinant forms were less effective; similarly, native haemoglobin gave 43% protection in cattle but the recombinant failed. Native paramyosin was also effective in cattle but it failed in sheep, while GST showed variable results in both hosts, and similar failure was observed when peroxiredoxin was tested in *F. gigantica* (reviewed in Toet *et al.*, 2014). Native adult cathepsins showed protection values ranging from 33 to 69% in cattle and sheep, and the recombinant forms worked in cattle but failed in goats (reviewed in Toet *et al.*, 2014). More recently, juvenile cathepsins B and L were tested in rodent models, resulting in a narrower protection range of between 43 and 66% (reviewed in Meemon & Sobhon, 2015).

Our laboratories have focused mostly on the development of vaccines against fasciolosis based on peptidases and anti-oxidant enzymes. According to their performance in preliminary trials, we have selected for further testing the exopeptidase leucine aminopeptidase (LAP) and, from the second group, thioredoxin-glutathione reductase (TGR). The first is the most promising candidate so far, while the second highlights the difficulties in transferring results from different host models.

Vaccine development based on leucine aminopeptidase

Leucine aminopeptidase (*Fh*LAP) was initially characterized, isolated and purified from a detergent-soluble extract of adult liver flukes in the context of a screening effort to detect exopeptidase activities in parasite extracts, using amino acids coupled to 7-amido-4-methylcoumarin as fluorogenic substrates. Histochemistry and immunoelectron microscopy localized this enzyme to the gastrodermal cells lining the alimentary tract of the adult worm, being particularly abundant at the microvilli. *Fh*LAP showed broad amidolytic activity against fluorogenic substrates at pH 8.0, and its activity was increased by the divalent metal cations Zn^{2+}, Mn^{2+} and Mg^{2+} (Acosta *et al.*, 1998).

When native *Fh*LAP (100 μg) was used as a vaccine (mixed with Freund's adjuvant) in Corriedale sheep it induced high levels of protection, alone or in combination with cathepsin Ls – *Fh*CatL1 and *Fh*CatL2 – two major cysteine proteinases derived from excretory/secretory products of adult worms. Vaccinated animals in the *Fh*LAP group had an 89% decrease in worm burden compared to the control group. The sheep that received a trivalent mixture of *Fh*LAP, *Fh*CatL1 and *Fh*CatL2 also showed a significant protection level (79%), which was higher than the non-significant protection observed with the divalent *Fh*CatL1/*Fh*CatL2 mixture (60%) (Piacenza *et al.*, 1999). In the *Fh*LAP vaccine group, 4 out of 6 sheep harboured no flukes in their livers, which is unusual for liver-fluke vaccine trials and highlights the striking efficacy of LAP in sheep. Although the anti-*Fh*LAP IgG antibodies elicited in sheep inhibited enzymatic activity, we found no statistically significant inverse correlation between antibody titres against *Fh*LAP and worm burdens in any of the vaccinated groups.

Moreover, analysis of serum aspartate aminotransferase (AST) and c-glutamyl transferase (GGT) levels revealed that AST levels were elevated in the *Fh*LAP group (i.e. evidence of damage to liver cells), but GGT levels were normal (i.e. no evidence to suggest damage to the bile ducts in this group). These results strongly suggested that immune-mediated killing of migrating flukes occurred in the liver parenchyma before the immature flukes reached the bile ducts. This makes sense as fully developed mature flukes live inside the immune-privileged site of the bile ducts.

The enzyme was cloned and functionally expressed as a thioredoxin fusion protein in bacteria, with similar biochemical properties as the native enzyme and confirmed by MALDI-TOF mass spectrometry (Acosta *et al.*, 2008). *Fh*LAP is a homohexameric enzyme of the M17 metalloprotease family conserved in bacteria, plants, unicellular eukaryotes and all multicellular animals (MEROPS peptidase database; merops.sanger.ac.uk). The M17 phylogenetic analysis demonstrates that all metazoan M17 LAPs fall into three well-defined clusters. Interestingly, *Fh*LAP and all flatworm orthologous enzymes lie in just one of the clusters devoid of enzymes from their vertebrate hosts, while the mammalian paralogues are found in the other two clusters. This differential organization between parasite and host enzymes strengthens the potential of these enzymes as candidates for specific drug design or their use as vaccines. Consistently, in the first trial with the recombinant enzyme, subcutaneous vaccination of New Zealand rabbits with r*Fh*LAP in Freund's adjuvant induced a high (78%) protective immune response (Acosta *et al.*, 2008).

More recently in a large vaccination trial in Corriedale sheep, r*Fh*LAP was formulated with five different adjuvants. Immunization with *rFh*LAP induced a significant 49–87% reduction of fluke burdens in all vaccinated groups compared to adjuvant control groups. Interestingly, all vaccine preparations elicited specific mixed IgG1/IgG2 responses independently of the adjuvant used. Additionally, morphometric analysis of recovered liver flukes showed no significant size modifications in the different vaccinated groups, suggesting that the flukes that survived the protective immune response developed at a normal rate in

the host (Maggioli *et al.*, 2011a). It will be of interest to determine why a small proportion of flukes (10–20%) can escape the highly protective immune response induced by the LAP vaccine.

In mammalian cells LAP is believed to play a significant role in the post-proteasomal degradation of cell proteins. Hence, participation in the last stages of host protein digestion was proposed for *Fh*LAP. The protective mechanism induced by *Fh*LAP vaccine is difficult to explain, due to the intracellular localization of the enzyme. In agreement with the hidden antigen status, very low anti-*Fh*LAP titres are detected in naturally infected animals and only traces of LAP activity are found in excretory/secretory (ES) products of adult *F. hepatica*. In contrast, *Fh*LAP was strongly recognized by a group of sera from confirmed human patients in a two-dimensional electrophoresis analysis of ES products (Marcilla *et al.*, 2008). More recently, *Fh*LAP has been detected prominently in extracellular vesicles, called exosomes, derived from cultured adult worms, particularly in those excreted by the digestive tract of the parasite (Cwiklinski *et al.*, 2015b). Altogether, these data suggest that at least part of the LAP detected in E/S could be released from gut exosomes. On the other hand, no other aminopeptidases have been detected in the secretome of adult worms and, since no universal dipeptide transporters were found in the genome of the liver fluke, digestion of host proteins, such as haemoglobin or albumin, must proceed until single amino acids are released, before being introduced through amino-acid transporters into gastrodermal cells. Q22

Vaccine based on TGR

In flatworm parasites (trematodes and cestodes), but not in free-living platyhelminths, the seleno-protein TGR appears to be the only enzyme responsible for recycling both thioredoxin and glutathione (GSH), due to the lack Q23 of glutathione reductase and thioredoxin reductase (TR) Q24 in these parasites. Moreover, phylogenetic analysis showed that flatworm TGRs represents a clade with no known orthologues on mammalian TRs or TGR (Salinas *et al.*, 2004). The crucial function of TGR in parasite redox homeostasis was confirmed when potent TGR inhibitory compounds induced the *in vitro* killing of *Schistosoma mansoni* schistosomules (Kuntz *et al.*, 2007; Simeonov *et al.*, 2008), *Echinococcus granulosus* protoscoleces Q25 and *F. hepatica* NEJs (Ross *et al.*, 2012). Indeed, Q26 TGR is now a lead target for development of novel antischistosomal drugs. In this context, thioredoxin reductase activity from a detergent-soluble extract of *F. hepatica* was initially isolated and characterized. Due to its glutaredoxin activity it was suggested that the purified protein could in fact be a TGR showing glutathione and thioredoxin specificities. More recently, a TGR of *F. hepatica* was cloned and functionally expressed in *Escherichia coli*, and found to be identical to the enzyme originally labelled as thioredoxin reductase (Maggioli *et al.*, 2011b). The enzyme was initially immunolocalized in testes and tegument of the adult fluke (Maggioli *et al.*, 2004), and, more recently, a proteomic analysis found TGR in the secreted proteome (Wilson *et al.*, 2011). In a preliminary trial 50 µg r*Fh*TGR inoculated with Freund's adjuvant in rabbits induced 96% protection compared to the adjuvant control group. Based in this encouraging outcome, two consecutive trials were conducted in Hereford calves. In the first trial r*Fh*TGR was administered in combination with Freund's incomplete adjuvant (FIA) in a three-inoculation scheme on weeks 0, 4 and 8, and in the second trial r*Fh*TGR was given mixed with Adyuvac 50 or alum as adjuvants on weeks 0 and 4. In both cases calves were challenged with metacercariae 2 weeks after the last inoculation. Our results demonstrated that two or three doses of the vaccine induced a non-significant reduction in worm counts of 8.2% (FIA), 10.4% (Adyuvac 50) and 23.0% (alum) compared to adjuvant controls, indicating that r*Fh*TGR failed to induce protective immunity in challenged calves. All vaccine formulations induced a modest mixed IgG1/IgG2 response but no booster was observed after challenge. No correlations were found between antibody titres and worm burdens (Maggioli *et al.*, 2016). This failure highlights the poor predictive value of vaccination trials against ruminant parasites following the use of small mammals as models. Q27

Conclusions

While it is generally accepted that fasciolosis is widespread in livestock in South America, it has failed to attract the attention of policy makers in most of the countries in the region, particularly those in charge of designing and implementing control programmes in the agricultural sector of the economy. The insidious nature of the infection conspires against the recognition of the problem by the public sector, despite the well-established academic knowledge of losses due to reduction in feed conversion, fertility, milk output and anaemia, and drug-related costs. Q28

In addition, when compared to the situation of gastrointestinal nematodes, where drug resistance is a familiar problem faced by livestock farmers, the emerging phenomenon of drug resistance in fasciolosis is too novel and focal to be recognized as relevant. In this context, abusive use of drugs, errors in dosing or livestock management might have helped the emergence of resistance to different drugs in several parts of the continent.

The isolation and characterization of the drug-resistant variants that are emerging in South America are needed, and the genetic characterization of these is warranted. Fortunately, novel genomic information is available, and genetic and genomic approaches are being developed that might provide clues in this search.

Novel forecasting tools are emerging, using available regional or nationwide indicator data, such as liver condemnation in abattoirs, associated with geographical and climate data, and they might allow the elaboration of better long-term control measures. A point of concern that needs to be addressed is the dispersion of the disease in feral species that might act as reservoirs.

The identification of key enzymes that differ from those present in their hosts has provided a novel framework in which to search for vaccination strategies, with promising results. The integration of these efforts, and the generation of research networks focused on these issues, might start

to provide answers about a disease that has conquered the continent.

Acknowledgements

We would like to thank Maria Jose Rodriguez Cajarville for her contribution to the collection of information and her valuable comments regarding native host species.

Financial support

Q29

Conflict of interest

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