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A prospective view of animal and human Fasciolosis

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SUMMARY

Fasciolosis, a food-borne trematodiasis, results following infection with the parasites, Fasciola hepatica *and* Fasciola gigantica*. These trematodes greatly affect the global agricultural community, infecting millions of ruminants worldwide and causing annual economic losses in excess of US $3 billion. Fasciolosis, an important zoonosis, is classified by WHO as a neglected tropical disease with an estimated 17 million people infected and a further 180 million people at risk of infection. The significant impact on agriculture and human health together with the increasing demand for animal-derived food products to support global population growth demonstrate that fasciolosis is a major One Health problem. This review details the problematic issues surrounding fasciolosis control, including drug resistance, lack of diagnosis and the threat that hybridization of the* Fasciola *species poses to future animal and human health. We discuss how these parasites may mediate their long-term survival through regulation and modulation of the host immune system, by altering the host immune homeostasis and/or by influencing the intestinal microbiome particularly in respect to concurrent infections with other pathogens. Large genome, transcriptome and proteomic data sets are now available to support an integrated One Health approach to develop novel diagnostic and control strategies for both animal and human disease.*

Keywords Fasciola hepatica*, immune modulation, innate immunity, microbiome, serodiagnosis*

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INTRODUCTION

Among the major neglected tropical diseases (NTD) of humans are a group that result from infection with the trematode parasites *Schistosoma mansoni*, *S. japonicum*, *S. haematobium*, *S. mekongi* and *S. bovis* (the blood flukes), *Paragonimus westermani* (the lung fluke) and *Clonorchis sinensis*, *Opisthorchis viverrini*, *Fasciola hepatica* and *F. gigantica* (the liver flukes). Collectively these diseases afflict >1 billion people and cause >600 million disability adjusted life years (DALYs) (1–3). Human infections are predominantly found in Africa, South America, North and South Asia, China and Korea (1).

Infections with the liver and lung flukes occur by ingestion, and hence, these are often classified separately as food-borne trematodiases (see 1–4). In this review, we examine the liver flukes *Fasciola hepatica* and *F. gigantica* that are the most important trematodes afflicting the global agricultural community. However, in the last 25 years alone fasciolosis has emerged as an important zoonosis and NTD with an estimated 17 million people infected and about 180 million people living in endemic areas at risk to infection (5–7).

Fasciola hepatica, usually termed the temperate fluke, is found worldwide, on every inhabited continent, while, *Fasciola gigantica*, the tropical fluke is found in tropical areas of Asia and Africa. The life cycle is essentially the same for both *Fasciola* species and differs only in the intermediate host snail species (8). Eggs are passed in the faeces of infected mammalian hosts and deposited into the environment, typically pastures and grazing areas near a body of water. Following a period of maturation and activation by temperature and light, the eggs hatch releasing miracidia, which actively seek out the snail intermediate host. The species of snail that act as intermediate host (*Galba truncatula* infected by *F. hepatica* and the African *Radix natalensis* and the Eurasian *Radix auricularia* infected by *F. gigantica*) differ for each parasite

species and exhibit marked differences in their geographical distribution (9, 10). Within the snail, the parasite undergoes a clonal expansion through radiae and cercerial stages. The cercariae are released from the snails and encyst on vegetation as metacercariae that can remain viable for months. Domestic animals pick the disease up by eating contaminated grass, while human infections occur following the ingestion of infected edible aquatic salad vegetation, typically found near the infected animals, such as watercress (11, 12) or through the consumption of metacercariae-contaminated water (10). Upon ingestion of infected vegetation, the parasites emerge from their cysts in the intestine, as newly excysted juveniles (NEJ) that then traverse the intestinal wall into the peritoneal cavity before migrating to the liver capsule and parenchyma. Following a period of approximately 7-8 weeks, the parasites migrate into the bile ducts where they develop into sexually mature adults, releasing 20 000–24 000 eggs per fluke per day (13).

DIAGNOSIS OF HUMAN LIVER FLUKE INFECTION

The exact prevalence of human fasciolosis is most likely underestimated due to the lack of epidemiological surveys performed in potentially endemic areas. Approximately 50% of human infections are asymptomatic and are therefore not reported (14). Diagnosis of the remaining infections can vary depending on the detection method used. Faecal egg counts (FEC) are routinely used for diagnosis of animal infections, with the FLOTAC system described by Cringoli and colleagues exhibiting particularly high levels of sensitivity and accuracy (15). For human infections, these methods can be inaccurate as they rely on a chronic infection comprising of mature adult flukes in the bile ducts. Parasite burden and the sporadic nature of egg deposition leading to miss timing of faecal sampling are also weaknesses of this technique for human diagnosis. ELISA-based methods can be used as alternatives for FEC, with several available that determine anti-*Fasciola* antibodies to proteins found within the parasite secretome. Numerous ELISAs have been reported based on the most abundantly secreted groups of proteases, the cathepsin L cysteine proteases. Gonzales-Santana and colleagues (16) have developed a recombinant cathepsin L-based ELISA test specific for human *F. hepatica* infections that showed 99·9% sensitivity and 99·9% specificity. The cathepsin L proteases are also the focus of the capture ELISA developed by Mezo *et al.* (17; MM3-SERO), which is based on a monoclonal antibody MM3 that binds to both cathepsin L1 and L2 proteases (18). An ELISA method focussing on saposin-like protein-2 has also been developed, although the levels of sensitivity and specificity of 87% and 99%, respectively (19), are lower than that reported by Gonzales-Santana *et al.* (16). Similar ELISA tests are available for *F. gigantica* using a variety of different antigens: (a) Fas1 and Fas2 cysteine proteases, resulting in 91·9% and 89·1%, sensitivity and specificity, respectively (20); (b) sandwich ELISA using fatty acid binding protein, resulting in 94·7% sensitivity and 84·62% specificities (21); (c) 27 kDa circulating antigen from sera of infected individuals gave >93% sensitivity and specificity (22). A lateral flow test (SeroFluke) was developed by Martinez-Sernandez and colleagues using samples obtained from *F. hepatica*-infected patients in Spain and Portugal, for use with serum or whole blood samples, which requires minimal training (23). However, ELISAs capable of distinguishing active infection by *F. hepatica* and *F. gigantica* are needed for diagnosis in areas of geographical overlap of the two species, particularly if access to the adult flukes for genotyping is not available.

HYBRID AND/OR INTROGRESSED POPULATIONS OF LIVER FLUKE

Fasciola has the ability to self-fertilize, cross-fertilize and in some cases undergo parthenogenesis. These flukes are typically diploid as shown by a recent study in the UK (24), although triploid and mixoploid isolates have been observed in one animal infection in cattle in the UK (*F. hepatica*, Cullumpton strain, triploid; 25), and in Asian flukes of China, Japan, Vietnam and Korea (26–31). The triploid and mixoploid parasites are often aspermic, relying on parthenogenesis for continuation of the life cycle.

In areas of Japan, Vietnam and Korea, parasites cannot be classified as *F. hepatica* or *F. gigantica* using morphometrics due to the variety of intermediate forms that exist. Moreover, molecular analysis of mitochondrial genes and intergenic genome sequences (ITS2) has found that these intermediate forms are hybrid species. This analysis identified individuals with nuclear DNA of one species, but the mitochondrial genome of the other species as well as individuals with copies of genes derived from both species (28–31). The ability of these species to hybridize and/or introgress will play a role in genetic diversity within populations (32) and has the potential for crossover of anthelminthic resistance between the two species. Furthermore, hybridization may also have serious implications for people living in areas where the two species co-localize as this could result in the emergence of more pathogenic *Fasciola* isolates. A recent study by Valero and colleagues (33) showed that when directly compared in infections of Guirra sheep, which have similar susceptibilities to both species, *F. gigantica* was found to be more pathogenic than

F. hepatica. Also, a recent analysis of human infections in Vietnam has revealed that hybrids are also able to infect people (34).

To date, *Fasciola* hybrids have been identified using mitochondrial genes, which act as robust markers due to their maternal inheritance. As mitochondrial genes are unlinked to the nuclear genome, introgression of these markers has often been shown to be greater than nuclear encoded markers (35). That said, these markers alone may not accurately reflect whether true hybridization/introgression occurs within *Fasciola,* due to different mutation rates across the genome. The availability of the *F. hepatica* genome has driven the development of larger panels of markers including SNPs (36) and microsatellites (37), which have shown that the *F. hepatica* genome is highly heterogenous. In depth analysis of these hybrids can now be carried out across the genome to elucidate whether hybridization and/or introgression is occurring between Asian *F. hepatica* and *F. gigantica* and at what level. Most importantly, there is a need to investigate the impact hybridization and/or introgression could have on human infections, especially as more cases of human *F. gigantica* infections are being reported, to determine whether the hybrid flukes are more or less pathogenic than their parents.

EMERGENCE OF DRUG RESISTANT PARASITES: A THREAT TO ANIMALS AND HUMANS

Control of *Fasciola* in ruminants is reliant on the use of anthelmintic drugs, particularly triclabendazole (TCBZ), which targets both the tissue damaging immature stages and mature adult stages of the parasites. TCBZ treatment failure and/or resistance are increasingly reported for *F. hepatica* infections in ruminants across Europe, South America and Australia (38, 39) but fortunately, to date, no cases of *F. gigantica* TCBZ resistance have been reported. Treatment of human fasciolosis also relies on the use of TCBZ, which was commercially available as Egaten (Novartis Pharma AG), through a joint venture with WHO. However, it is still not licensed for use in several countries (40), such as USA and Canada, where TCBZ is only available to US licensed physicians under approval from the Centres for Disease Control and Prevention (CDC) and the Food and Drug Adminstration (FDA) on a case-by-case basis (41). Other drugs have also been used to treat human *F. hepatica* infections, including nitazoxanide, originally FDA approved for protozoal infections which was shown to have varying efficacy levels depending on the study, ranging between 40% and 100% (42). As with the ruminant infections, TCBZ failure has been found in humans, with the first case being reported in 2012 in the Netherlands of a sheep farmer who became infected with *F. hepatica* as a result of casual chewing of contaminated grass (43). Following the failed treatment with TCBZ in this case, nitazoxanide was used but with no effect, indicating that nitazoxanide was not effective against these potentially TCBZ-resistant parasites. Gulhan and colleagues (44) have also reported a case of a child in Turkey that was infected with *F. hepatica* and, did not respond to TCBZ treatment, although this report did not speculate as to the mode of infection with a potentially TCBZ-resistant isolate. Similarly, Cabada and colleagues (45) have reported a group of patients, infected by ingesting watercress in the Cusco region of Peru that failed to respond to multiple courses of triclabendazole. As with the Winkelhagen *et al.* (43) case report, following TCBZ treatment failure, the patients were prescribed nitazoxanide, which only appeared to cure one patient, further emphasizing that nitazoxanide is not effective against TCBZ-resistant parasites. As TCBZ resistance becomes more prevalent in *F. hepatica* endemic regions, it is very possible that more human cases of infection with TCBZ-resistant *F. hepatica* parasites will be reported which poses a real problem for human treatment.

IMMUNE RESPONSES AND IMMUNITY TO LIVER FLUKE

The immune response induced during natural infection has been well characterized in ruminants for *F. hepatica* although there are increasing studies on *F. gigantica* that report similar findings (46–50). During the acute stages of infection, cattle exhibit a mixed immune response with elevated IL-10, TGF-β, IL-4 and IFN-γ. However, as infection progresses Th2/Treg immune responses become more dominant (51). During the later chronic stages, Treg cells release cytokines that inhibit inflammatory Th1/Th2 cytokines; PBMCs isolated from *F. hepatica* - infected cattle produced enhanced levels of IL-4 and IFN-γ cytokines when cultured *in vitro* in the presence of TGF-β and IL-10 neutralizing antibodies (51). This immune profile is similar in sheep infected with *F. hepatica* as they also present a mixed Th1/Th2 cytokine profile in the spleen at week 3 after infection and as infection progresses enhanced gene expression of Th2 but not Th1 cytokines is observed (52). Interestingly, although an overall systemic Th2 immune response dominates, different cytokines are expressed at different anatomical locations; in sheep, IL-5 can be detected in the hepatic lymph nodes, while IL-10 is primarily observed in the spleen (53–55), whereas in goats, IFN-γ

and high levels of IL-4 can be detected in both the hepatic lymph node and liver (56).

While it has been reported that Indonesian thin-tailed sheep can resist infection to *F. gigantica,* no natural or experimental hosts exhibit resistance to *F. hepatica* infection, and successful trickle infection in cattle demonstrates a lack of concomitant immunity (57–59). This suggests that the ability of *F. hepatica* to successfully infect a broad spectrum of mammalian hosts across the globe (e.g. cattle, sheep, goats, buffaloes, kangaroos, capybara, camelids and humans, see (4, 10) can be attributed partly to the development of effective immune-modulatory mechanisms to prevent the normal protective response and thus ensure the parasites longevity. The potent suppression of host Th1 immune responses during active infection of both natural hosts and experimental rodent models has been attributed to the development of a strong regulatory/Th2-type immune response (51, 54, 56, 60–66).

While Th2 and regulatory T-cell cytokines are important in downplaying host protective Th1 responses during infection with *F. hepatica,* it seems that the parasite also influences various cells of the innate immune response. Firstly, in experimental mouse models, $CD11c^+$ dendritic cell (DC) populations are increased during *Fasciola* infection displaying an immature phenotype with lower expression of co-stimulatory markers (CD40, CD80 and CD86), MHC class II, increased expression of CCR5 and are hyporesponsive to TLR activation (67). These cells express enhanced levels of intracellular IL-10 and *ex vivo* suppress the secretion of antigen specific IL-17 and IFN-γ from naïve DO11.10 OVA TCR Tg $CD4^+$ T cells independent of IL-10 and TGF-β (68). Secondly, the induction of macrophages with a regulatory/M2 phenotype is common in both large animals and rodents infections (69–72). This switch occurs within the first 3 days of murine infection and similar to DCs activated by *Fasciola* antigens, M2 macrophages are hyporesponsive to TLR ligands, suggesting a reduced ability to promote the differentiation of host Th1 immunity. In addition, it has been shown that M2 macrophages isolated from mice during infection with *F. hepatica* promote the polarization of Th2 cells (69, 70). Thirdly, there is a significant increase in the number of mast cells observed at the site of infection and in the gut mucosa (73–77). While mast cells are critical to the expulsion of gut helminths (78), their role in *Fasciola* infection is not clear, although we hypothesize that given that mast cells have an important role in wound healing and tissue remodelling (79), they are recruited to combat the extensive tissue damage caused by migratory flukes (80).

It also appears that during infection of mice with *F. hepatica,* T cells are induced to enter an anergic state as markers of anergy (GRAIL, EGR2, ICOS and ITCH) were observed in $CD4^+$ T-cell populations (81) and may explain why these cells become hyporesponsive to antigen stimulation in the late stages of infection (82). This anergic state, as shown by decreased cytokine responses and reduced proliferative activity, could be reversed with the addition of IL-2 to cultures (81). The presence of anergic T cells is yet to be demonstrated in ruminants (and humans); however, the lack of IL-2 reported in the local HLN of infected sheep supports such a mechanism of immune inactivation (54, 55).

ALTERED IMMUNE HOMEOSTASIS DURING *FASCIOLA* INFECTION

The induction of wound-healing immune mechanisms (Th2, M2 macrophages, mast cells) by *F. hepatica* is clearly important to protect vital tissues, particularly the liver, from damage caused by the migratory activity of the parasite (83–86), while the regulation/suppression of protective pro-inflammatory type 1 immune responses is central to the promotion of the host's tolerance to the parasite, supporting its long-term survival (87, 88). However, helminth parasites are rarely the sole infecting organism within a mammalian host, particularly in endemic regions where co-infections are common (89). Therefore, immune suppressive effects of *F. hepatica* are not confined to the host-parasite relationship but likely have broader implications to the induction of type 1 immune responses necessary to mediate resistance (induced by infection or vaccination) to major coexisting pathogens. For example, cattle infected with *F. hepatica* were more susceptible to infections with *Salmonella dublin* and took longer to clear the bacteria than nonhelminth-infected animals (90). Similarly, concurrent infection of mice with *F. hepatica* and *Bordetella pertussis* resulted in a prolonged bacterial lung infection (60).

Importantly, epidemiological surveys indicate that hyperendemic regions (>10%) for infection with *Fasciola* overlap with the geographical distribution of human microbial pathogens implying a significant impact on human health. For example, aside from fasciolosis, the most common childhood illnesses among the Aymara people of the Northern Altiplano of Bolivia are upper respiratory infections such as whooping cough and tuberculosis (91). These children are also infected with a range of other protozoal and helminth parasitic infections, with a significant positive association for *F. hepatica* and *Giardia intestinalis* co-infections (92). *Fasciola* is also emerging as a human pathogen in communities of Iran's Guilan Province (93, 94) that are already afflicted by malaria and tuberculosis (95, 96). Although there are currently no studies on the immune status of humans co-infected with

Fasciola and other pathogens, clinical studies with other helminths (97–101) suggest that modulation of host responses by helminth infection increases susceptibility to microbial pathogens and impairs vaccine efficacy. More extensive longitudinal human studies are required to fully determine the effect of *Fasciola* infection on the pathogenesis of microbial pathogens within endemic populations.

Beyond this specific impact on immune response to concurrent infections, there is a growing body of evidence suggesting that the constant presence of helminth parasites (particularly intestinal soil transmitted species) within the human population since prehistoric times has strongly shaped the evolution of the human immune system (102). Indeed, the need to overcome the regulatory mechanisms exerted by helminths appears to have resulted in compensatory adjustments to immune-related genes (103, 104). Accordingly, in populations where parasitic infections are no longer endemic, there is an increased prevalence of inappropriate immune responsiveness to auto-antigens and allergens and the concomitant development of autoimmune/inflammatory diseases (105, 106). The epidemiological pattern of fasciolosis is more varied than that seen for intestinal helminth infection. While low and stable levels of *Fasciola* exist within small, defined populations, sporadic outbreaks are associated with climatic changes that boost the life cycle of the parasite and/or intermediate snail host (107–109). Therefore, it is presently unclear whether infection with either *F. hepatica* or *F. gigantica* provides any such immune benefit to its host.

The possibility that helminth infection influences immune homeostasis by mediating changes to intestinal microbiota has not been extensively explored, even though it is now clear that this commensal community profoundly impacts the homeostasis of innate and adaptive immune responses and thus the development of immune-mediated disease (110–116). Changes in gut microbiota, attributed to the use of antibiotics, particularly in childhood, have been correlated with incidences of multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and allergy (116, 117). Furthermore, experimental studies show that changes to the intestinal microbiota of mice strongly influence the development of allergy, inflammatory bowel disease, rheumatoid arthritis, experimental autoimmune encephalitis and type 1 diabetes (118–123). Controlled infections of animals have shown that the presence of intestinal parasites results in changes to the composition and abundance of intestinal microbiota species (124–127). However, the use of anthelminthics suggests that the continuing presence of the parasite may be required for sustained changes to the bacterial community (128).

A primary mechanism by which the intestinal microbiota regulate immune responses is proposed to be through the production of immune modulating metabolites such as short-chain fatty acids (SCFA; 129, 130). The most abundant of these are butyrate, propionate and acetate produced by the bacterial fermentation of plant-derived nondigestable polysaccharides, like cellulose, in the gut (131, 132). Treatment of both murine macrophages and dendritic cells with bacterial SCFAs is associated with a decrease in the production of pro-inflammatory cytokines such as IL-12 and TNF and increased expression of IL-10 (129, 133, 134). Similarly, human peripheral blood mononuclear cells exposed to SCFAs display a reduced ability to produce TNF and IFN-γ in response to treatment with lipopolysaccharide (135). Furthermore, SCFAs appear to regulate production of pro-inflammatory cytokines in nonimmune cells such as Caco-2 cells (136). In addition, these SCFAs also contribute to the induction of regulatory T cells, either directly through interaction with the G-protein-coupled receptor GPR43 (137) or indirectly via activation of regulatory macrophages, which in turn generate a population of FoxP3+ T cells (121). It is interesting that parasite-associated microbiota produce an increased amount of SCFAs, which have been shown to mediate an increase in the proportion of lung regulatory T cells and as a result attenuate allergic airway inflammation (138). Although some studies in humans confirm that there is a difference in the gut microbiome community and the production of SCFA in helminth-infected individuals (139–141), the data are not consistent across populations, likely due to sample size, differences in diet and other yet unknown confounding factors.

F. hepatica spends a short time in the gut during infection and is typically localized to the duodenum (8), which suggests that this parasite may not significantly influence the composition of the gut microbiota. Instead, *Fasciola* parasites take a number of weeks migrating through the liver of its mammalian host before ultimately residing in the bile duct for many years from where they can mediate systemic immune modulation; the adult parasite is an obligate blood feeder, an activity likely to result in the continued release of parasite excreted/secreted (ES) products into the host circulation. Like SCFAs, these parasite ES products actively suppress the production of pro-inflammatory cytokines by most cells of the innate immune system, resulting in the systemic switch towards a regulatory/Th2 immune environment (67, 69, 70, 142).

Additionally, the presence of the parasite appears to influence the composition of bile acids (143). Bile acids act as signalling molecules through either TGR5 or FXR to regulate intestinal homeostasis via the inhibition of inflammation, prevention of pathogen invasion and maintenance of tissue integrity (144, 145). Activation of TGR5 inhibits NFκB and thus reduces the production of pro-

inflammatory cytokines such as IL-6 and TNF by macrophages and Kupffer cells (144–146). While signalling via FXR also represses NFκB-dependent transcription resulting in an anti-inflammatory response (129, 130, 144, 145, 147), activation of this receptor also increases the expression of several genes associated with antibacterial actions such as IL-18 (148). Notably, infection with *F. hepatica* results in significant increases in the production of bile acids (149) and IL-18 was recently identified as the most significantly up-regulated interleukin in the liver of infected sheep (52). Such preliminary observations encourage further characterization of the composition of bile acid during acute and chronic infection with *F. hepatica* in animals and humans and an assessment of the ability of these to regulate the functions of innate and adaptive immune cells as well as influence the microbiome of the gut into which they are passed.

ADVANCES IN -OMIC TECHNOLOGY WILL HELP FILL THE GAPS IN OUR KNOWLEDGE

In the past decade, there has been major advancement in the available '-omics' data for trematode parasites (150–153), which has allowed robust comparative genome analyses between these species. Data sets are now available for both *F. hepatica* and *F. gigantica* which has greatly expanded our knowledge of these parasites, particularly regarding life cycle stages that have been historically difficult to study at the molecular level due to sample sizes. These recent technological developments have allowed more in depth analysis of these two *Fasciola* species to further our understanding of fluke biology and how they infect and persist within their hosts. Several secretome data sets are available for both species (154–158), which for *F. hepatica* are being complimented by analysis of the exosome component of the secreted proteins (159, 160) as well as glycan analysis of these proteins (161; Ravida *et al.*, unpublished), allowing in depth analysis of those proteins directly interacting with the host. A complete set of transcriptomes from the life cycle stages present within the definitive host, ranging from metacercariae to mature adult flukes are now available, allowing analysis of the extensive differential expression that occurs within this host, particularly as the parasite migrates through the liver (36). These transcriptomes have been mapped onto the recently published *F. hepatica* draft genome (36). Together this analysis has revealed that *F. hepatica* has one of the largest known pathogen genomes, at 1·3 Gbp that currently cannot be explained simply by whole genome duplication or expansion of repeated regions. The genome also shows high levels of polymorphism, allowing for the potential of dramatic genetic adaptation to new environments and molecules. Analysis of the draft genome has clarified several gene families of interest for vaccine and drug target development, such as the cathepsin cysteine proteases (L & B families), the asparaginyl endopeptidases (legumains) and the ABC transporters, which have greatly expanded and diversified biochemically and functionally.

Analysis of the differential expression across the *F. hepatica* life cycle has shown that the parasite undergoes rapid metabolic development, particularly during the early stages of parasite migration through the host, which may be notable for future drug development. Increased levels of gene expression were induced as the parasite enters and migrates through the liver. This is coupled with extensive parasite growth that may possibly be driven by neoblast-like stem cells (162, 163). This study is further complimented by the proteomic analysis of the secretome of these life cycle stages, which has revealed in particular that the majority (70%) of the secretome of the early NEJ stages is represented by 10 proteins (Cwiklinski and Dalton, unpublished). Elucidating the function of these secreted proteins and their relevant abundance within the secretome is key to furthering our understanding of how the parasite interacts and manipulates its host.

Similarly, for *F. gigantica*, transcriptome data are available for the adult stage parasites, which when compared with *F. hepatica* data revealed that the predicted proteins showed between 80 and 90% homology (based on E-value $1E^{-15}$ and E-value $1E^{-05}$ cut-offs; 164). These two species have been consistently shown to have high levels of similarities at the morphological and life cycle level as well as at the gene transcription level. Comparative analysis of the *F. gigantica* genome currently being sequenced (Trematode.net, 165) with the *F. hepatica* genome is critical for our comprehension of these two species and their hybrids to define differences that may govern species-specific pathogenesis.

Similarly, more robust analysis can now be carried out for the phylum Trematoda, to reveal how these parasites have developed their host/tissue preferences and immune evasion strategies and how this relates to their transmission patterns and distribution. Particularly for the liver flukes, namely *C. sinensis*, *O. viverrini* and *Fasciola* spp., such comparative genomics could illuminate the species-specific strategies of surviving within the same environment (bile duct). Furthermore, these studies should unravel the mode of action of the *Fasciola*-specific anthelmintic TCBZ and explicate why praziquantel, which acts on most trematodes, has no effect on *Fasciola*.

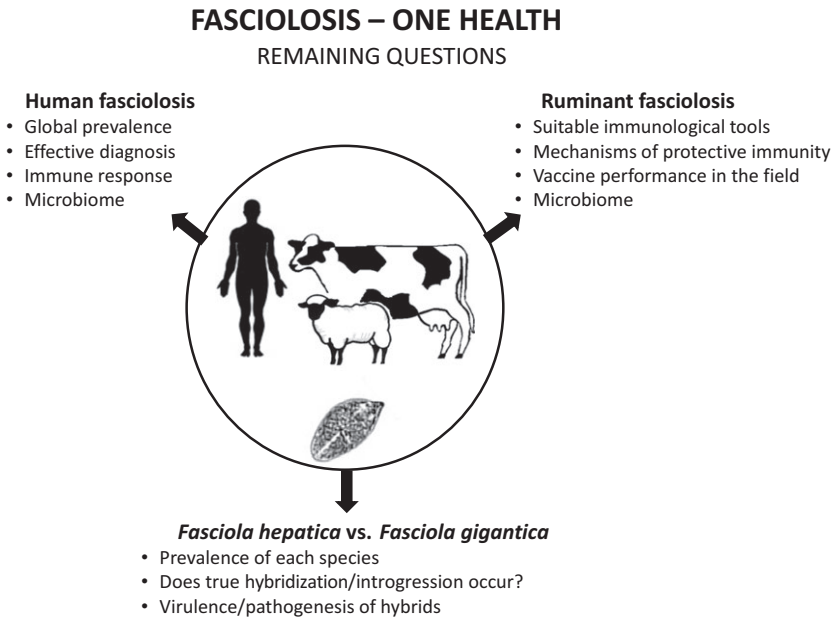

Figure 1 Graphical representation of the remaining questions associated with Fasciolosis as a 'One Health' problem.

CONCLUSION

Diseases caused by *F. hepatica* and *F. gigantica* in humans have emerged as an important NTDs, mainly in poor rural regions in Africa and Asia. We still do not know the full extent or epidemiological distribution of diseases caused by either of these two parasites, nor do we fully understand their virulence and pathogenicity in humans. Cross-fertilization between the species threatens the spread of hybrids with unknown capacity to infect and cause damage in animals and humans, and therefore, we urgently require new species-specific tests for diagnosis and surveillance purposes. The spread of triclabendazole-resistant parasites is also of concern because it is the only drug that targets the tissue damaging juvenile stage, and is the only available effective treatment for human infections. While immunological studies are gradually defining immune mechanisms of infection, pathogenesis and protection, these are predominantly confined to animal studies and can only be assumed to apply to humans. Nevertheless, old and recent results strongly suggest that infection with *Fasciola* species does leave the host, animal and human, susceptible to co-infection with other pathogens; this affect may be mediated via immune modulation, immunosuppression, immune polarization and/or by altering the composition of the microbiome (gut and/or bile).

The relevance of fasciolosis as a 'One Health' foodborne zoonosis will grow if we do not come up with new control measures. Vaccines for animal liver fluke disease should have a major impact on human infection, but despite excellent progress, these are still some years away. Molecules actively secreted as soluble components or in extracellular vesicles are critical to how *Fasciola* interacts and modulates the host immune response and are considered prime vaccine candidates (reviewed in 67, 160, 166, 167). Development in the 'omics' technologies is keeping research on *Fasciola* abreast with the broader field of parasitology and pathogen biology, and complete data sets (genome, transcriptome, proteome, glycome) will become available to researchers in the near future. However, many gaps in our knowledge (e.g. mechanisms of protective immunity, the relevance of the microbiome in parasite resistance and protection, vaccine performance in the field etc.) and available tools (e.g. immunologicals such as cytokine arrays, microarrays and multiplex cytokine qPCRs) still exist and require our collective efforts to fill (Figure 1). Moreover, there is a serious dearth of information regarding the global prevalence of human fasciolosis as well as an understanding of immune response to infection and the pathogenesis associated with the migration of the parasite through the liver and its residence in the bile duct.

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