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Research paper

Low allelic diversity in vaccine candidates genes from different locations sustain hope for *Fasciola hepatica* immunization

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ABSTRACT

Fasciola hepatica is a trematode parasite that causes fasciolosis in animals and humans. Fasciolosis is usually treated with triclabendazole, although drug-resistant parasites have been described in several geographical locations. An alternative to drug treatment would be the use of a vaccine, although vaccination studies that have been performed mainly in ruminants over the last 30 years, show high variability in the achieved protection and are not yet ready for commercialisation. Since *F. hepatica* exhibits a high degree of genomic polymorphism, variation in vaccine efficacy could be attributed, at least partially, to phenotypic differences in vaccine candidate sequences amongst parasites used in the challenge infections. To begin to address this issue, a collection of *F. hepatica* isolates from geographically dispersed regions, as well as parasites obtained from vaccination trials performed against a field isolate from Uruguay and the experimentally maintained South Gloucester isolate (Ridgeway Research, UK), were compiled to establish a *F. hepatica* Biobank. These collected isolates were used for the genetic analysis of several vaccine candidates that are important in host-parasite interactions and are the focus of the H2020 PARAGONE vaccine project (https://www.paragoneh2020.eu/), namely FhCL1, FhCL2, FhPrx, FhLAP and FhHDM. Our results show that *F. hepatica* exhibits a high level of conservation in the sequences encoding each of these proteins. The consequential low variability in these vaccine candidates amongst parasites from different geographical regions reinforces the idea that they would be suitable immunogens against liver fluke isolates worldwide.

1. Introduction

Fasciola hepatica is the causative agent of fasciolosis, a parasitic disease of ruminants that seriously affects farm productivity worldwide as a result of livestock morbidity and mortality, as well as being an important zoonotic parasite of man (Cwiklinski et al., 2016; Carmona and Tort, 2017; Mehmood et al., 2017). The annual losses related to pathologies caused by fasciolosis have been reported to be 3 billion USD (Spithill et al., 2012). While triclabendazole is the most effective drug treatment, *F. hepatica* has rapidly developed drug resistance resulting in the widespread threat to livestock production systems (Kelley et al., 2016). Therefore, the development of an effective vaccine is paramount and would represent the most appropriate and sustainable way forward in the control of fasciolosis (Dalton et al., 2013).

Since the early 1990 s, a growing number of vaccine trials in livestock have evaluated the efficacy of candidate antigens from *F. hepatica*. Among them, different parasite secreted antigens such as cathepsin L peptidases 1 and 2 (FhCL1, FhCL2), the antioxidant peroxiredoxin (FhPrx) and the gut-associated exopeptidase leucine aminopeptidase (FhLAP) have been selected as vaccine candidates due to their importance in host-parasite interactions (Dalton et al., 2013; Toet et al., 2014). These studies highlighted that high levels of variability in vaccine efficacy occur between trials which likely results from differences between the antigen source, the adjuvants used and the host species vaccinated. Variation between animals from the same or different breeds was also observed. In general, native antigens were more effective than recombinant vaccines, and with the exception of FhLAP combination of antigens perform better than single antigen formulations (see an overview of vaccine data in Table 1).

Following the sequencing of the *F. hepatica* genome, high levels of genetic polymorphism were observed, particularly in the chemosensory and neurodevelopmental pathways which might account for adaptations to the host environment and the capacity for rapid evolution (Cwiklinski et al., 2015). Further genome sequencing of liver flukes

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Table 1
Efficacy of single or combination vaccines against *Fasciola hepatica*.

Antigen	Source	Host	Schedule[a]	Adjuvant	Efficacy[b]	Reference
FhCL1	Adult E/S	Cattle	10–500 μg X 3	FCA/FIA	38.2–69.5%	Dalton et al. (1996)
			200 μg X 3		42.5%	
		Sheep	100 μg X 2		33%	Piacenza et al. (1999)
	Recombinant	Cattle	200 μg X 2	Montanide[c]	47.2%	Golden et al. (2010)
				Montanide[d]	49.2%	
		Goat	100 μg X 2	Quil A	0%	Pérez-Ecija et al. (2010)
					38.7%	Buffoni et al. (2012)
					0%	Zafra et al. (2013)
				Montanide[c]	0%	Pacheco et al. (2017)
	Mimotope	Goat	1×10^{13}pp	Quil A	46.9–79.5 %	Villa-Mancera et al. (2014)
FhCL1 + Hb	Adult E/S	Cattle	200 μg X 3	FCA/FIA	51.9%	Dalton et al. (1996)
FhCL2		Sheep	100 μg X 2	FCA/FIA	34%	Piacenza et al. (1999)
FhCL2 + Hb		Cattle	200 μg X 3	FCA/FIA	72.4%	Dalton et al. (1996)
				FCA/FIA	72.4%	Mulcahy et al. (1998)
				FIA	11.2%	
				FCA/FIA	29%	Mulcahy et al. (1999)
FhCL1 + CL2		Sheep	100 μg X 2	FCA/FIA	60%	Piacenza et al. (1999)
		Cattle	200 μg X 3	FCA/FIA	55%	Mulcahy et al. (1999)
FhCL1 + CL2 + LAP	Adult E/S, SOM	Sheep	100 μg X 2	FCA/FIA	79%	Piacenza et al. (1999)
FhCL1 + Prx + Sm14	Recombinant	Goat	100 μg X 2	Quil A	10.1%	Buffoni et al. (2012)
FhLAP	Adult SOM	Sheep	100 μg X 2	FCA/FIA	89.6%	Piacenza et al. (1999)
	Recombinant	Rabbit	100 μg X 2	FCA/FIA	78%	Acosta et al. (2008)
		Sheep	100 μg X 2	FCA/FIA	83.8%	Maggioli et al. (2011)
				Alum	86.7%	
				Adyuvac 50	74.4%	
				DEAE-D	49.8%	
				Ribi	49.5%	
FhPrx	Recombinant	Goat	100 μg X 2	Quil A	33.1%	Mendes et al. (2010)
					33.1%	Buffoni et al. (2012)

Adult E/S or SOM, *F. hepatica* adult worm excreted/secreted or somatic products. FCA/FIA, Freund's complete/incomplete adjuvant.
[a] Vaccination dose (μg, micrograms; pp, phage particles number) and boosts number.
[b] Only referred to the reduction of worm numbers in vaccinated and infected animals, compared with infected and non-vaccinated animals.
[c] Montanide ISA70VG.
[d] Montanide ISA206VG.

from two American locations also found polymorphisms between the *F. hepatica* isolates (McNulty et al., 2017). Both studies were based on sequencing several individual parasites but opened the path to population genetic approaches, a much needed follow-up of the helminth genomic era (Wit and Gilleard, 2017). Analysis of UK isolates based on neutral markers (microsatellites) confirmed substantial variation within *F. hepatica* populations (Beesley et al., 2017), which complements the population genetics studies of liver fluke populations carried out using ribosomal and mitochondrial markers to unravel geographical variations (reviewed by Teofanova et al., 2012). Although marked genetic heterogeneity between liver fluke populations is now well recognised, an association between *F. hepatica* haplotypes and specific phenotypic traits has yet to be made (reviewed in Zintl et al., 2015).

To further the development of vaccines against a range of economically important parasitic pathogens of livestock, the EU H2020 funded consortium PARAGONE (https://www.paragoneh2020.eu/) has brought together liver fluke researchers with an aim to develop a multivalent vaccine against *F. hepatica*. Our current vaccine candidates include those molecules that have shown potential in previous studies, including cathepsin L proteases (FhCL1), leucine aminopeptidase (FhLAP) and peroxiredoxin (FhPrx), in addition to assessing the *F. hepatica* helminth defence molecule (FhHDM) as a vaccine candidate. Specifically, this study evaluates FhHDM as a recombinantly expressed protein, which complements recent sheep vaccination trials by the Prof. Ubeira group using native and synthetically synthesised FhHDM (Martínez-Sernández et al., 2017; Orbegozo-Medina et al., 2018).

An important task of the project is to investigate the potential genetic variability of these vaccine targets within different *F. hepatica* isolates that may be a cause of variability in efficacy results between vaccine trials. Accordingly, we sourced *F. hepatica* from geographically dispersed liver fluke populations for genetic analysis and compared these results with an analysis of the liver fluke isolates that we used in our vaccine trials, namely a field isolate from Uruguay and the laboratory-maintained South Gloucester isolate from Ridgeway Research, UK. The collection of *F. hepatica* isolates sourced as part of this study is now housed in a *F. hepatica* Biobank and is publically available.

2. Materials and methods

2.1. Parasite material and sample processing

A comprehensive biobank for *F. hepatica* was established at the Institute of Natural Resources and Agrobiology of Salamanca (IRNASA-CSIC) (Salamanca, Spain), with the aim of collecting representative samples of *F.hepatica* from different geographical locations, hosts and variable drug susceptibility/resistance. Samples were sourced from geographically dispersed regions collected at local abattoirs and stored individually in RNAlater (Sigma–Aldrich). Samples from the PARAGONE *F. hepatica* vaccine trials were also included in the biobank from cattle experimentally infected with a field isolate from Uruguay and sheep experimentally infected with the laboratory maintained South Gloucester isolate (Ridgeway Research). The samples used for this study are detailed in Table 2.

All samples were processed upon arrival to the biobank following the European rules for handling and traceability of biologicalsamples. In brief, adult liver flukes stored in RNAlater were transversally sliced in half and used for (a) extraction of genomic DNA (Nucleospin Tissue, Magerey Nagel), and (b) extraction of RNA and subsequent cDNA synthesis (PureLink RNA Minikit, Thermo Fischer Scientific; Kit First Strand cDNA Synthesis, Roche), according to the manufacturer's instructions. The extracted genomic DNA and RNA were assessed for quality and quantity by OD at 260/280 nm and by gel electrophoresis.