International Journal for Parasitology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Fasciola hepatica demonstrates high levels of genetic diversity, a lack of population structure and high gene flow: possible implications for drug resistance

Nicola J. Beesley [a,*], Diana J.L. Williams [a], Steve Paterson [b], Jane Hodgkinson [a]

[a] *Veterinary Parasitology, Institute of Infection and Global Health, University of Liverpool, Liverpool L3 5RF, UK*
[b] *Centre for Genomic Research, Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK*

ARTICLE INFO

Article history:
Received 15 July 2016
Received in revised form 1 September 2016
Accepted 7 September 2016
Available online xxxx

Keywords:
Fasciola hepatica
Population genetics
Anthelmintic resistance
Diversity
Self-fertilisation
Gene flow
Microsatellites

ABSTRACT

Fasciola hepatica, the liver fluke, is a trematode parasite of considerable economic importance to the livestock industry and is a re-emerging zoonosis that poses a risk to human health in *F. hepatica*-endemic areas worldwide. Drug resistance is a substantial threat to the current and future control of *F. hepatica*, yet little is known about how the biology of the parasite influences the development and spread of resistance. Given that *F. hepatica* can self-fertilise and therefore inbreed, there is the potential for greater population differentiation and an increased likelihood of recessive alleles, such as drug resistance genes, coming together. This could be compounded by clonal expansion within the snail intermediate host and aggregation of parasites of the same genotype on pasture. Alternatively, widespread movement of animals that typically occurs in the UK could promote high levels of gene flow and prevent population differentiation. We identified clonal parasites with identical multilocus genotypes in 61% of hosts. Despite this, 84% of 1579 adult parasites had unique multilocus genotypes, which supports high levels of genotypic diversity within *F. hepatica* populations. Our analyses indicate a selfing rate no greater than 2%, suggesting that this diversity is in part due to the propensity for *F. hepatica* to cross-fertilise. Finally, although we identified high genetic diversity within a given host, there was little evidence for differentiation between populations from different hosts, indicating a single panmictic population. This implies that, once those emerge, anthelmintic resistance genes have the potential to spread rapidly through liver fluke populations.

1. Introduction

Fasciola hepatica is a trematode parasite that causes disease of economic importance in sheep and cattle (Bennett and Ijpelaar, 2005; Schweizer et al., 2005), with an estimated 250 million sheep and 350 million cattle at risk worldwide (Hillyer and Apt, 1997). A zoonosis, it is classed by the World Health Organisation as a neglected tropical disease endemic in human populations in parts of South America, western Europe and Iran (Mas-Coma, 2005; World Health Organisation, 2007, http://www.who.int/neglected_diseases/diseases/en/). Over the last 15–20 years, the diagnosis of *F. hepatica* infection in European livestock has increased (Caminade et al., 2015; https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/458616/vida-cattle-07–14.pdf; https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/458618/vida-sheep-07–14.pdf), possibly due to changing climate, changing farming practices including animal movement and land use, and the emergence of resistance to the drug of choice, triclabendazole (van Dijk et al., 2010; Fairweather, 2011a; Fox et al., 2011; Caminade et al., 2015). Resistance of *F. hepatica* to triclabendazole was first reported in sheep in Australia in 1995 (Overend and Bowen, 1995), and is now frequently reported across Europe and South America (Moll et al., 2000; Gaasenbeek et al., 2001; Álvarez-Sánchez et al., 2006; Mooney et al., 2009; Olaechea et al., 2011; Daniel et al., 2012; Ortiz et al., 2013). It is considered to be a substantial threat to the current and future control of *F. hepatica* (Kelley et al., 2016).

Population genetic analyses are key to understanding the origin, evolution and spread of resistance genes in populations and are thus a vital component of anthelmintic resistance studies

* Corresponding author at: Veterinary Parasitology, Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool, Liverpool L3 5RF, UK.
E-mail address: nbeesley@liverpool.ac.uk (N.J. Beesley).

http://dx.doi.org/10.1016/j.ijpara.2016.09.007
0020-7519/

(Gilleard and Beech, 2007). They allow us to identify management factors influencing the migration of resistance genes, and so help to mitigate against their spread. It is recognised that the husbandry and management of different farms have the potential to affect the population structure of parasites (Grillo et al., 2007) by influencing the movement of the definitive host and, therefore, *F. hepatica* parasites. Additionally, the age and production system for an animal influences the extent to which it has been exposed to *F. hepatica* on pasture and to what extent it may have been treated with anthelmintics.

A number of aspects of *F. hepatica* biology have the potential to influence genetic diversity and population structure and therefore impact on the spread of genes, including those responsible for anthelmintic resistance (Hodgkinson et al., 2013). Firstly, it is known that clonal expansion of *F. hepatica* occurs within the snail intermediate host, *Galba truncatula* (Thomas, 1883; Krull, 1941). Therefore, there is the potential for multiple metacercariae of the same origin and genotype to exist on pasture, and parasites with the same multilocus genotype (MLG) have been found within, and shared between, definitive hosts (Vilas et al., 2012). Secondly, as a hermaphrodite, *F. hepatica* can self- and cross-fertilise. Self-fertilisation is a form of inbreeding which has the potential to influence allele frequency in a population. If anthelmintic resistance is a recessive trait, a high level of self-fertilisation means there is the potential for resistant alleles to spread more rapidly. Thirdly, clonal expansion in the snail, combined with low levels of infection in the snail population as a whole, could pose a bottleneck to gene flow and lead to population structuring. Finally, *F. hepatica* has a wide host range, infecting multiple species of domestic and wild animals (Parr and Gray, 2000; Vignoles et al., 2001, 2004; Arias et al., 2012). This may allow the flow of genes amongst livestock species and maintain a reservoir of genetic diversity in wild animals. In addition, adult *F. hepatica* in the definitive host can be long-lived (Durbin, 1952), and their reproductive capacity may be present for many years in untreated animals.

An understanding of *F. hepatica* genetic diversity has implications for the development and validation of new methods of control. Knowledge of the provenance, infectivity, pathogenicity and resistance status of laboratory isolates is important (Hodgkinson et al., 2013). Laboratory maintained isolates of *F. hepatica* are frequently used in research, including in drug and vaccine trials (Fairweather, 2011b), but are not representative of field isolates. For example, the Cullompton isolate is aspermic and triploid (Fletcher et al., 2004), the Sligo isolate exhibits abnormal spermatogenesis (Hanna et al., 2008), and the Fairhurst isolate is highly homogenous (Walker et al., 2007).

Previously we have shown that the British *F. hepatica* population naturally infecting sheep and cattle is diploid, spermic and predominantly reproduces by sexual reproduction (Beesley et al., 2015). Here, we present the largest population genetic study to date for *F. hepatica*, involving the genotyping of 1579 adult parasites. Adult *F. hepatica* samples were collected from three countries (Scotland, England and Wales) from two definitive host species (sheep and cattle), and MLGs were produced using our panel of microsatellite markers (Cwiklinski et al., 2015a). A proportion of hosts harboured multiple, genotypically identical parasites. However, overall, we found substantial genetic variation within populations infecting a given host and high levels of genetic diversity in the liver fluke population as a whole, but little differentiation between populations infecting sheep and cattle. Our data indicate a lack of geographic or host species structuring in *F. hepatica* from Great Britain and high gene flow, which could promote the emergence and spread of drug resistance in a population. The results of this study may be relevant to other areas where widespread movement of livestock is practised.

2. Materials and methods

2.1. Populations of F. hepatica

Adult *F. hepatica* were recovered from the livers of 44 naturally infected sheep between November 2012 and April 2013, from two abattoirs (Wales and central England, UK). Similarly, parasites were recovered post mortem from 31 cattle livers between October 2013 and January 2014, from an abattoir (Wales, UK). A total of 950 parasites were genotyped from sheep and 629 from cattle (Table 1). The Rapid Analysis and Detection of Animal Related Risks (RADAR), Animal and Plant Health Agency (APHA, UK, https://www.gov.uk/government/organisations/animal-and-plant-health-agency) provided information on the origin of cattle livers. Adult parasites were isolated from the bile ducts and incubated for 2 h at 37 °C in 1–2 ml of DMEM with 120 μg ml^{-1} gentamicin and 120 μg ml^{-1} amphotericin B to allow purging of intestinal contents and eggs. Parasites were snap frozen and stored at −80 °C.

2.2. Preparation of DNA template and microsatellite genotyping

A small section of each parasite, anterior to the ventral sucker, to avoid contamination with eggs or sperm, was used for DNA extraction. The tissue was divided into small pieces to ensure efficient lysis. DNA extraction was performed using a DNeasy Blood & Tissue Kit (Qiagen, UK) as per the manufacturer's instructions and DNA was diluted to 10 ng μl^{-1}.

A panel of 15 microsatellites previously validated with 46 adult *F. hepatica* (Cwiklinski et al., 2015a), was applied to each parasite DNA sample to generate an individual MLG. For efficiency the methodology was modified for a multiplex approach; the Type-it Microsatellite PCR kit (Qiagen) was used according to the manufacturer's instructions (Cwiklinski et al., 2015a). The 15 loci were grouped as follows: (i) Fh_1, Fh_6, Fh_13, Fh_15 annealing temperature 55 °C; (ii) Fh_2, Fh_3, Fh_5, Fh_8, annealing temperature 57 °C; (iii) Fh_9, Fh_10, Fh_11, Fh_14, annealing temperature 57 °C; and (iv) Fh_4, Fh_7 and Fh_12, annealing temperature 59 °C. PCR products were visualised using SYBR Safe DNA stain (Life Technologies, UK) on a 1.5% agarose gel. PCR products were diluted 25-fold in HPLC-grade water (Sigma–Aldrich, UK), and sequenced using an ABI PRISM 3100 Genetic Analyser capillary electrophoresis system (Life Technologies) (Cwiklinski et al., 2015a). Fragment sizes were determined using Peak Scanner v2.0 software (Life Technologies).

2.3. Population genetic analyses

Allele frequencies were determined using CERVUS 3.0.7 (Kalinowski et al., 2007; available from www.fieldgenetics.com) and genotype frequencies were determined using GENEPOP 4.2.1 (Rousset, 2008; available from http://kimura.univ-montp2.fr/~rousset/Genepop.htm). Null allele frequency was determined using CERVUS 3.0.7 (Kalinowski et al., 2007). Loci Fh_1, Fh_3, Fh_4, Fh_7, Fh_8 and Fh_14 were identified as having greater than 5% frequency of null alleles (Table 2), therefore these loci, together with locus Fh_9 which produced inconsistent traces, were excluded from the remaining population genetic analyses.

Average heterozygosities were determined for each locus using Arlequin 3.5.1.3 (Excoffier and Lischer, 2010). Unbiased heterozygosity was calculated using GenClone 2.0 (Arnaud-Haond and Belkhir, 2007). Heterozygosity was determined for each individual parasite based on the proportion of loci that were heterozygous. Mann–Whitney *U* tests were performed using Minitab 17. GenClone 2.0 (Arnaud-Haond and Belkhir, 2007) was used to identify repeated MLGs (defined as two or more parasites sharing the same

Please cite this article in press as: Beesley, N.J., et al. *Fasciola hepatica* demonstrates high levels of genetic diversity, a lack of population structure and high gene flow: possible implications for drug resistance. Int. J. Parasitol. (2016), http://dx.doi.org/10.1016/j.ijpara.2016.09.007