Comparison of molecular markers used for population genetic analyses of *Fasciola gigantica* from Pakistan

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Introduction

Fasciola hepatica and *Fasciola gigantica*, are the causative agents of fasciolosis, an economically important disease of people and their livestock worldwide. *Fasciola* spp. are typically restricted geographically, with *F. hepatica* distributed in more temperate areas and *F. gigantica* favouring tropical climates, however there are areas where the two species co-exist, such as Pakistan, resulting in the potential for species hybridisation. This has implications for the severity of disease, and potential spread of genetic loci associated with drug resistance within liver fluke populations.

Several molecular markers have been developed for analyses and confirmation of *Fasciola* spp., based on ribosomal, mitochondrial and nuclear regions of the liver fluke genomes. Using these markers, studies have shown that geographically dispersed liver fluke populations display high levels of genetic diversity.

Aim:

- To determine the *Fasciola* spp. present in cattle and buffalo across Pakistan.
- Compare four molecular markers available for *Fasciola* spp. analyses.
- Assess the level of genetic diversity in these *Fasciola* spp. samples based on mitochondrial sequences.

Methods

- Adult parasites (n=595) were collected from buffalo and cattle across four provinces in Pakistan (Baluchistan, Gilgit and Skardu, Khyber Pakhtunkhwa, Punjab).
  - 4-7 parasites recovered from each animal.
  - DNA extracted using the Qiagen DNeasy Blood and Tissue kit.
- Four molecular markers were used.
  - Phosphoenolpyruvate carboxykinase (*pepck*) [1]
  - Fatty acid binding protein (*fabp*) [2]
  - Random Amplified Polymorphic DNA (RAPD) [3]
  - NADH dehydrogenase (*nad1*) [4]
- *pepck*, *fabp* and RAPD PCR products amplified from individual adult parasite samples were visualised on an agarose gel.
- *nad1* PCR products from pooled samples from individual animals were sequenced using Illumina MiSeq2 and the data analysed using the mothur pipeline [5].

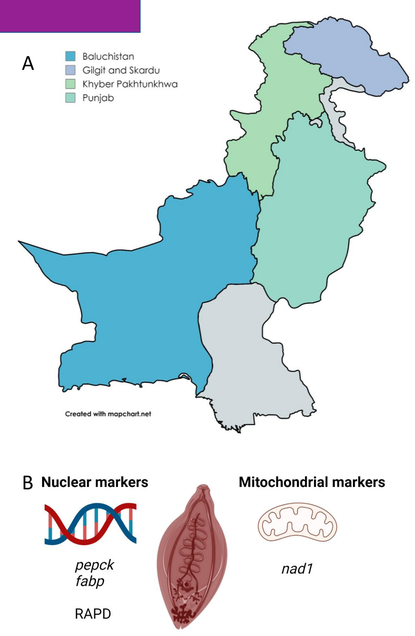

Fig 1. A. Map of Pakistan highlighting the four provinces from which adult parasites were recovered. B. Graphical representation of the type of molecular markers used in the study.

Results

PCR results:

- All the molecular marker PCRs were optimised and carried out with *F. hepatica* and *F. gigantica* DNA samples as positive controls,
  - Positive controls defined based on the geographical location the samples were sourced from – *F. hepatica*: UK isolate; *F. gigantica*: African isolates.
- *pepck*: PCR products were amplified from all 595 samples, corresponding to the 510 bp PCR product size associated with *F. gigantica*.
- *fabp*: PCR products corresponding to the 190 bp product size associated with *F. gigantica* were only amplified from 321 samples (54%). The remaining samples (274 parasites; 46%) were negative, indicating potential sequence differences in the *fabp* primer sequences.
- RAPD: The *Fasciola* species classification of the 274 samples negative in the *fabp* PCR was confirmed using the RAPD PCR [ref]. RAPD PCR products were amplified for all the 274 samples, which corresponded to *F. gigantica*.

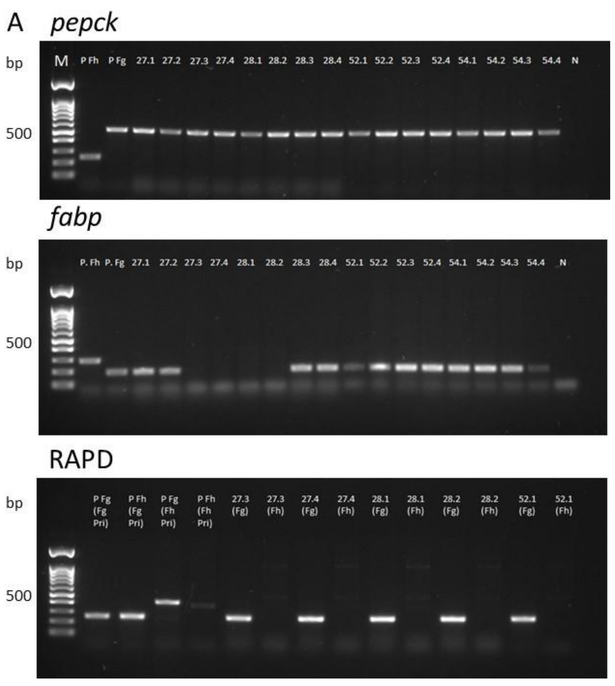

Fig 2. *Fasciola* spp. molecular markers.
A. Agarose gel electrophoresis of the *pepck*, *fabp* and RAPD PCR amplicons. Examples from the Gilgit and Skardu province. Positive controls: *F. hepatica*, P Fh; *F. gigantica*, P Fg. N: Negative control. M: 100 bp ladder.
B. Table highlighting the proportion of samples across the four provinces for which the *fabp* marker which could not be amplified.

B. Proportion of samples negative in *fabp* PCR	
Baluchistan	61/126; 48%
Gilgit and Skardu	16/52; 29%
Khyber Pakhtunkhwa	107/252; 42%
Punjab	90/165; 55%

Sequence analysis of *nad1* amplicons:

- DNA from parasites recovered from an individual animal (Table 2) were pooled in equimolar concentrations. The *nad1* PCR was carried out on 100 ng/ul per sample.

Table 2	Number of animals	Adult parasites/animal
Baluchistan	18; 16 buffalo; 2 cattle	7
Gilgit and Skardu	13; 11 buffalo; 2 cattle	4
Khyber Pakhtunkhwa	33; 15 buffalo; 18 cattle	5
Punjab	36; 23 buffalo; 13 cattle	7

- The sequences were aligned with *F. gigantica* and *F. hepatica nad-1* reference sequences available from the NCBI database to confirm the *Fasciola* spp. classification. Consistent with the PCR data, all the samples were classified as *F. gigantica.*
- Haplotype analysis revealed a predominance for single haplotypes across the animal samples. As the samples represent pooled samples of the 4-7 parasites/animal, this highlights that animals were infected with a genetically similar source of metacercariae, but that this differed between animals. Representative data in Fig. 3 and Table 3.
- One haplotype (H1) dominated across all four provinces.

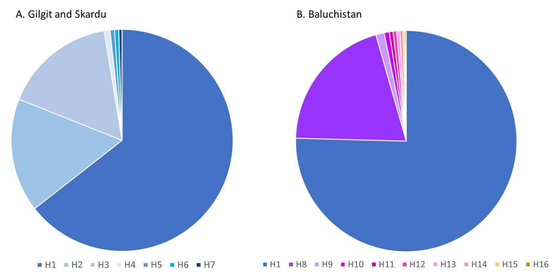

Fig 3. Haplotypes represented in the (A) Gilgit and Skardu and (B) Baluchistan provinces. Representative of the predominant sequences in the total sequences generated for the animals per province.

Table 3. Predominant haplotypes per animal, represented as percentage of sequences.

Gilgit and Skardu	Predominant haplotype (%)	Baluchistan	Predominant haplotype (%)
G1	H3 (70%); H4 (6%)	B1	H1 (66%); H9 (9%)
G2	H3 (66%); H6 (6%)	B2	H8 (84%)
G3	H2 (76%)	B3	H8 (92%)
G4	H2 (71%)	B4	H1 (61%); H16 (3%)
G5	H1 (81%)	B5	H1 (67%)
G6	H1 (80%)	B6	H1 (75%)
G7	H1 (86%)	B7	H1 (45%); H14 (4%)
G8	H1 (90%)	B8	H1 (60%); H12 (4%)
G9	H1 (88%)	B9	H8 (645); H11 (5%)
G10	H1 (56%); H5 (5%)	B10	H8 (65%); H13 (5%)
G11	H1 (60%); H7 (6%)	B11	H1 (72%); H10 (6%)
G12	H1 (80%)	B12	H1 (70%); H15 (7%)
G13	H1 (83%)	B13	H1 (88%)
		B14	H1 (71%)
		B15	H1 (54%); H14 (3%)
		B16	H1 (77%)
		B17	H1 (76%)
		B18	H1 (74%); H9 (8%)

Conclusions

- All 595 parasites were confirmed as *F. gigantica* using both nuclear and mitochondrial markers.
- Results indicate potential genetic diversity in *fabp* gene sequence, that is not associated with geographic location/parasite isolate.
- Sequence analysis of the mitochondrial *nad1* gene revealed a predominance for single haplotypes within liver fluke parasites infecting individual animals, consistent with other *Fasciola* spp. genetic studies in Pakistan [4].
- Study verifies the high level of genetic diversity observed in *F. gigantica* parasites in Pakistan.
- Inconsistencies can be observed between the *Fasciola* spp. molecular markers.
- Further analysis is required to determine the utility of the *fabp* maker.
- Analyses of genetic diversity in liver fluke populations requires multi-locus markers.

Acknowledgements & References

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