T-RFLP for Identification of Spirorchiid Flukes in Green Sea Turtle Tissues: Application for investigations in disease epidemiology and pathology

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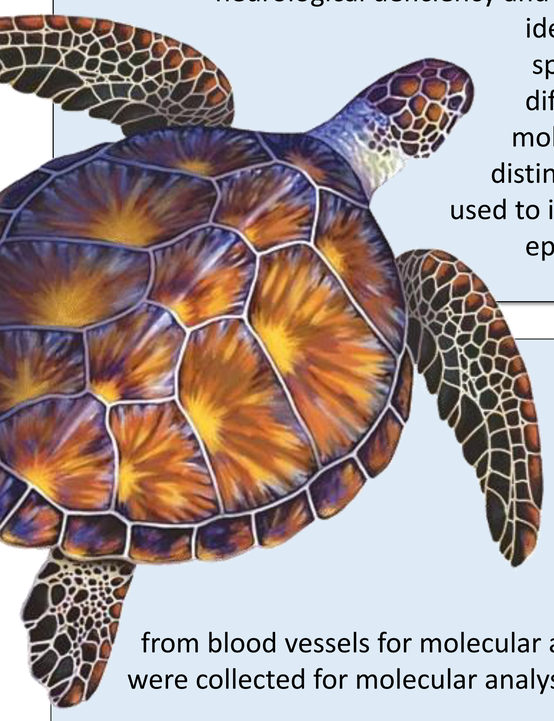
Introduction

Spirorchiid blood flukes are important parasites of sea turtles, playing a role in strandings and mortalities globally and causing up to 42% of deaths in south-east Queensland's green sea turtles (*Chelonia mydas*) (Flint, 2010). Their ova are regularly found in most tissues, where they cause inflammatory responses ranging from mild to severe. Of particular interest are neurological lesions in the brain, which have been associated with neurological deficiency and death. However, ova cannot be morphologically identified beyond genus level, making detection of species specific trends in epidemiology and pathology difficult. In order to overcome this problem, a molecular assay was developed to detect and distinguish between spirorchiid ova in tissues and was used to investigate and compare the pathogenicity and epidemiology of each genus and species.



Methods

Sample collection

A total of 51 *C. mydas* were collected between Townsville and the Gold Coast (Queensland, Australia) and underwent post-mortem examination. Adult spirorchiids were collected from blood vessels for molecular and morphological characterisation, and tissues were collected for molecular analysis and histopathology.

Assay design

Sequence data from the collected spirorchiids was used to design primer sets to target specific genera present in the region. Multiplex PCR was used to detect the presence or absence of each genus, following which singleplex PCRs were performed using genus/genera-specific fluorescently tagged (6-FAM) primer pairs. Final PCR products were digested using restriction endonucleases to produce size distinguishable fragments for each species (**Figure 1**).

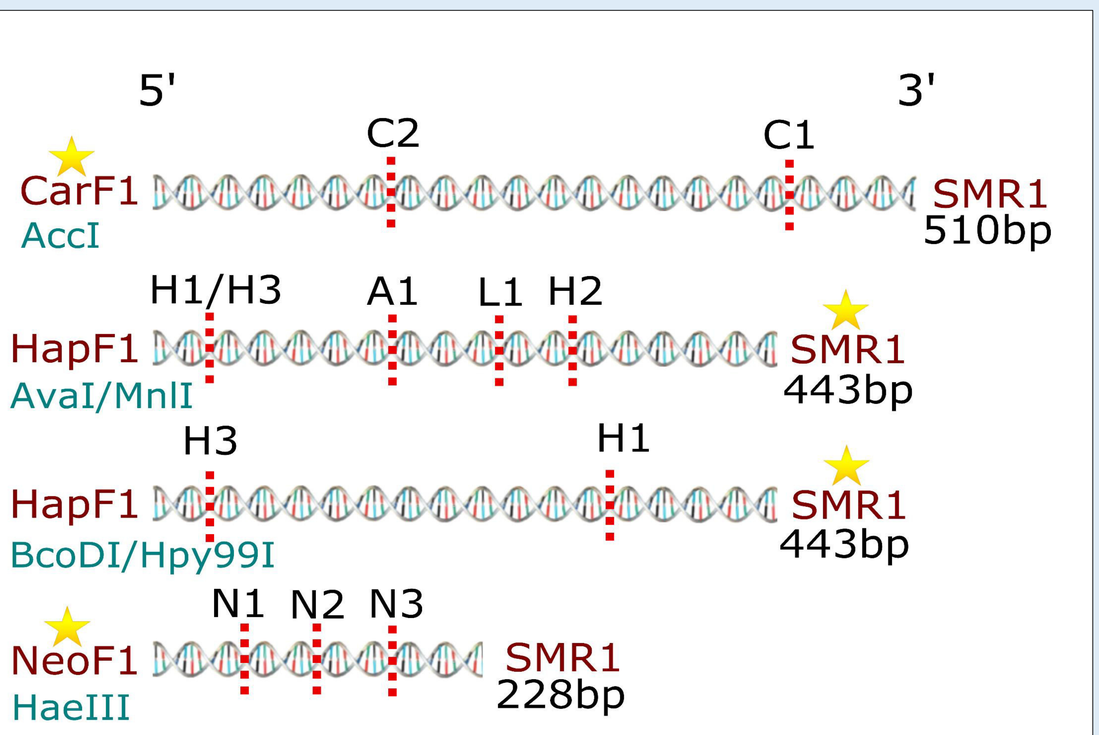


Figure 1. T-RFLP assay to detect spirorchiids in green sea turtle tissues. Maroon denotes primer names. Blue denotes restriction endonucleases. Red dashed lines indicate restriction sites for each target species, denoted by H1/H2/H3 etc. Yellow star denotes primer with fluorescent 6-FAM tag.

Results were visualised by capillary electrophoresis (**Figure 2**).

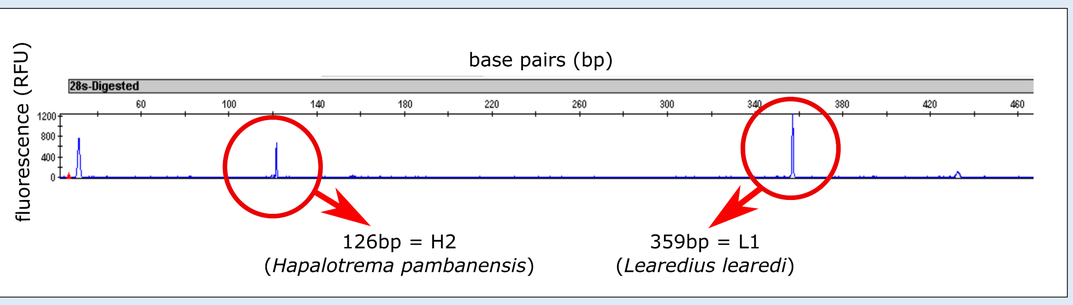


Figure 2. Electropherogram output of capillary electrophoresis. Fluorescence peaks indicate the size of DNA fragments present. Abbreviations: bp = base pairs. RFU = relative fluorescence units.

Statistical analyses

The association between granulomas in the brain and spirorchiid infection type (single or multi species) used multivariable generalised linear model (GLM) adjusted for the effect of age, sex, and body condition. In other organs, Fisher's exact test (95% confidence interval) or Mann-Whitney U-test was used. All analyses were performed using STATA version 13.

Results

Assay performance

- The T-RFLP method was applied to 151 tissue samples and successfully differentiated between twelve spirorchiid genotypes.
- It was more sensitive than visual diagnosis, detecting infections in 28 of 32 tissues that were negative on histology.

Spirorchiids

- Present in 96.7% of samples
- *Neospirorchis* Genotype 2 the most common species, found in 93% of samples
- Highest spirorchiid diversity in the spleen and pancreas; lower diversity in the brain
- Diversity in kidney and liver inversely proportionate to host age, but no clear effect of age/sex/body condition in other organs.

Pathology

- Granulomas of varying severity were the most common lesions and affected most organs (**Figure 3**).
- In the brain, they were more likely to occur where ova from multiple spirorchiid species were present.

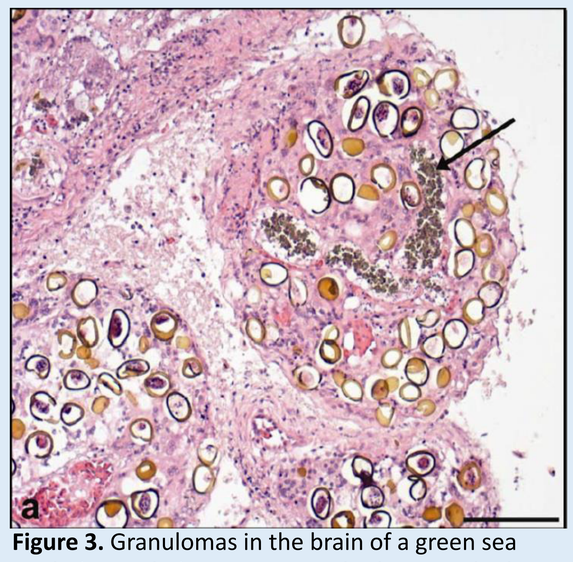
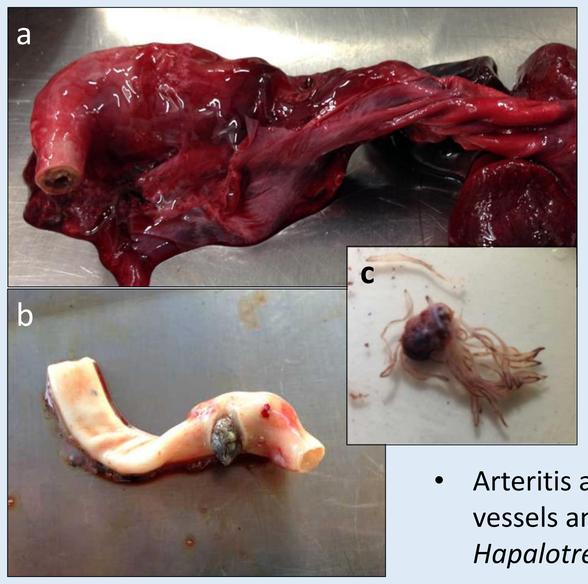


Figure 3. Granulomas in the brain of a green sea turtle (*C. mydas*) containing multiple spirorchiid eggs. An adult parasite (arrow) is also present. (Flint, 2010)



- Arteritis and thrombi were also common in the great vessels and were usually associated with adult *Hapalotrema* or *Learedius* spp. (**Figure 4**).

Figure 4. Lesions of the aortas of green sea turtles: a) severe arteritis and thrombus formation associated with adult *Hapalotrema postorchis* b) Free thrombus in situ within the vessel lumen with attached *H. postorchis* c) thrombus and flukes following removal from vessel.

Discussion and Conclusions

- The T-RFLP method successfully identified spirorchiid ova to species level for the first time, and proved to be reliable, efficient and accessible.
- It demonstrated improved sensitivity and specificity over traditional microscopic methods and therefore contributes improved data to epidemiological and pathology studies.
- Spirorchiids were found to be almost universally present in stranded turtles from Queensland.
- Age appears to play a factor in susceptibility to infection, potentially due to lack of acquired immunity.
- Further large scale studies will be required to confirm the relationship between age, body condition and diversity of spirorchiid infection, and are likely to confirm that younger, chronically ill turtles are more likely to become severely infected.
- The correlation of infection type (which species and how many) and severity with environmental factors is likely to be important in determining the factors driving severe infection.
- The investigation of options for ante-mortem diagnostic tools should be a future priority, leading to field surveys of wild turtle populations and enabling monitoring of treatment and recovery in rehabilitation.

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