Characterisation of Community-Derived *Hymenolepis* Infections in Australia

Marion G. Macnish

BSc. (Medical Science) Hons

Division of Veterinary and Biomedical Sciences

Murdoch University

Western Australia

This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

2001

I declare that this thesis is my own account of my research and contains as its main work which has not been submitted for a degree at any other educational institution.

…….

(Marion G. Macnish)

Abstract

Hymenolepis nana is a ubiquitous parasite, found throughout many developing and developed countries. Globally, the prevalence of *H. nana* is alarmingly high, with estimates of up to 75 million people infected. In Australia, the rates of infection have increased substantially in the last decade, from less than 20% in the early 1990's to 55 - 60% in these same communities today. Our knowledge of the epidemiology of infection of *H. nana* is hampered by the confusion surrounding the host specificity and taxonomy of this parasite. The suggestion of the existence of two separate species, *Hymenolepis nana* von Siebold 1852 and *Hymenolepis fraterna* Stiles 1906, was first proposed at the beginning of the 20th century. Despite ongoing discussions in the subsequent years it remained unclear, some 90 years later, whether there were two distinct species, that are highly host specific, or whether they were simply the same species present in both rodent and human hosts. The ongoing controversy surrounding the taxonomy of *H. nana* has not yet been resolved and remains a point of difference between the taxonomic and medical literature.

The epidemiology of infection with *H. nana* in Australian communities is not well understood as the species present in these communities has never been identified with certainty. It is not clear which form of transmission commonly occurs in Australia, whether the *H. nana* 'strain/species' present in the north-west of Western Australia is present in human and rodent hosts, or whether humans harbour their own 'strain/sub-species' of *Hymenolepis*. Furthermore, it is not known whether mice are a potential zoonotic source for transmission of *Hymenolepis* to human hosts. In this study, 51 human isolates of *H. nana* were inoculated into highly susceptible laboratory rodent species. However, these failed to develop into adult worms in all instances, including when rodent species were chemically and genetically immunosuppressed. In addition, 24 of these human isolates were also cross-tested in the flour beetle intermediate host, *Tribolium confusum*. Of these, only one isolate developed to the cysticercoid stage in beetles, yet when inoculated into laboratory rodents, the cysticercoids also failed to develop into adult stage.

Since isolates of *H. nana* infecting humans and rodents are morphologically indistinguishable, the only way they can be reliably identified is by comparing the parasite in each host using molecular criteria. In the current study, three regions of ribosomal DNA, the small subunit (18S), the first internal transcribed spacer (ITS1) and the intergenic spacer (IGS) were chosen for genetic characterisation of *Hymenolepis* spp. from rodent and human hosts from a broad geographic range. In addition, a mitochondrial gene, the cytochrome *c* oxidase subunit 1 (C01) gene and a non-ribosomal nuclear gene, paramyosin, were characterised in a number of *Hymenolepis* isolates from different hosts.

A small PCR fragment of 369 bp, plus a larger fragment of 1223 bp, were sequenced from the 18S gene of reference isolates of *H. nana* and the rat tapeworm *H. diminuta.* Minimal sequence variation was found in the two regions of the 18S between these two morphologically distinct, phylogenetically recognised species, *H. nana* and *H. diminuta*, and this indicated that the 18S gene was too conserved for further genetic characterisation of isolates of *H. nana* from different hosts.

A large number of human isolates of *H. nana* (104) were characterised at the ITS1 using PCR-restriction length fragment polymorphism (PCR-RFLP). The profiles obtained were highly variable and often exceeded the original size of the uncut fragment. This was highly suggestive of the existence of ribosomal spacers that, whilst identical in length, were highly variable in sequence. To overcome the problems of the variable PCR-RFLP profiles, further characterisation of the ITS1, by cloning and sequencing 23 isolates of *H. nana,* was conducted and this confirmed the existence of spacers which, although similar in length (approximately 646 bp), differed in their primary sequences. The sequence differences led to the separation of the isolates into two clusters when analysed phylogenetically. This sequence variation was not, however, related to the host of origin of the isolate, thus was not a marker of genetic distinction between *H. nana* from rodents and humans. Indeed, the levels of variability were often higher within an individual isolate than between isolates, regardless of whether they were collected from human or mice hosts, which was problematic for phylogenetic analysis. In addition, mixed parasite infections of *H. nana* and the rodent tapeworm *H. microstoma* were identified in four humans in this study, which was unexpected and surprising, as there have been no previous reports in the literature documenting humans as definitive hosts for this parasite. Further studies are required, however, to determine if the detection of *H. microstoma* in humans reflects a genuine, patent infection or an atypical, accidental occurrence.

Sequencing of the mitochondrial cytochrome *c* oxidase 1 gene (C01) in a number of isolates of *Hymenolepis nana* from rodents and humans identified a phylogenetically supported genetic divergence of approximately 5% between some mouse isolates compared to isolates of *H. nana* from humans. This provided evidence that the mitochondrial C01 gene was useful for identifying genetic divergences in *H. nana* that were not resolvable using nuclear loci. Despite a morphological identity between isolates of *H. nana* from rodent and human hosts, the genetic divergence observed between isolates at the mitochondrial locus was highly suggestive that *H. nana* is a species complex, or "cryptic" species (= morphologically identical yet genetically distinct). In addition, whilst not supported by high bootstrap values, a clustering of the Australian human isolates into one uniform genetic group that was phylogenetically separated from all the mouse isolates was well supported by biological data obtained in this study. To

confirm the phylogeny of the C01 tree a small segment of the nuclear gene, paramyosin, was sequenced in a number of isolates from humans and rodents. However, this gene did not provide the level of heterogeneity required to distinguish between isolates from rodent and human hosts. The high sequence conservation of the paramyosin gene characterised in this study did not refute the finding that *H. nana* may be a cryptic species that is becoming host adapted. It simply did not provide additional data to that already obtained.

A DNA fingerprinting tool, PCR-RFLP, of the ribosomal intergenic spacer (IGS), was developed in this study in order to evaluate its usefulness in tracing particular genotypes within a community, thus determining transmission patterns of *H. nana* between rodent and human hosts. Analysis of the IGS of numerous *H. nana* isolates by PCR-RFLP identified the presence of copies of the IGS that, whilst similar in length, differed in their sequence. Similar to that observed in the ITS1, the existence of different IGS copies was found in both rodent and human isolates of *H. nana*, thus the variability was not evidence of the existence of a rodent- or human-specific genotype. Evaluation of the intergenic spacer (IGS) as a fingerprinting tool suggests that this region of DNA is too variable within individuals and thus, cannot be effectively used for the study of transmission patterns of the tapeworm *H. nana* between different hosts.

In summary, it appears that the life cycle of *H. nana* that exists in remote communities in the north-west of Western Australia is likely to involve mainly 'human to human' transmission. This is supported by both the biological and genetic data obtained for the mitochondrial locus in this study. The role of the intermediate hosts, such as *Tribolium* spp., in the *Hymenolepis* life cycle is still unclear, however it would appear that it may be greatly reduced in the transmission of this parasite in remote Australian communities. In the future, it is recommended that further genetic characterisation of faster evolving mitochondrial genes, and/or suitable nuclear genes be characterised in a larger number of isolates of *H. nana*. The use of techniques which can combine the characterisation of genotype and phenotype, such as proteomics, may also be highly valuable for studies on *H. nana* from different hosts.

-oOo-

TABLE OF CONTENTS

Acknowledgements

The moment has arrived……..I am finally sitting down with a weak cappuccino at my favourite Café in Fremantle contemplating how I can adequately express the feelings that I have in finally reaching this point – the thesis hand in. I am also wondering how I can adequately express my heartfelt gratitude to a growing list of people who have helped make it all possible.

First and foremost, I would like to thank the two people who have nurtured and guided me throughout my entire PhD – my supervisors Andrew Thompson and Una Morgan. They never failed to provide me with welcome advice and positive thinking. Thankyou so much to both of you.

It doesn't seem possible to get through this process without large amounts of technical and academic help. My most sincere appreciation is extended to Dr. Jerzy Behnke in the UK. Jerzy was an invaluable source of many things – worm specimens from field trips in Portugal, numerous taxonomy references and preserved histology specimens of Hymenolepis, the images of which you will find in the following pages. Jerzy has also been a major springboard for many of my crazy ideas and suggestions. I am eternally grateful for his patience in reading and responding to my long-winded emails. I would like to sincerely thank Russ Hobbs for his fantastic efforts in constructing gel photos and microscope images that are also presented in this thesis. He has made a great contribution with his brilliant computer drawing skills. Thanks also go to Russ for the long discussions we had on sticky taxonomy issues!

I would like to thank Paul Monis in South Australia who generously helped with the phylogenetic analysis of my sequencing results. Thanks also to Clare Constantine and Alan Lymbery who helped me to understand what it all means.

I tried hard not to leave a path of destruction behind me as I emerged into the last crucial weeks before finishing but I am quite certain that those who helped me format the text and diagrams hope I never do this to them again! Pat Marshall was asked to do the impossible – and managed. She turned 50 separate files into one large document and managed to keep them there, despite the computer, not because of it. I can't thank her enough for her patience and even-temper throughout. My two buddies Louise Pallant and Carolyn Read were an ongoing source of support, food, jokes and hugs to prop me up on the final formatting and print run. Thanks heaps guys!!

I would like to extend a warm appreciation to the best group anyone could wish to work with – the Parasitology/Pharmacology group at Murdoch University. A special thanks to Aileen Elliot for passing on any Hymenolepis specimens that came through the department. It's been great to be part of the team.

As I sit here sipping my cappuccino I realise I can now get into the shower at night and not have to hop back out again to jot down that "perfect sentence" that has eluded me the entire evening. What a joy to realise I no longer need to take pen and paper into the bathroom. Ditto for when I sink under the doonah only to realise I've just composed an entire paragraph that works. I no longer have to get out of bed to write it all down!

When things got really tough and Andy started suggesting I should go home, pour myself a glass of red wine and try re-writing a couple of chapters. Meanwhile, I'm secretly realising that Andy has no idea that this whole thesis has been written with a glass of red wine at hand. OK, so as Louise suggested, I should go home, drink a strong cup of coffee, sober up and re-write that damn chapter. I hope you can tell which ones have been written on strong coffee!

Lastly, I would like to convey to the entire world what an amazing family I have. I swear I could not have got through this without them. Never again will we spend entire weekends agonising over where commas and semi colons should go in sentences. To my mother Fay, my father Ian, my sisters Barbara, Jenny and Anne and my two brothers Kim and Graham

This thesis is dedicated to them all.

Publications

Part of the work presented in this thesis has been accepted for publication or presented at scientific conferences in the following form:

Abstracts of Papers Presented at Conferences

Macnish, M. G., Morgan, U. M. and Thompson, R. C. A. (1998). Molecular characterisation of *Hymenolepis nana* in Aboriginal communities in north west Western Australia. In *73rd Annual Meeting of the American Society of Parasitologists*, p44. August 16-20, Kona, Hawaii.

Macnish, M. G., Morgan, U. M., Monis, P. T., Behnke, J. M. and Thompson, R. C. A. (1999). Genetic characterisation of three DNA loci of *Hymenolepis nana*. In *Annual Scientific Meeting of the Australian Society for Parasitology Inc.*, p26. September 26-30, Yeppoon, Queensland, Australia.

Macnish, M. G., Morgan, U. M., Behnke, J. M. and Thompson, R. C. A. (2000). Evidence for the zoonotic transmission of the rodent tapeworm *Hymenolepis microstoma* to humans. In *Joint meeting of the New Zealand Society for Parasitology and Australian Society for Parasitology*, p67. September 25-29, Wellington, New Zealand.

Journal Articles

Macnish, M. G., Morgan, U. M., Behnke, J. M. and Thompson, R. C. A. (2001)**.** Failure to infect laboratory rodents with humans isolates of *Hymenolepis nana*. *Journal of Helminthology **In Press***.

Macnish, M. G., Morgan, U. M., Monis, P. T., Behnke, J. M. and Thompson, R. C. A. (2001)**.** A molecular phylogeny of nuclear and mitochondrial sequences in *Hymenolepis nana* (Cestoda) supports the existence of a cryptic species. *Parasitology Submitted for publication* .

Macnish, M. G., Ryan, U. M., Behnke, J. M. and Thompson, R. C. A. (2001)**.** Detection of the rodent tapeworm *Rodentolepis Hymenolepis microstoma* in humans. A new zoonosis? *International Journal for Parasitology Submitted for publication*.