Chapter 7: Conclusions and Future Research

7.1 Overview

An estimated 200 million people in world wide suffer from schistosomiasis most of whom live in sub-Saharan Africa followed by Asia, North Africa, and South America [8]. Most schistosome infections are caused by *S. mansoni*, *S. haematobium* or *S. japonicum*, with two other species (*S. intercalatum* and *S. mekongi*) contributing less to the case load [6]. The global burden of death and chronic disability of schistosomiasis is high – with an estimated 20 million suffering severe disease [8]. Although an effective anti-schistosome drug, praziquantel, is available, rapid reinfection occurs following treatment, with schistosomiasis continuing to be a major public health problem in many tropical areas [9, 15]. Additionally, there is a concern that schistosome resistance to praziquantel may be inevitable [9]. Since there are no anti-schistosome vaccines currently available, the identification of vaccine targets, and new drug therapies and diagnostic methods are now a priority [15].

Schistosomiasis japonica remains a significant public health issue in North-East and South-East Asia, particularly in China and the Philippines. The causative species, *S. japonicum* occurs as several distinct geographical strains that have been identified from Taiwan, Indonesia, Japan, China and the Philippines [61]. As discussed in Chapter 1 (section 1.4) the Chinese (SJC) and Philippine (SJP) forms of *S. japonicum* exhibit a number of morphological and other phenotypic differences, including pre-patent period (26 and 28 days, SJC and SJP respectively), tegument topography, adult worm size (SJC longer than the SJP), virulence (SJC is more pathogenic) and the sub-species of snail intermediate host infected [59-61]. Until the studies described herein,

major genotypic or gene expression differences have not been identified between the two strains [61].

7.2 Major findings presented and future research directions

7.2.1 Microarray construction

The major goal of this thesis was to contribute to the design of a microarray platform and use it to explore the transcriptome of *S. japonicum*. As discussed in Chapter 3 (section 3.1), a 22,575 feature 60-mer oligonucleotide microarray was constructed using two extensive EST public datasets for *S. japonicum* [25] and *S. mansoni* [58]. The design has increased the coverage of the transcriptome of both *S. mansoni* and *S. japonicum* species to an estimated 80–90%, based on the number of hybridizing probes and estimated transcriptome size [105]. Moreover, this is the first commercially manufactured resource for studying schistosomes that incorporates the flexibility of design to change the probe sequences *in silico*. Additionally, *S. mansoni* probes may be used for differential analysis of same *S. japonicum* species due to equal sequence mismatch, being reciprocally applicable for *S. mansoni* studies [4, 105].

The constructed microarray will continue to be a valuable resource for further expression studies. The next generation of this microarray will see an expansion of the number of probes currently on the array. The new design will have four microarrays per slide each with more than 44,000 features [193]. The original design will be modified by reviewing all previous microarray data obtained from the array described here. The probe data will be placed into three major categories; (A) hybridized and differentially expressed (p-value $\leq$0.05); (B) hybridized but not differentially expressed and; (C) continual non-hybridization. The latter group of probes will not be included in the new

microarray format. Two options then remain; either include new contigs that have been recently published to create 44,000 features, or duplicate the 22,000 new and remaining probes. The new microarray chip kit may also contain "spike-ins", so that where a mixture consisting of a sample and two "spike-ins" (Agilent, Santa Clara, USA) (comprising 10 *in vitro* synthesised, polyadenylated transcripts in predetermined ratios) are co-hybridized onto a microarray containing "spike-in" probes. Subsequently, expected comparative log ratios will then be used to monitor a microarray workflow for linearity, sensitivity and accuracy [194]. It should be emphasised that any new design of the chip will not negate the current resource which will continue to provide a much wider coverage of the *Schistosoma sp*. transcriptome than any other reported schistosome microarray [63, 195, 196].

7.2.2 Strain related expression

There are several *S. japonicum* strains in south east Asia; these include strains from China, Japan, Indonesia, Philippines and Twain [10]. I have hypothesized that there are major differences at the expression levels between these strains of *S. japonicum*, which may explain the known morphological and other phenotypic differences. In order to explore this, the schistosome microarray described in Chapter 3 was used to profile the strain-associated differential gene expression between the Chinese (SJC) and Philippine (SJP) forms of *S. japonicum*. The analysis showed that 593, 664 and 426 probes were differentially expressed between the two geographical strains, either as mix-sexed adults, male worms and female worms. This differential expression included probe sequences that may encode proteins that are import to the evolution, virulence and differential drug efficacy of schistosomes. As discussed in Chapter 4 (section 4.3.6) this new information will provide new insights into aspects of the biology of *S. japonicum*,

such as an understanding of its unique dioecious state. Additionally it will provide a fulcrum for understanding the transcriptional basis of the phenotypic differences evident between the two strains. Moreover, the strain associated probes identified here serve as starting points for further research into defining better chemotherapeutic and vaccine targets against Philippine and Chinese *S. japonicum*.

Future studies may include comparative investigation of the expressed probes between *S. japonicum* Taiwanese (SJT) and Chinese strains. The schistosome from Taiwan is unlike other strains, in that it is zoophilic; that is it uses mammals other than humans as definitive hosts [197]. Although the Taiwan schistosome can infect humans it does not produce eggs [197]. The reasons for this are unknown; microarray analysis of the expression levels between the SJT and SJC strains could provide an explanation for this variation in host specificity. As discussed in Chapter 4 (section 4.3.2) SJC is more pathogenic than SJP. Further studies focusing on the up-regulation of *S. mansoni* probes TC 7634 [homologue to p48 eggshell protein] and TC7487 [similar to major egg antigen] in SJC may provide important information regarding the basis for this difference in pathogenicity. It is likely also that differences between SJC and SJT of these and other probes may provide further insight on the question of host specificity of the two strains.

7.2.3 Gender associated expression

As outlined in Chapter 5 (section 5.1) schistosomes, unlike other platyhelminths, are unusual because they are dioecious instead of being hermaphroditic [156]. Schistosome sex is genetically determined in the zygote at fertilisation and is propagated through the sporocyst stage in the molluscan intermediate host [156]. This sexual dimorphism is associated with a range of important biological phenomena which are likely reflected in

gene expression patterns. Accordingly, I utilised the microarray resource to profile the gender-associated differential gene expression between male and female adults of Chinese and Philippine strains of *S. japonicum* (Chapter 4). Gender associated expression experiments revealed that 1,163 male- and 1,016 female-associated probes were differentially expressed in SJP whereas 1,047 male- and 897 female-associated probes were differentially expressed in SJC (Chapter 5). The probes discussed in Chapter 5 (section 5.3) have expanded significantly the list of gender-associated genes in adult *S. japonicum* compared to previous findings [15] and reflect the quality of the microarray resource used.

Future studies may expand on the differential gene expression between male and female adult schistosome worms. Recent work by Fitzpatrick and Hoffmann [196] showed that differential gene expression is activated by coupling between mature and immature *S. mansoni* worms. Their microarray experiments were additionally able to show the identification of genes that are required for adult worm maturation [196]. The same experimental design could be used for SJC or SJP worms, providing new information on aspects of schistosome biology, particularly the up-regulation of relevant genes following the pairing of male and female worms [196].

7.2.4 Selected genes of interest

Eight contigs of interest and their differential expression in four life stages including male and female adult, egg, miracidia and cercariae were investigated using real time PCR (Chapter 6, section 6.1). The analysis was able to show fluctuating expression patterns of the selected genes throughout the schistosome life cycle. This approach combining microarray and subsequent real time PCR analysis can be used to: (a) putatively identify gender-specific genes (section 6.3.3); (b) increase knowledge of the

basis of the mode of action of current anti-schistosome drugs, while putatively identifying new chemotherapeutic targets (section 6.3.4); and (c) show differential gender and life cycle expression patterns of new putative vaccine targets [187] (section 6.3.5). The current study has used microarray analysis of differential life cycle expression to identify three putative vaccine targets, namely Contigs 5299, 7515 and 8540. Additional work will need to be undertaken on the characterisation of these probes. This will include obtaining their full DNA sequence, expression of recombinant protein, generation of antibodies to determine protein localisation and assessing finally their vaccine potential in vaccine-challenge experiments.

Sukhdeo and Sukhdeo [198] described the many facets of optimal habitat selection by helminths within their hosts. Parasites do not contain a complex nervous system that could allow them to differentiate between sub-optimal and optimal habitats [198]; instead, they respond only to a few features of the host [198]. For schistosomes this would include vein morphology, and it is likely that their responses to this host environment would necessarily be accompanied by gene expression; such responses could be studied using total RNA samples amplified in the same way as described by Chai M, McManus D P, McInnes R, **Moertel L**, Tran M, Loukas A, Jones M K and Gobert G N. [109] to be probed by the current or modified microarray chip. In order to provide potential vaccine targets, life-cycle studies could include the mammalian host stages, the skin stage, and lung and liver schistosomula [109]. Additional stages for analysis could include immature adults [199] and adults from 15 days and six weeks post challenge. In order to differentiate expression patterns between mature and immature adults, single sex infections could be perfused at four and six weeks post challenge [196]. Overall, this analysis would provide a comprehensive overview of gene expression within the schistosome life cycle.

Gobert G N, McInnes R, **Moertel L P**, Nelson C, Jones M K, Hu W and McManus D P. [105] were able to show major differences between Chinese *S. japonicum* and *S. mansoni* in reference to probes with a putative gene ontology (GO). This was in contrast to GO comparisons of the Chinese and Philippine *S. japonicum* where only minor differences were evident (Chapter 4 section 4.3.3). An up-date of gene ontology description of the schistosome probes may provide novel insights into parasite development, signalling processes, host interactions and immune evasion strategies [105]. This may be done through a review of previous and future data generated by the current microarray using GeneSpring. In future comparative microarray experiments of the complete schistosome life cycle, a gene tree could be generated using QT clustering or "principle component analysis" (PCA) [121, 122] (see Chapter 3 section 3.4). The GO classifications associated with probes in this gene tree could be combined with the data from Gobert G N, **Moertel L P** and McManus D P. [63] to provide a putative classification of uncharacterised probes.

7.3 Conclusions

The aims of this study were to; (1) Contribute to the design of a custom made microarray based on ESTs of *S. mansoni* and *S. japonicum*; (2) Identify differential gene expression between the Chinese and Philippine strains of *S. japonicum*; (3) Additionally show differential gene expression between the sexes of the Chinese and Philippine strains of *S. japonicum*.; and (4) Use real time PCR to further explore the differential expression of selected genes in different life stages of Chinese *S. japonicum*. These aims were achieved and the oligonucleotide microarray described in this thesis now provides the basis for analysis of genomic data from both *S. mansoni and S. japonicum*.

In summary, the current study has demonstrated considerable differences in the gene expression profiles of the Chinese and Philippine strains of *S. japonicum.* This information will provide new insights into the biology of *S. japonicum* and provide a fulcrum for understanding the transcriptional basis of the phenotypic differences evident between the two strains. The list of gender-associated genes in adult *S. japonicum* was also greatly expanded, emphasising the quality of the microarray resource used [4]. The life cycle expression analysis described in Chapter 6 (section 6.3) represents the first use of real time PCR analysis to investigate differential expression of biologically relevant genes selected by microarray analysis. The eight target contigs serve as a basis for further investigation of differential gene expression during the schistosome life cycle and for studying sexual dimorphism between adult worms. Continual refinement and annotation of the microarray will aid in furthering such studies that will build on the gene expression profiles already obtained. It is anticipated that such work will aid in the identification of new vaccine and/or drug targets against schistosomiasis.