CHARACTERISATION OF *CRYPTOSPORIDIUM* GROWTH AND PROPAGATION IN CELL FREE ENVIRONMENTS

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

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DECLARATION

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

Annika Estcourt (previously Boxell)

ACKNOWLEDGEMENTS

Well finally! 8 years, almost to the day, from the very start of commencing on this journey, here it is, the finished product complete with blood, sweat and tears! Many times I have imagined the feeling of finally handing in and the celebrations I would have with family and friends when my PhD came to fruition.

If I had a dollar for every time one of my friends, family or work colleagues asked me over the last four years 'how is your thesis going?' or 'have you handed in yet?' I would be very wealthy! As much as these questions occasionally hit a raw nerve, I would like to thank every one of you who kept on me as this has driven me bit by bit to put the finishing touches on my thesis in amidst concentrating on my new career path and being side tracked with life's up and downs and my favorite hobby – horses!

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ABSTRACT

Cryptosporidium is an enteric parasite that has a global impact on the health and survival of millions of people and animals worldwide. The resistant oocyst stage of the organism's life cycle is excreted in the faeces of infected animals and humans and can contaminate sources of drinking water. *Cryptosporidium* currently represents the major public health concern of water utilities in developed nations as the oocysts produced by *Cryptosporidium* are extremely hardy, easily spread via water, resistant to chlorine and are difficult to inactivate or remove from water intended for consumption without the use of filtration.

Recent developments in the in vitro cultivation have revealed that *C. parvum* can complete its life cycle in media devoid of host cells, which highlights the paucity of knowledge about the developmental biology of this parasite. This finding supports other biological and phylogenetic analysis suggesting that *Cryptosporidium* has a closer affinity with gregarines than with the coccidia. This thesis investigates the growth and propagation of *Cryptosporidium* in cell free environments.

A current limitation of host cell-free cultivation is the difficulty involved in visualising the life-cycle stages as they are very small in size and morphologically difficult to identify and dispersed throughout the media. This is in contrast to conventional cell culture methods for *Cryptosporidium*, where it is possible to focus on the host cells and view the foci of infection on the host cells. Three specific and three non-specific techniques for visualising *C. parvum* life-cycle stages in cell-free culture were compared; antibody staining using anti-sporozoite and anti-oocyst wall antibodies (Sporo-Glo™ and Crypto Cel), fluorescent in-situ hybridization (FISH) using a *Cryptosporidium* specific rRNA oligonucleotide probe and the non-specific dyes; texas red, carboxyfluorescein

diacetate succinimidyl ester (CFSE) and 4,6' diamino-2-phenylindole dihydrochloride (DAPI). Results revealed that a combination of Sporo-Glo™ and Crypto Cel staining resulted in easy and reliable identification of all life cycle stages.

This thesis reports for the first time the completion of the life cycle of *C. hominis* in cell-free culture and multiplication of the parasite via qPCR. Individual life cycle stages were characterised using *Cryptosporidium*-specific antibody staining (Sporo-GloTM) and fluorescent in situ hybridisation (FISH) staining on cultures inoculated with excysted oocysts and purified sporozoites. In both cultures, *C. hominis* successfully proliferated and completed its life cycle, however development in cultures inoculated with purified sporozoites lagged behind cultures inoculated with excysted oocysts. Some novel findings of the study include the visualisation of pairing and multiple associations between various developmental stages in a process similar to syzygy and the formation of *Cryptosporidium* stages (trophozoites and meronts) inside the oocysts without excystation. qPCR analysis revealed a 5-6-fold amplification of parasite DNA. Future studies are required to improve the amplification of the parasite.

Chapter 5 describes the complete development of all life cycle stages of *Cryptosporidium parvum* (cattle genotype) in water. This is the first report in which *Cryptosporidium* is shown to develop and complete its life cycle in water. Amplification of parasite numbers in water was quantified using oocyst counts and quantitative PCR (q-PCR). Daily monitoring by microscopy revealed that some oocysts, when placed in tap or rain water and incubated at 4, 15, 24 and 37°C, would excyst, releasing sporozoites resulting in continuation of the life cycle and production of new oocysts. Manual oocyst counts and qPCR analysis at days 0, 3, 6, 9 and12, when compared with the initial inoculum, showed a small but significant increase in oocyst numbers and oocyst equivalents, respectively.

A study was also conducted to determine if meront and merozoite life cycle stages derived from *C. parvum* oocysts incubated in rain water at 24°C were infective to neonatal ARC/Swiss mice. Each mouse was inoculated with approximately 25,000 meronts and merozoites, meronts were counted as '1' stage. An estimated 1.1 $x10^6$ oocysts were recovered from 6 mice, therefore on average of 183,333 oocysts per mouse was recovered. This represents a 7.3 fold increase from the number of stages, which were inoculated into the mice. This study provided some evidence that meront and merozoite life cycle stages are capable of causing infection in neonatal mice. This is the first report of life cycle stages of *Cryptosporidium* other than the oocyst causing infection, however further research is required to confirm this.

The finding of this thesis will greatly assist in our understanding of the developmental biology of *Cryptosporidium*, its position within the Apicomplexa and its relationship to gregarine protozoa.

PUBLICATIONS AND CONFERENCES

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Estcourt, A., Hijjawi, N., Cox, P., Ryan, U., Thompson, R.C. 2011. Survival and amplification of *Cryptosporidium parvum* life cycle stages in water.

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