CHAPTER 8

General Discussion, Conclusions and Recommendations

8.1 Identification and molecular characterization of pathogenic, mildly pathogenic and benign *Theileria* spp. of the South African buffalo

The 18S rRNA gene is part of the ribosomal functional core and is exposed to similar selective forces in all living organisms (Moore and Steitz, 2002). Generally rRNA gene sequences are easy to access and their repetitive arrangement within the genome provides excess amounts of template DNA for PCR (Meyer et al., 2010). It is therefore one of the most frequently used molecular markers for PCR amplification, molecular epidemiology and characterization, and phylogenetic studies of living organisms (Meyer et al., 2010).

The results of our molecular epidemiology study on the 18S rRNA genes of *Theileria* parasites of the South African buffalo using the RLB hybridization assay have confirmed the findings of previous studies that buffalo are commonly co-infected with different *Theileria* spp., namely, *T. parva*, *Theileria* sp. (buffalo), *T. mutans*, *T. velifera* and *T. buffeli* (Allsopp et al., 1993; Gubbels et al., 1999; Oura et al., 2004). Molecular characterization of partial (V4 hypervariable) (Chapter 6) and full-length (Chaisi et al., 2011; Chapters 3, 4, 5) 18S rRNA gene sequences of *Theileria* spp. of the African buffalo indicated extensive sequence variation within the 18S rRNA genes of these species. Co-infections with several genotypes of a single species were also common.

Based on the results of the partial and full-length 18S rRNA sequences, we described the occurrence of at least 13 distinct 18S rRNA *Theileria* genotypes of the African buffalo (Chapters 3, 4, 5, 6). Seven of these genotypes differed from known or previously reported sequences (Chapter 6) and were recently reported as novel (unique) by Mans et al. (2011). Our study was limited to the analysis of a smaller number of samples from buffalo, while Mans et al. (2011) analysed many more samples from both buffalo and cattle. The phylogenetic positions of some of these genotypes, and the fact that they show distinct host, and geographical differences, indicates that they may represent new species. However, additional biological and morphological data are needed in order to confirm this statement.

Although we don't know the actual mechanisms that bring about the observed diversity in the 18S rRNA gene, in their review, Deitsch et al. (1997) indicated that gene sequence diversity may be due to random mutation of nucleotides during sexual reproduction or through inter- and intra-genetic recombination and the random assortment of alleles during sexual reproduction. Studies on the ITS region of *Theileria* spp. of cattle indicated higher levels of genetic variation in the ITS region in the pathogenic *Theileria* species (*T. annulata* and *T. parva*) than in the mildly pathogenic and non-pathogenic species (Collins and Allsopp, 1999; Aktas et al., 2007). This has been attributed to higher levels of parasitemia during acute disease, leading to the ingestion of greater numbers of organisms by the tick and therefore a more diverse pool during gametogenesis in the tick vector which then results in a greater opportunity for recombination to occur during sexual reproduction in the tick vector (Collins and Allsopp, 1999). Aktas et al. (2007) also indicated that it is possible that a higher rate of parasite proliferation in the pathogenic species increases the likelihood of random mutation.

The effect of the 18S rRNA gene heterogeneity on the epidemiology of theileriosis in South Africa is unknown and should be investigated in order for the development of more efficient control strategies for this disease. Although *T. mutans* is generally regarded as a benign species, some strains of this species have been associated with severe disease in cattle (Young et al., 1978; Paling et al., 1981; Saidu, 1981). It would therefore be interesting to see whether there in an association between *T. mutans* genotypes and disease.

8.1.1 Implications of the sequence variation of the V4 hypervariable region of the 18S rRNA gene on the diagnosis of pathogenic and non-pathogenic *Theileria* species of the African buffalo

The RLB hybridization assay by Gubbels et al. (1999) is a practical tool for the simultaneous detection and differentiation of *Babesia* and *Theileria* spp. in infected animals. We designed two new RLB probes, designated *T. mutans*-like 1 and *T. mutans*-like 2/3, for the specific detection of these novel genotypes in order to determine their prevalence in the South African cattle and buffalo populations (Chapter 4). However, the new probes cross-reacted with the *T. mutans* target DNA despite the 3 nucleotide differences between the new probes and the original *T. mutans* sequence.

In order to discriminate between the different *T. mutans* genotypes, we recommend that new RLB probes should be designed from a more variable area of the 18S rRNA gene which is located upstream of the current probe area (Chapter 4). Similarly, new RLB probes could be designed for

all the novel genotypes identified in this study, and for *Theileria* sp. strain (MSD) in order to determine their prevalence in the South African buffalo and cattle populations. Alternatively, a real-time PCR assay could be developed for genotyping as quantitative real-time PCR (qPCR) assays are known to be more sensitive than the RLB assay in the identification of *Theileria* spp. from various hosts (Sibeko et al., 2008, Bhoora et al., 2009, Chaisi et al., 2011).

Although the RLB hybridization assay has previously been used for the identification of new *Theileria* spp. and species variants, cross-reactions occur between some of the currently used probes with certain genotypes (Mans et al., 2011; personal observation), resulting in false positives. It is also not possible to use the RLB assay to identify novel species or species variants in mixed infections, which commonly occur in cattle and buffalo, as a positive signal of a known species does not indicate the possibility of the presence of a novel genotype (Chapter 4; Mans et al., 2011). The latter problem can be resolved by random sequencing of the 18S rRNA gene (Mans et al., 2011).

Our study and others (Mans et al., 2011; Pienaar et al., 2011a) have indicated that despite the extensive sequence variation within the 18S rRNA gene of *Theileria* spp., only *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) can compromise the sensitivity of the 18S rRNA hybridization assay of Sibeko et al. (2008) for the detection of *T. parva* in mixed infections. This problem has been partially overcome by the recent development of the Hybrid II assay (Pienaar et al., 2011b). Although the primers used in the Hybrid II assay amplify both *Theileria* sp. (buffalo) and *T. parva*, two distinct melting peaks are obtained for these species and *Theileria* sp. (bougasvlei) is not amplified by the new primer set. The Hybrid II assay is therefore more specific than the hybridization assay in the diagnosis of *T. parva* infections in cattle and buffalo. A different target gene that sufficiently differentiates between these species should be used to develop a more sensitive *T. parva* assay (Chapter 3; Chaisi et al., 2011).

8.2 Molecular characterization and phylogeny of *T. buffeli*-like and *T. sinensis*-like genotypes of the African buffalo (*Syncerus caffer*) based on their 18S rRNA gene and internal transcribed spacer (ITS) sequences

Classification of the *Theileria buffeli/T. sergenti/T. orientalis* group of benign parasites of cattle and buffalo is complicated and confusing. These species have a cosmopolitan distribution, and although they have previously been identified from cattle and buffalo in South Africa, very little is known about these organisms in this country. Chapter 5 therefore provides useful genetic information

towards the proper phylogeny and classification of this group based on their 18S rRNA and ITS gene sequences. Although the phylogenetic groupings of the *T. buffeli*-like ITS sequences were consistent with those of the 18S rRNA gene sequences for clones obtained from samples originating from the Hluhluwe iMfolozi Park, a direct comparison of 18S rRNA gene and ITS sequences amongst clones was not possible due to mixed infections. An integrated phylogenetic study of sequence data from different genetic markers might provide additional phylogenetic information for defining species, subspecies and/or strains of this complex group of benign bovine *Theileria* spp. (Aktas et al., 2007).

8.3 Evaluation of the cox III qPCR assay for the simultaneous identification and differentiation of *Theileria* spp. in buffalo

In an effort to develop a more sensitive diagnostic assay for *T. parva* infections in cattle, Janssens (2009) developed two qPCR assays based on the cox III gene. The first assay simultaneously identifies and differentiates, by melting curve analysis, between all known *Theileria* spp. of cattle. This assay was later modified to include the identification of *Theileria* sp. (buffalo) in buffalo samples. Both buffalo and cattle are of socio-economic importance in South Africa, they share the same *Theileria* spp. and therefore diagnostic tools should target the identification of these species in both animals. Our results (Chapter 7) indicate that the modified cox III assay is sensitive and specific in detecting *T. parva* infection from cattle and buffalo samples as indicated by Janssens (2009), and an added advantage over existing diagnostic assays for *T. parva* is that it can simultaneously identify and differentiate this pathogen from the other co-infecting *Theileria* spp. However, extensive sequence variation observed within the cox III gene of the other *Theileria* spp. of buffalo renders the assay unsuitable for the identification of these parasites in buffalo in South Africa. The novel cox III gene sequences could be used to improve the specificity of the assay for the identification and differentiation of all known *Theileria* spp. and their variants in buffalo. It will be important to characterize the cox III gene of *Theileria* spp. of cattle in South Africa before a more specific assay is developed.

This study highlights the complexities involved in the diagnosis of *T.parva* in cattle and buffalo in South Africa, and provides invaluable information towards the classification of the complex *T. buffeli*/*T. sergenti*/*T. orientalis* group of benign species. The sequence data generated from this project will allow for the development of a more specific and sensitive diagnostic assay for *T. parva* which will assist the veterinary regulatory authorities in the control of Corridor disease in South Africa

8.4 References

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