CHAPTER 7

General Discussion and Conclusion

For as the heavens are higher than the earth, so are my ways higher than your ways, and my thoughts than your thoughts. **Isaiah 55:9**
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"As long as you are going to be thinking anyway, think big." **Donald Trump**

7.1 Improvement of molecular diagnosis of *T. parva* infections

Real-time PCR technology has revolutionized molecular diagnostics allowing analysis of total PCR product accumulated in a reaction. This, combined with the use of minute amounts of input DNA, confers superior sensitivity when compared to conventional PCR techniques. In addition, real-time PCR ensures accurate detection of specific targeted nucleic acid sequences by allowing differentiation of PCR products of the same size without the need for post-amplification analysis, which usually risks contamination and is time consuming. These qualities of real-time PCR technology are essential for diagnosis of *T. parva* infections, especially when *T. parva* DNA has to be detected from carrier animals. Hence real-time PCR was exploited in this study to improve the molecular diagnosis of *T. parva* in South Africa. While other molecular tests evaluated in this study, namely RLB, PCR/probe and *cox*III PCR-RFLP, required 2.5 (~37.5 ng) to 5.0 (~75 ng) µl of input DNA, the real-time PCR assay showed superior sensitivity and could detect target DNA using only 1.0 µl (~15 ng) of input DNA. Increasing the input DNA to 2.5 µl improved the sensitivity by 20% making the real-time PCR assay the most sensitive molecular assay developed for *T. parva* DNA detection to date, detecting a piroplasm parasitaemia as low as 8.79×10^{-4} % with 100% certainty.

Using melting curve analysis, real-time PCR technology can be used to differentiate between specific and non-specific PCR products, because of differences in the PCR products' melting temperature (T_m). In this way, it is even possible to distinguish between amplicons with a single nucleotide difference. Achieving this form of discrimination is impossible with other molecular assays, especially when there is high sequence similarity between the target product and the non-specific PCR product and even more so when the non-specific PCR product is the same size as the target product. This was the challenge with the real-time PCR assay developed in this study, which amplified *Theileria* sp. (buffalo) DNA in addition to the targeted *T. parva* DNA. Amplification of *Theileria* sp. (buffalo) DNA could not be avoided as the 18S rRNA gene sequence of this species is very similar to that of *T. parva.* However, the assay successfully discriminated between the PCR products of the two *Theileria* species because they have different T_ms at the region where the probe is designed. Only *T. parva* amplicons were detected by the *T. parva*-specific probe subsequent to melting curve analysis; the real-time PCR assay was therefore specific for detection of *T. parva* infections. To avoid the amplification of *Theileria* sp. (buffalo) DNA the use of a different target gene with significant differences between *Theileria* sp. (buffalo) and *T. parva* will have to be explored

as differences in the 18S rDNA variable region are not sufficient to improve the assay based on this gene.

Contamination remains a major problem in diagnostics; most molecular methods require post amplification handling of PCR products for confirmation of a positive result which increases the risk of contamination that can result in the reporting of false positive results. This is a serious challenge because it is almost impossible to differentiate between a true positive and a false positive result. In South Africa, one of the most important applications of tests for detection of *T. parva* infections is in the regulation of "disease-free" buffalo breeding projects, and false positive results can have an enormous financial implication. "Disease-free" buffalo have become a sought-after commodity, but, once reported positive for *T. parva* infection, these animals lose their value and the game farmer's investment in the animals is threatened. Thus, inaccurate results are to be avoided wherever possible. The contamination-controlled system offered by the real-time PCR assay greatly reduces incidents of contamination while allowing rapid processing of samples and increases the number of samples that can be analyzed in a day by three fold or more; these are much desired qualities of a diagnostic assay, especially when large numbers of samples are to be processed as is more often the case with the "disease-free" buffalo breeding projects in South Africa.

The reproducibility of the real-time PCR assay and the consistency in interpretation of test results by different personnel has resulted in the successful transfer of the technology to the ARC-OVI laboratory, a national laboratory with a mandate from the South African Department of Agriculture, Forestry and Fisheries (DAFF) to perform tests for *T. parva* infections in cattle and buffalo blood samples.

7.2 Molecular characterization of South African *T. parva* parasites

7.2.1 Evidence of cattle-type p67, p104 and PIM alleles in *T. parva* parasite populations in South Africa

The cattle-derived *T. parva* stocks, Muguga, Marikebuni and Boleni, characterized by Nene *et al*. (1996), all had the same p67 allele with a 129 bp deletion, while there was no deletion in the central region of the gene in the buffalo-derived stock 7014. The PIM gene from these same stocks was relatively homogenous for the cattle-derived *T. parva* stocks and heterogeneous for buffalo-derived stocks (Toye *et al*., 1995). p104 sequences were assigned as alleles 1, 2, 3 and 4 for the sequence obtained from *T. parva* Muguga, *T. parva* Marikebuni, *T. parva* Boleni and *T. parva* 7014, respectively (Skilton *et al*., 2002). It is believed that *T. parva* parasites are originally buffalo parasites and that there is a heterogeneous population of parasites circulating among buffalo, only some of which can be transmitted within the cattle population (Toye *et al*., 1995; Nene *et al*., 1996). In an attempt to identify cattle-type p67, p104 and PIM alleles in South African *T. parva* parasite populations, both samples from cattle and buffalo were investigated in this study.

7.2.1.1   Identification of cattle-type alleles from cattle T. parva *samples*

In this study, cattle-type p67, p104 and PIM alleles were identified from three *T. parva* samples obtained from cattle from a farm near Ladysmith in the KwaZulu-Natal Province. These cattle-type alleles were almost identical to those previously identified in a cattle-derived *T. parva* stock, *T. parva* Muguga, which causes ECF in Kenya, East Africa. It was an unexpected finding to identify Muguga-like cattle-type alleles from these animals, since ECF was not diagnosed in animals on this farm, and there have not been reports of ECF in South Africa since its eradication in the early 1950s. Between 2002 and 2004, there were seasonal outbreaks of what was initially suspected to be babesiosis on the Ladysmith farm, as the farm is located outside the designated *T. parva*-infected area (Thompson *et al*., 2008). Thompson *et al*. (2008) reported the presence of *T. parva* infections on this farm and attributed the source of infection to possible contact with infected buffalo or *T. parva*-infected ticks, but such contact could not be confirmed. The epidemiology of the disease on this farm was complicated by the fact that during the 2003 and 2004 outbreaks, the entire herd was treated with tetracyclines, and regularly and intensively dipped against ticks. Apparently healthy,

T. parva-positive animals were identified both in the study by Thompson *et al*. (2008) and in the present study. It is a known fact that cattle that survive *T. parva* infections can become carriers of the parasite, and furthermore, carriers may arise from being immunised and after treatment with anti-theilerial drugs (Dolan, 1986; Irvin *et al*., 1989; Bishop *et al*., 1992). Our results might provide evidence for the selection of a subpoplution of *T. parva* parasites through cattle-to-cattle transmission of *T. parva* on the Ladysmith farm, resulting in parasites with cattle-type p67, p104 and PIM alleles.

p67, p104 and PIM alleles obtained from *T. parva* samples from several other animals from this farm were either buffalo-type or 'novel' (or mixed-type in the case of PIM) suggesting that the parasite population circulating on this farm may have originated from buffalo. Although contact between these animals and *T. parva*-infected buffalo could not be established, findings in this study suggest that this event may have occurred. The Hluhluwe-iMfolozi 'signature' obtained in PIM PCR-RFLP profiles from cattle samples from the Ladysmith farm, similar to that obtained from buffalo samples from Hluhluwe-iMfolozi game park, provides support for the hypothesis that there may have been contact between cattle on this farm and *T. parva*-infected buffalo.

While our results suggest that there may have been transmission of *T. parva* from buffalo to cattle on the Ladysmith farm, possibly followed by cattle-to-cattle transmission, the data obtained in this study cannot be used to establish the route of infection, i.e. if the parasite was transmitted from buffalo-to-cattle, cattle-to-cattle or cattle-to-buffalo.

7.2.1.2 *Identification of cattle-type alleles from buffalo* T. parva *samples*

No alleles identical to *T. parva* cattle-type alleles were obtained from buffalo samples analyzed in this study. It is possible that the pool of buffalo that were investigated was not large enough to allow detection of all possible *T. parva* alleles that occur in buffalo. Therefore, a larger pool of samples will have to be characterized in order to determine if there are cattle-type alleles in buffalo. Although cattle-type alleles identical to those previously reported (Toye *et al.,* 1995; Nene *et al*., 1996; Skilton *et al*., 2002) were not identified from buffalo samples that were characterized in this study, variants of p67 allele 1 as well as variants of p104 allele 1 (both cattle-type alleles associated with cattle-derived *T. parva* stocks), initially identified from the cattle-derived *T. parva* Muguga stock (a cattle stock that can cause fatal disease) were obtained from *T. parva* samples collected from buffalo. This finding suggests that parasites possessing cattle-type p67 and p104 alleles may not be

exclusively associated with cattle-derived *T. parva* parasites. While these results could suggest that there may have been transmission of cattle-derived *T. parva* parasites to buffalo, they could also be indicative of an ancestral buffalo-derived *T. parva* subpopulation with characteristics that might aid the parasite to establish in cattle. The importance of these parasites in the epidemiology of theileriosis in South Africa needs to established. Furthermore, it will be important to determine whether parasites that possess these variants can cause disease in cattle; this could be achieved by means of tick transmission experiments.

It is still not clear if the parasite that caused ECF in South Africa in the early part of the 20th century was transmitted to buffalo during the ECF epidemic, or whether South African strains of *T. parva* could eventually become adapted to cattle and cause ECF. To date, there are no reports where the source of infection was determined in cases where both cattle- and buffalo-derived *T. parva* parasites occur, and more reliable markers to distinguish between cattle-derived and buffalo-derived parasites would be needed in order to perform such experiments. There are also no reports indicating whether cattle-derived parasites can be transmitted to buffalo. Such transmisson, if it did occur, could play an important role in the evolution of genetic polymorphism in *T. parva* parasites and in the selection for genetically variant *T. parva* strains. It would be necessary to perform tick transmission studies to show whether *T. parva* parasites that are adapted to cattle could be transmitted to buffalo.

7.2.2 Extensive genetic diversity among South African *T. parva* parasites

Most sequences obtained from the three antigen genes, p67, p104 and PIM, analysed in this study were not identical to the previously reported sequences (Toye *et al*., 1995; Nene *et al*., 1996; Skilton *et al*., 2002), demonstrating extensive genetic diversity among South African *T. parva* parasite populations. Diversity can be either generated by random mutation of nucleotides during asexual reproduction and selection of changes that confer a biological advantage or sexual reproduction through intra- and inter-genetic recombination and the random assortment of alleles (Gubbels *et al.*, 2000). There is no evidence from this study to conclusively implicate any of these mechanisms in the diversity that was observed and the generation of novel alleles. Two different novel p67 alleles, 3 and 4, were identified in addition to the previously reported alleles (Nene *et al*., 1996). Although the novel alleles were only identified from samples that were obtained from buffalo in South Africa, allele 3 was obtained from a Zambian field isolate (Zam 5) from a naturally infected bovine diagnosed with ECF (Geysen, 2000). This result suggests that cattle-derived *T. parva* parasites,

especially those that can cause ECF, do not exclusively contain p67 allele 1 as previously believed.

Sequence stability may differ in different domains in the genome, which implies that some genes may be reasonably conserved while others are more prone to variation (Bishop *et al*., 1997). While p67 and p104 gene sequences examined in this study were relatively conserved, and most variation appeared to be due to single nucleotide polymorphisms, the PIM gene was highly polymorphic as evident from the multiple deletions and insertions in the central variable region. Furthermore, in addition to the known PIM sequence types (Toye *et al*., 1995), 'mixed'-type PIM sequences, suggestive of recombination between cattle-type and buffalo-type PIM alleles, were identified. There was no evidence from data obtained in this study to suggest that mixed-type PIM sequences were due to PCR artifacts generated by *in vitro* cross-over events. In the tick vector, sexual reproduction between different strains of *T. parva* could result in recombinant progeny. The PIM gene is known to evolve very rapidly (Toye *et al*., 1995). Shared sequences between genes may arise as a result of common ancestry, convergent evolution, recombination or gene conversion (McDevitt, 1995; Klein and O'hUigin, 1995; Parham *et al*., 1995). According to Geysen *et al*. (2004), recombination and gene conversion may be involved in the generation of shared sequences in the PIM gene. The 'mixed' PIM sequences identified in this study appeared to be recombinants of cattle- and buffalo-type sequences. Whatever mechanism is involved, the alteration of amino acid sequences is perceived as an evolutionary advantage (Bishop *et al*., 1997); there is evidence that frequent genetic exchange has the potential to generate parasites that have high antigenic diversity (Morrison, 2007). Findings from this study have shown that the sequence heterogeneity within the *T. parva* antigen genes, p67, p104 and PIM, is greater than previously thought. It will be interesting to establish the significance of this genetic diversity in relation to the antigenic diversity of the parasite and the epidemiology of the disease.

While an integrated phylogenetic study of the sequence data obtained from all three genes might be used to further elucidate the evolution of *T. parva* strains in cattle and buffalo, the data from the present study was unfortunately not suitable for this purpose because of mixed infections with different *T. parva* strains in a single sample. More than one sequence was obtained for each of the three genes from most field samples examined in this study. It was therefore not possible to match sequences obtained from each of the three genes to a single parasite. Such a study could be done in future by making parasite clones from field isolates

and characterizing all three genes from individual clones to obtain data that could then be integrated and used to establish the evolution of different parasite strains.

7.3 Conclusion

Similar to other diagnostic tests, the real-time PCR assay developed in this study has challenges that still need to be addressed to improve on the performance of the assay. The sensitivity of the *T. parva*-specific real-time PCR assay is still compromised in cases of mixed infections with *Theileria* sp. (buffalo), especially when the ratio of *Theileria* sp. (buffalo) to *T. parva* is high. Increasing input DNA or extracting total DNA from a larger volume of infected blood may increase the probability of detecting the relevant parasite DNA template in the real-time PCR assay. The specificity of the real-time PCR assay could not be further improved using the 18S rRNA gene because of the high similarities between *Theileria* sp. (buffalo) and *T. parva* in this gene sequence. For a more specific test it is advisable that a different gene with enough variation at species level should be targeted. Until then, the real-time PCR test reported here remains the most sensitive and specific molecular test for detection of *T. parva* infection.

Findings from this study have shown that *T. parva* parasites with cattle-type p67, p104 and PIM alleles were present on a farm near Ladysmith in South Africa. However, ECF pathology was not diagnosed in these animals. The PIM PCR-RFLP profiles and amino acid sequence data provided evidence that the source of *T. parva* infections in other cattle on the Ladysmith farm was infected buffalo originating from Hluhluwe-iMfolozi. This supports the suspicion of the involvement of *T. parva*-infected buffalo in the events that resulted in an outbreak in the Ladysmith farm (Thompson *et al.*, 2008). No gene sequences identical to cattle-type p67, p104 and PIM were identified from *T. parva* samples obtained from buffalo; however, variants of cattle-type p67 and p104 alleles were identified.

Extensive genetic diversity amongst South African *T. parva* populations in buffalo was evident as shown by identification of novel p67 alleles and PIM 'mixed'-type alleles. The existence of these 'new' alleles will make it difficult to use these antigen genes to characterize South African *T. parva* isolates because it has not yet been established if these isolates are associated with cattle- or buffalo-derived parasites. Although p67, p104 and PIM gene profiles and sequences have been previously used successfully to characterize *T. parva* parasites in East Africa (Bishop *et al.*, 2001), identification of markers that are differentially

expressed between cattle- and buffalo-derived *T. parva* parasites will be required for successful characterization of *T. parva* parasites in South Africa. The presence in South African *T. parva* field samples of alleles associated with *T. parva* parasites responsible for classical ECF remains a concern and the significance of the different alleles, particularly the novel variants, in the epidemiology of theileriosis in South Africa should be established.

7.4 References

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