

**Improvement of diagnostics for
Trypanosoma equiperdum infecting
equines in South Africa**

MA Mlangeni

 **orcid.org 0000-0002-8651-0014**

Thesis submitted in fulfilment of the requirements for the degree *Doctor of Philosophy Zoology* at the North-West University

Promoter:	Prof MMO Thekiso
Co-promoter:	Prof M Syakalima
Co-promoter:	Dr M Taioe

Graduation May 2019

26849984

DEDICATION

To my daughter Rethabile Mlangeni, my family and to myself.

ACKNOWLEDGEMENTS

Acknowledgments first and foremost, goes to my Father almighty GOD, Jesus Christ and the Holy Spirit (the Holy Trinity), for making this journey endurable as well as provision for strength and wisdom to endure all the struggles in my everyday life. Creator of heaven and earth, father of the fatherless, You have always been there for me throughout my study, you proved to be my strong tower. “You said in your word: You will never leave me nor forsaken me” (Deutronomy 31:6 & Hebrews 13:5). Indeed, You have been with me through the mountains and valleys “wena Jehovah Shammah””. You created heavens and earth and all that is in it. All the glory, honour and power are yours BABA.

I would like to express my sincere gratitude and gratefulness to the following people and institutions for their contribution and support towards the completion of this study:

My promoter Prof. Oriel Thekiso, through your mentorship and support you have helped me grow from anxious and unsure student into a more confident and hardworking individual. Thank you Prof. for your undying patience, believing in me and providing me this opportunity. I have learned so much from you. My co-promoter Prof Syakalima for your supervision, guidance, time and understanding.

Dr. Moeti (co-promoter) for assistance and mentoring me. Thank you Dr. T, for your patience, words of encouragement and always lending an ear. You know how hard it was really for me but you have never given up.

Mme Jane Mlangeni, my mom, my yonkinto, my caterpillar, my strong tower, my everything, thank you for your prayers, love, emotional and financial support as well as for believing in me always. You didn't understand or know what I'm actually doing but you have always been there for me. Dankie mommy.

My younger brother and my sister, Papi and Anna for their support, words of encouragement and believing in me always. Thank your daily support, encouragement, love, and confidence. Thank you.

Moeketsi and Palesa, “my nephew and niece” thanks for your motivation and reminding me always that they are watching and looking up to me.

My “kid” Rethabile, for watching and looking up to be like mommy and do more. Every time when I thought of giving up, you reminded me that there is someone out there who’s looking up to me.

Many, many thanks to all of the current and past members of the parasitology lab, Paballo, S bonginhlanhla (Sbo), Sanchez aka Lehlohonolo, Bridget, Anna, Dr. ThankGoD, Dr Nthatisi, Setjhaba, Dr. Lisemelo, Siphamandla, and Clara, without all of you, I would have never made it, you guys have played a tremendous role in my life bona ke ya le phahamisetsa. Thank you all for your inputs and help over the years. I will miss our weekly journal clubs to discuss and present the progress of our work.

My friends: Rethabile, Makgauta, Lebo, Dineo, Oratilwe, Sbo, Sanchez, Bridget, Anna, Paballo, Bonolo, Mzy, Audrey, Natash, Godfrey, Abraham (Abes), you guys have been my greatest friends and I wish you all the best of luck in your careers. Thank you for always having open doors and listening ears every time I needed to talk. Thank you for your prayers as well

Aunty Jersey Leboela for your financial support, love and prayers.

The Mokhele’s and Tsomo’s families for their prayers, words of encouragement and support. They are family friends.

Mme Mo Apostola Mofokeng, my Pastor Annah, Mme Litabe and Christian Faith Church members, for your prayers, words of encouragement, love and support.

My ENC prayer and Word of encouragement groups: Thank you for your prayers, love and support.

The microbiology group for allowing me to use their lab, everyone who played a role to some extent.

This study was funded by National Research Foundation (NRF) and NWU financial student support: Scarce Skills bursary and NWU bursary for financial support.

PHILIPPIANS 1:6

**BEING CONFIDENT IN THIS, THAT HE WHO BEGAN A GOOD WORK IN YOU WILL
CARRY IT ON TO COMPLETION UNTIL THE DAY OF CHRIST JESUS.**

LUKE 1:45

**BLESSED IS SHE WHO HAS BELIEVED THAT THE LORD WOULD FULFILL HIS
PROMISES TO HER!"**

2 TIMOTHY 1:7

**FOR GOD HAS NOT GIVEN US A SPIRIT OF FEAR, BUT OF POWER AND OF LOVE
AND OF A SOUND MIND.**

JEREMIAH 29:11

**FOR I KNOW THE PLANS I HAVE FOR YOU," DECLARES THE LORD, "PLANS TO
PROSPER YOU AND NOT TO HARM YOU, PLANS TO GIVE YOU HOPE AND A
FUTURE.**

1 PETER 2:10 (MSG)

THE DIFFERENCE HE MADE FOR ME –

FROM NOTHING TO SOMETHING, FROM REJECTED TO ACCEPTED!

ABSTRACT

Dourine is a sexually transmitted disease of equines caused by *Trypanosoma equiperdum*. Dourine has worldwide distribution and is an economically important veterinary disease. There is little to no active research on dourine in South Africa despite the high number of reported cases in various provinces. The OIE recommended diagnostic technique is a serological assay referred to as complement fixation test which confirms exposure to infection. The lack of simple and reliable diagnostic methods is an obstruction in the effective control of diseases. It is still difficult to entirely distinguish all *Trypanozoon* species. Therefore, the aim of this study was to develop DNA based diagnostic assays including conventional polymerase chain reaction (conPCR), real-time PCR (qPCR) and loop-mediated isothermal amplification (LAMP) for the detection of *Trypanosoma equiperdum* infections in South African equids.

Primer sets and probes were designed from the repetitive insertion mobile element (RIME) gene. The three assays namely conPCR, qPCR and LAMP specifically amplified *T. equiperdum* DNA when tested against other parasites which co-infect equines. However, the specificity of qPCR was not stable and required analyses using melting curves. The detection limit of conPCR and LAMP for serially diluted DNA was 1×10^{-5} and 1×10^{-7} for conPCR and LAMP which is equivalent to 1 and 0.001 trypanosome cells/ml respectively, while the SYBR green and probe based qPCR had 1×10^{-5} detection limit which is equivalent to 1 trypanosome/ml.

The conPCR, qPCR and LAMP assays were used to screen DNA extracted from blood collected from horses and donkeys in South Africa. The detection performance of LAMP was higher than that of real-time qPCR, conPCR with 70.8%, 52.1% and 62.5% respectively. Data obtained from this study show that LAMP, conPCR and qPCR assays can be a useful supplementary tools to clinical signs and microscopical diagnosis of *T. equiperdum* infections in equines in South Africa.

Keywords: Dourine, LAMP, PCR, qPCR, *Trypanosoma equiperdum*, South Africa

RESEARCH OUTPUTS

Malitaba A. Mlangeni, Moeti O. Taioe, Nthatisi I. Molefe, Michelo Syakalima, Moratehi Mefane, Matthew Nyirenda, Lehlohonolo Mefane, Keisuke Suganuma, Thuy Thu Nguyen, Noboru Inoue, Oriël M.M. Thekisoë, 2018. Loop-mediated isothermal amplification (LAMP) assay for rapid diagnosis of *Trypanosoma equiperdum* in South Africa. 14th International Parasitology Congress, EXCO DAEGU, South Korea, 19-24 August 2018 (ORAL).

Malitaba A. Mlangeni, Moeti O. Taioe, Moratehi Mefane, Matthew Nyirenda, Lehlohonolo Mefane, Thuy Thu Nguyen, Noboru Inoue, Oriël M.M. Thekisoë, 2017. Detection of *Trypanosoma equiperdum* infections in horses and donkeys in South Africa by PCR and ELISA. 3rd International Congress on Parasites of Wildlife, Skukuza, Kruger National, Park, 24-27 September 2017 (ORAL).

Malitaba A. Mlangeni, Moratehi Mefane, Matthew Nyirenda, Lehlohonolo Mefane, Igarashi Ikuo, Oriël M.M. Thekisoë, 2016. Molecular Detection of *Anaplasma*, *Babesia*, *Neorickettsia* and *Theileria* infections in horses and donkeys in South Africa. 45th conference of the Parasitological Society of Southern Africa, Cape Town, South Africa. 28-31 August 2016 (ORAL).

TABLE OF CONTENTS

DEDICATION.....	I
ACKNOWLEDGEMENTS.....	II
ABSTRACT.....	V
RESEARCH OUTPUTS.....	VI
CHAPTER 1 INTRODUCTION.....	1
1.1 Equine population in South Africa.....	1
1.2 Dourine.....	1
1.3 Statement of the problem.....	3
1.4 Aim.....	4
1.5 Objectives.....	4
1.5.1 To develop a conventional PCR assay for detection of <i>T. equiperdum</i> infections in South African equines.....	4
1.5.2 To develop a real-time PCR (qPCR) assay for detection of <i>T. equiperdum</i> infections in South African equines.....	4
1.5.3 To develop a loop-mediated isothermal amplification (LAMP) assay for detection of <i>T. equiperdum</i> infections in South African equines.....	4
1.6 Hypothesis.....	4
1.7 Thesis outline.....	4
REFERENCES.....	6
CHAPTER 2 LITERATURE REVIEW.....	10

2.1	General overview of dourine.....	10
2.2	Distrubion of dourine	10
2.3	Pathogenesis	11
2.4	Transmission of <i>Trypanosoma equiperdum</i>.....	12
2.5	Clinical signs of dourine	12
2.6	Treatment of dourine.....	13
2.7	Economic importance of dourine	13
2.8	Diagnosis of trypanosomes.....	13
2.9	Serological techniques	15
2.9.1	Complement fixation test	15
2.9.1.1	Dourine complement fixation test.....	17
2.9.2	Immunofluorescence antibody test for trypanosomes	18
2.9.2.1	Immunofluorescence antibody test for dourine (OIE, 2013)	18
2.9.3	Enzyme-linked immune sorbent assay	20
2.10	Molecular techniques.....	23
2.10.1	Conventional polymerase chain reaction (conPCR).....	25
2.10.2	Real-time polymerase chain reaction (qPCR)	26
2.10.3	Loop-mediated isothermal amplification (LAMP).....	26
	REFERENCES.....	28
	 CHAPTER 3 CONVENTIONAL PCR FOR DETECTION OF <i>TRYPANOSOMA</i> <i>EQUIPERDUM</i> INFECTIONS IN SOUTH AFRICAN EQUIDS	 37
3.1	Introduction	37

3.1.1	Polymerase chain reaction.....	37
3.1.2	Polymerase chain reaction mechanism.....	38
3.1.3	Polymerase chain reaction technique for trypanosomes	39
3.2	Objective of the study	40
3.3	Materials and Methods	41
3.3.1	Horse and donkey blood samples.....	41
3.4	Genomic DNA Isolation	42
3.5	Conventional PCR using F3 and B3 primers	42
3.5.1	PCR specificity	43
3.5.2	Reaction sensitivity	43
3.5.3	Evaluation of the conventional PCR detection efficiency.....	43
3.6	Agarose gel electrophoresis.....	43
3.7	Statistical analysis.....	44
3.8	Results	45
3.8.1	Sensitivity and specificity of conPCR using F3 and B3 primers.....	45
3.8.2	Conventional PCR analyses of field blood samples	46
3.9	Discussion	48
	REFERENCES.....	51
	CHAPTER 4 REAL-TIME PCR ASSAY FOR DETECTION OF <i>TRYPANOSOMA</i>	
	<i>EQUIPERDUM</i> INFECTIONS IN SOUTH AFRICAN EQUIDS	56
4.1	Introduction	56
4.1.1	Real time PCR (qPCR) assay.....	56

4.1.2	The mechanism and expected reaction steps in real-time PCR	58
4.1.2.1	Amplification efficiency (e)	60
4.1.2.2	Linearity (R ²).....	60
4.1.2.3	Sensitivity	61
4.1.2.4	Specificity	61
4.1.3	Real-time PCR for trypanosomes	61
4.2	Aim of the study	62
4.3	Materials and Methods	63
4.3.1	Real-time PCR assay	63
4.3.1.1	Optimization Real time reaction	64
4.3.1.2	Reaction sensitivity using both dyes (SYBR and PrimeTime)	64
4.3.1.3	Reaction specificity and evaluation of the assays	65
4.4	Conventional PCR using RIME 1 primers	65
4.4.1	Analytical sensitivity and specificity of RIME1 primers	65
4.4.2	Comparison of real-time PCR assay with conPCR techniques	66
4.5	Statistical analysis.....	66
4.6	Results	68
4.6.1	Real-time PCR optimization and standardization	68
4.6.5	Comparison between qPCR and conPCR assays	78
4.7	Discussion	80
	REFERENCES.....	83

CHAPTER 5 LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR DETECTION OF <i>TRYPANOSOMA EQUIPERDUM</i> INFECTIONS IN SOUTH AFRICAN EQUIDS	89
5.1 Introduction	89
5.1.1 Loop-mediated isothermal amplification (LAMP) assay.....	89
5.1.2 Mechanism and expected reaction steps of LAMP	91
5.1.3 LAMP detection for trypanosomes	94
5.2 Aim of the study	94
5.3 Materials and Methods	95
5.3.1 Loop-mediated isothermal amplification (LAMP) assay.....	95
5.3.1.1 Design of LAMP primers.....	95
5.3.1.2 Detection of LAMP product.....	96
5.3.1.3 Sensitivity and specificity of LAMP.....	96
5.3.1.4 Comparison of LAMP reaction assay with PCR assay	97
5.3.1.5 Agarose gel electrophoresis	98
5.4 Statistical analysis.....	98
5.5 Results	99
5.5.1 Development of loop-mediated isothermal amplification (LAMP) assay	99
5.5.1.1 The specificity of RIME2 LAMP assay	101
5.5.1.2 The sensitivity of RIME2 LAMP and conventional PCR	103
5.5.1.3 Field samples screened using RIME2 LAMP primers	105
5.5.1.4 Comparison between RIME 2 LAMP and conPCR assays.....	109
5.6 Statistical analysis.....	109
5.7 Discussion	111

REFERENCES..... 115

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS 121

6.1 Conclusion..... 121

6.2 Recommendations..... 123

REFERENCES..... 124

LIST OF TABLES

Table 3.1: Summary of <i>Trypanosoma equiperdum</i> overall infections in horse and donkey DNA tested samples by PCR: $p < 0.0084$	46
Table 4.1. Results of donkey and horse DNA samples screened using RIME1 conPCR $p > 0.05$	76
Table 4.2. Results of the comparison between the two assays qPCR and conPCR for RIME 1 primers $p > 0.05$	78
Table 4.3. Overall infection obtained from the two assays qPCR and RIME 1 conPCR for the detection of <i>T. equiperdum</i>	79
Table 4.4. Symmetric measures between qPCR and RIME 1 conPCR	79
Table 5.1. RIME 2 LAMP primers.....	96
Table 5.2: Shows temperatures of melting peaks for optimization.....	99
Table 5.3. Shows temperatures of melting peaks for specificity	101
Table 5.4. Shows temperatures of melting peaks for RIME2 LAMP sensitivity	103
Table 5.5. Results of Donkey and Horse DNA samples screened using RIME2 LAMP assay	106
Table 5.6. RIME 2 LAMP and conPCR amplification results for <i>T. equiperdum</i> infections across the sampled Provinces ($p < 0.04$)	109
Table 5.7. Results of the comparison of RIME 2 LAMP and conPCR for the detection of <i>T. equiperdum</i> ($p < 0.0001$).....	110
Table 5.8. Symmetric Measures between RIME 2 LAMP and PCR assays.....	110

LIST OF FIGURES

Figure 2.1: Illustration of the Complement fixation test	17
Figure 2.2: General steps for performing ELISA (For interpretation of the references to color in this text, the reader is referred to the web version of the article). Adapted and modified from resource number.....	22
Figure 3.1: South African map indicating provinces where the equine samples were collected.....	41
Figure 3.2: Gel image of 1% agarose gel electrophoresis showing specificity and sensitivity of <i>Trypanosoma equiperdum</i> . M: Ladder (1kb), 1. DDW, 2. <i>T. equiperdum</i> , 3. <i>T. brucei brucei</i> , 4. <i>T. evansi</i> , 5. H-DNA for specificity, 6. 10^{-1} , 7. 10^{-2} , 8. 10^{-3} , 9. 10^{-4} , 10. 10^{-5} , 11. 10^{-6} , 12. 10^{-7} , 13. 10^{-8} for <i>T. equiperdum</i> sensitivity, 14. DDW. PCR had a detection limit of 10^{-5} which is equivalent to 1 trypanosome/ml.....	45
Figure 3.3: Gel image of 1% agarose gel electrophoresis of <i>Trypanosoma equiperdum</i> . M: Ladder (1kb), 1. DDW, 2. <i>T. equiperdum</i> , 3. P1, 4. P2, 5. P3, 6. P8, 7. H1, 8. H2, 9. H5, 10. H8, 11. H10, 12. H14, 13. H26, 14. H28, 15. MP9, 16. MP10, 17. MP12, 18. MP13, 19. MP14, 20. MP46, 21. MP47, 22. MP51, 23. MP52, 24. MP62, 25. MP77, 26. MP87 & 27. H-DNA.....	47
Figure 3.4: BLASTn results showing the alignment of <i>T. brucei</i> and one of the sequences from this study which was from a horse sample from Free State Province. The subject sequence (<i>Trypanosoma brucei</i> (RIME) in rRNA gene: Accession no. K01801.1, it had a match of 95% on the query sequence (FS_H14 Equine) The black stars indicate trans-versions as well as transitions that occurred between sequences.....	47
Figure 4.1: Real-time PCR response curves. A threshold level is set sufficiently above background and the number of cycles required to reach threshold (C_t) are registered.....	59
Figure 4.2: Forward primer, probe and reverse primer design based on 1402 bp RIME genomic sequence, GenBank accession number K01801.1	63

Figure 4.3: Optimization amplification plot of RIME 1 real-time PCR primers using SYBR Green I. <i>T. equiperdum</i> DNA was used and different temperatures were selected (58, 60, & 62°C) no reaction was detected at 58°C in DDW.	69
Figure 4.4: Optimization melt-curve of RIME 1 real-time PCR primers using SYBR Green I. <i>T. equiperdum</i> DNA was used and different temperatures were selected (58, 60, & 62°C) no reaction was detected at 58°C in DDW.	69
Figure 4.5: SYBR Green I amplification plot of RIME 1 primers for <i>T. equiperdum</i> parasites.	70
Figure 4.6: SYBR Green I qPCR standard curve generated from linear region of each amplification curve of five 10-fold serially dilutions of <i>T. equiperdum</i> for RIME 1 primers.	70
Figure 4.7: SYBR Green I qPCR melt-curve analysis of RIME 1 primers for <i>T. equiperdum</i> parasites.	71
Figure 4.8: PrimeTime probe-based qPCR amplification plot of RIME 1 for <i>T. equiperdum</i> parasites.	71
Figure 4.9: Standard curve generated from five 10-fold serially dilutions of <i>T. equiperdum</i> for RIME 1 real-time PCR primers using PrimeTime a probe-based.	72
Figure 4.10: SYBR Green I qPCR sensitivity of RIME 1 primers for <i>T. equiperdum</i> parasites.	73
Figure 4.11: Melt-curve analysis for sensitivity of RIME 1 primers for <i>T. equiperdum</i> parasites.	73
Figure 4.12: Results of specificity for RIME 1 primers using SYBR Green I. DNA's from different pathogens were used together with <i>T. equiperdum</i> DNA as a positive control. No amplification was observed in non-target DNA and DDW.	74
Figure 4.13: Melt-curve analysis for specificity of RIME 1 primers. Different pathogens were used together with <i>T. equiperdum</i> DNA as a positive control. No amplification was observed in non-target DNA and negative control (DDW).	74

Figure 4.14: RIME1 primers sensitivity gel image of 1% agarose gel electrophoresis. M: Ladder (1kb), 1. DDW, 2. <i>Trypanosoma equiperdum</i> , 3. 10^{-1} , 4. 10^{-2} , 5. 10^{-3} , 6. 10^{-4} , 7. 10^{-5} , 8. 10^{-6} , PCR had a detection limit of 10^{-5} which is equivalent to 1 trypanosome/ml.....	75
Figure 4.15: RIME 1 primers specificity gel image of 1% agarose gel electrophoresis. M: Ladder (1kb), 1. DDW, 2. <i>Trypanosoma equiperdum</i> , 3. <i>T. brucei brucei</i> , 4. <i>T. evansi</i> , 5. <i>T. congolense</i> & 6. H-DNA.....	75
Figure 4.16: Gel image of 1% agarose electrophoresis showing amplified PCR products with amplicon size of 115 bp. M: Ladder (1kb), 1. DDW, 2. <i>Trypanosoma equiperdum</i> , 3. P1, 4. P2, 5. P3, 6. P8, 7. H1, 8. H2, 9. H5, 10. H8, 11. H10, 12. H14, 13. H26, 14. H28, 15. MP9 & 16. H-DNA. Red arrows indicating positive samples.....	77
Figure 4.17: BLASTn results showing the alignment of <i>T. brucei</i> and one of the sequences from this study which was from a horse sample from Free State Province. The subject sequence (<i>Trypanosoma brucei</i> RIME in rRNA gene: Accession no.: K01801.1, it had a match of 95% on the query sequence (FS_H14 Equine). The red stars show gaps between sequences.	77
Figure 5.1: LAMP hybridizing to specific sites of target gene	91
Figure 5.2: Principle of loop-mediated isothermal amplification (LAMP) method. (a) Primer design (b) starting structure producing step and (c) cycling amplification step	93
Figure 5.3: Forward primer, reverse primer, FIP and BIP primers design based on 1402 bp RIME genomic sequence, GenBank accession number K01801.1.....	95
Figure 5.4: A series of ten-fold dilutions of <i>T. equiperdum</i> DNA.....	97
Figure 5.5: Results for optimization, when the temperature was set at 65°C for 1hr. Produced positive responses.	100
Figure 5.6: Annealing curves for the amplified <i>T. equiperdum</i> in triplicates at ~88.8°C.....	100
Figure 5.7: Agarose gel electrophoresis showing optimization results of RIME2 LAMP primers using <i>T. equiperdum</i> DNA. M: DNA 1kb; L1: DDW, L2, L3 & L4 <i>T. equiperdum</i> , L5 & L6 DDW.	101

Figure 5.8: Specificity amplification at 65°C for 1 hr. The RIME2 LAMP assay was specific; no reactivity was recorded with non-target DNA (H-DNA) & DDW.....	102
Figure 5.9: Specificity annealing curves of the <i>Trypanosoma equiperdum</i> , <i>T. brucei brucei</i> and <i>T. evansi</i> produced a single peak, at 89.5°C, indicating similar amplicons.....	102
Figure 5.10: Gel agarose electrophoresis showing results of specificity of RIME2 LAMP primers M: DNA ladder (1kb), L1: <i>T. equiperdum</i> , L2: <i>T. brucei brucei</i> , L3: <i>T. evansi</i> , L4: H-DNA & L5: DDW.....	103
Figure 5.11: RIME 2 LAMP sensitivity 10 ⁰ , 10 ⁻¹ , 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ , 10 ⁻⁸ at 65°C for 1 hr. Amplification had a detection limit of 10 ⁻⁷ which is equivalent to 0.001 number of trypanosomes/ml.	104
Figure 5.12: RIME2 LAMP sensitivity annealing peaks generated in the real time LAMP assay for the amplified target resulting from different concentrations.	104
Figure 5.13: Gel agarose electrophoresis showing results of sensitivity of RIME2 LAMP primers M: DNA ladder (1kb), L1:10 ⁻¹ , L2: 10 ⁻² , L3: 10 ⁻³ , L4: 10 ⁻⁴ , L5: 10 ⁻⁵ , L6: 10 ⁻⁶ & L7: 10 ⁻⁷	105
Figure 5.14: RIME 2 LAMP Amplification plot at 65°C for 1 hr when field samples were screened. <i>Trypanosoma equiperdum</i> (positive control), D1 & D2 (donkey samples from North-West), MP (14, 77 &, 87 horse samples from Mpumalanga).....	107
Figure 5.15: Annealing (melt) curves of the field samples, some samples produced a single peak with <i>Trypanosoma equiperdum</i> at 89.5°C, indicating similar amplicons.....	107
Figure 5.16: Gel agarose electrophoresis showing results of RIME2 LAMP primers M: DNA ladder (1kb), L1: DDW (negative control), L2: <i>T. equiperdum</i> (positive control), L3: D1, L4: D10, L5: D11, L6: MP 77 & L7: MP87 donkey and horse samples from the sampled provinces.	108
Figure 5.17: The overall <i>Trypanosoma equiperdum</i> positives obtained using RIME 2 LAMP primers for all tested horse and donkey samples was 26 and 8 respectively across all the sampled provinces.....	108

CHAPTER 1 INTRODUCTION

1.1 Equine population in South Africa

The equine population in South Africa is estimated to be more than 450 000 individuals consisting of 300 000 horses, 150 000 donkeys, and 14 000 mules (Marlow, 2010; EU final Report, 2013). From this population, 20% of the horse population is registered (purebred) horses, of which about 20 000 are thoroughbred racehorses. Zebras (20 000) on the other hand are found almost exclusively in national parks and more than half of that population is confined to the Kruger National Park, whilst the remaining are in private parks (EU final Report, 2013). The equine industry reaches far and wide around the world, creating an international market place that depends on the rapid movement of horses and their biological products to and from distant lands (Ferraro *et al.*, 2006). The most common roles of horses and donkeys are transport, whether riding, pack transport or pulling carts and in some countries they may be utilized in farm cultivation and for other agricultural purposes. In certain countries they may contribute to threshing of grain, raising water, milling or other operations (Marlow, 2010; Starkey & Starkey, in press). Equines are very sensitive and vulnerable to infectious diseases and require very good management practices in comparison to other animal species (Khurana *et al.*, 2016).

Despite their, usefulness, significant contribution to the communities and the national economy, little attention is given to study the health aspects of working equids (Fikru *et al.*, 2015). Equine practise is a small but essential part of the wider concept of equine veterinary medicine. It can never be seen in isolation as it is concerned with and dependent on equine veterinary activities and ongoing worldwide research (Marlow, 2010). There are numerous prevailing and emerging equine pathogens some having zoonotic potential, and posing a threat to the public health (Khurana *et al.*, 2016).

1.2 Dourine

Dourine is one of the equine diseases listed by the World Organization for Animal health (OIE) as an internationally important animal disease (Suganuma *et al.*, 2017). Dourine is

caused by the protozoan parasite *Trypanosoma equiperdum* (Clausen *et al.*, 2003; Samper & Tibary, 2006; Lu *et al.*, 2007; OIE, 2015, Gizaw *et al.*, 2017). It has a cosmopolitan distribution (Brun *et al.*, 1998; Wei *et al.*, 2011; OIE, 2015) and considered endemic where it exists. Dourine can be found anywhere as the transmission of the disease does not require insect vectors that are influenced by climatic factors, but more importantly even in areas where mechanical or tsetse-transmitted trypanosomes are endemic. This disease poses a significant challenge to equine production (OIE 2015; Gizaw *et al.*, 2017). Dourine threatens equidae around the globe and is known in most countries of the world as a notifiable disease (Ahmed *et al.*, 2018). Unlike nagana and surra which require a vector to be transmitted, dourine is sexually transmitted amongst equids and foals are infected during birth or through ingestion of maternal milk, resulting in global health threats for all equines. Horses usually die from infection without treatment whereas donkeys and mules are more resistant than horses and may remain unapparent carriers (Gizaw *et al.*, 2017; Ahmed *et al.*, 2018). There are no vaccines available for the control of the disease and slaughtering of infected animals and controlling movement are the only control measures enforced by legislation (OIE, 2013; Gizaw *et al.*, 2017).

Dourine can be clinically detected on the basis of the following symptoms in infected equids: inflammation of genitalia, followed by oedema of subcutaneous tissues (silver dollar plaques) and paralyses (Ricketts *et al.*, 2011; Chin *et al.*, 2013; Luciani *et al.*, 2013). The long-term efficiency of treatment is uncertain (OIE, 2015). Parasitological diagnosis of dourine in chronically infected horses or donkeys is difficult, due to the uncertainty of finding parasites in the tissues and its fleeting presence in the bloodstream (Clausen *et al.*, 2003, Becker *et al.*, 2004; Gizaw *et al.*, 2017). The detection and diagnosis of parasite infections rely on several laboratory methods in addition to clinical symptoms observed and factors such as the clinical history, travel history and geographic location of animal are also considered for accurate diagnosis (Ndao, 2009). According to Liu (2008), microscopic examinations in combination with staining and immunological techniques are commonly simple and fast. However, not every pathogen can be identified by means of microscopy and many pathogens cannot grow outside their hosts. Countless boundaries of microscopy and serology-based assays have influenced parasitologists towards the use of gene amplification methods (Ndao, 2009). Serology has come to play a major role in the diagnosis of parasitic diseases in recent years (Voller *et al.*, 1976). Numerous molecular assays have been developed using a variety of technologies (Liu, 2008). In the

present study, various nucleic acid techniques were implemented that provide powerful alternative tools to overcome the limitations of traditional approaches, particularly those which rely on the amplification of nucleic acids (Gasser, 2006).

1.3 Statement of the problem

Dourine has been neglected by research, and current knowledge on the disease as well as the parasite is very poor despite its considerably high burden globally. Diagnostic techniques and identification of *T. equiperdum* still remains a challenge (Gizaw *et al.*, 2017). More accurate epidemiological information on trypanosome species would possibly serve as a good basis for revisiting established control protocols. This information would help in enhancing the current knowledge about the disease to the farmers and experts in the field. Donkeys and horses contribute in various ways to the socio-economic well-being of many communities in terms of transport, animal power, entertainment (Kumba *et al.*, 2003; Hagos *et al.*, 2010), beef cattle husbandry and as an important export commodity to the southern African market (Kumba *et al.*, 2003). Therefore, there is possibility of disease transmission and spread at the time of equine movement from one country to another (Prasad *et al.*, 2016). A rapid diagnosis of diseases and suitable treatment are important steps that promote optimal clinical outcomes and general public health (Prasad *et al.*, 2016).

The findings of a previous study (Mlangeni, 2016) revealed the presence of dourine by polymerase chain reaction (PCR) and Enzyme-linked immune-sorbent (ELISA) assays from horse and donkey blood samples. However, some samples were serologically positive but were seemingly aparasitemic by PCR. Unfortunately, parasitological techniques are known to lack sensitivity, especially for the detection of *T. equiperdum*, which is considered to be a tissue parasite rather than a blood parasite (Claes *et al.*, 2005). According to Konnai *et al.*, (2009), PCR is rapid and sensitive. Polymerase chain reaction has been acknowledged as one of the most specific and sensitive methods for the diagnosis of infectious diseases, and many applications of PCR for detecting pathogenic microorganisms have been reported (Desquesnes & Davila, 2002). Serological tests are efficient for large scale epidemiological surveys although they do not discriminate between current and past infections (Becker *et al.*, 2004; Thekiso *et al.*,

2007; Konnai *et al.*, 2009; Gizaw *et al.*, 2017). LAMP is a much more sensitive DNA based diagnostic assay which does not require expensive equipment and uses simple detection methods after completion of the reaction (Notomi *et al.*, 2000). However, LAMP is still to be exploited for *T. equiperdum* infection diagnostics.

1.4 Aim

To develop molecular diagnostic assays for detection of *Trypanosoma equiperdum* infections in equines in South Africa.

1.5 Objectives

1.5.1 To develop a conventional PCR assay for detection of *T. equiperdum* infections in South African equines

1.5.2 To develop a real-time PCR (qPCR) assay for detection of *T. equiperdum* infections in South African equines

1.5.3 To develop a loop-mediated isothermal amplification (LAMP) assay for detection of *T. equiperdum* infections in South African equines

1.6 Hypothesis

DNA based diagnostic assays can detect South African *T. equiperdum* infections from blood with high diagnostic efficiency.

1.7 Thesis outline

Chapter 1: It provides an overview on the current state of equine population in South Africa. In addition, it provides information on the roles of horses and donkeys, their

significant contribution, dourine, clinical significance, symptoms as well as the control of the disease. Furthermore, the problem statement, hypothesis, aim and objectives are also included in this chapter.

Chapter 2: Provides a detailed literature review on dourine including distribution, pathogenesis, transmission, treatment, economic importance and diagnosis of trypanosomes.

Chapter 3: This chapter reports on the development of conventional PCR targeting RIME gene for detection of *T. equiperdum* infections in South Africa.

Chapter 4: This chapter provides the information on the standardization, sensitivity, specificity and validation of real-time PCR targeting RIME gene for detection of *T. equiperdum* infections in South Africa.

Chapter 5: This chapter reports on the successful development of LAMP assay targeting RIME gene for detection of *T. equiperdum* infections in South Africa.

Chapter 6: This chapter provides a summary of the relevant conclusions of the overall study as well as recommendations for future research.

REFERENCES

- Ahmed, Y., Hagos, A., Merga, B., Van Soom, A., Duchateau, L., Goddeeris, B.M. & Govaere, J. (2018). *Trypanosoma equiperdum* in the horse – a neglected threat? *Vlaams Diergeneeskundig Tijdschrift*, 87:66–75.
- Becker, S., Franco, J.R., Simarro, P.P., Stich, A., Abele, P.M. & Steverding, D. (2004). Real-time PCR for detection of *Trypanosoma brucei* in human blood samples. *Diagnostic Microbiology and Infectious Disease*, 50:193–199.
- Brun, R., Hecker, H. & Lun, Z-R. (1998). *Trypanosoma evansi* and *T. equiperdum*, distribution, biology, treatment and phylogenetic relationship. *Veterinary Parasitology*, 95–107.
- Chin S.W., Uppal P.K., Premaalatha B., Chandrawathani P., Norazura A.H. & Ramlan M. (2013). Freedom status of dourine (*Trypanosoma equiperdum*) in Malaysia. *Malaysian Journal of Veterinary Research*, 4:61-64.
- Claes F., Ilgekbayeva G.D., Verloo D., Saidouldin T.S., Geerts S., Buscher, P. & Goddeeris, B.M. (2005). Comparison of serological tests for equine trypanosomiasis in naturally infected horses from Kazakhstan. *Veterinary Parasitology*, 131:221-225.
- Claes, F., Büscher, P., Touratier, L. & Goddeeris, B.M. (2005). *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends in Parasitology*, 21:316-321.
- Clausen, P-H., Chuluun, S., Sodnomdarjaa, R., Greiner, M., Noeckler, K., Staak, C., Zessin, K-H. & Schein, E. (2003). A field study to estimate the prevalence of *Trypanosoma equiperdum* in Mongolian horses. *Veterinary Parasitology*, 115:9–18.
- Desquesnes, M. & Davila, A. M. R. (2002). Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. *Veterinary Parasitology*, 109:213-231.
- European Commission, (2013). In order to evaluate the animal health controls in place in relation to export of equidae to the EU, with particular reference to African horse sickness. *Final Report of an Audit Carried out in South Africa*, 4-31.

Ferraro, G.L. (2016). Global health and the sport horse. Edited by Meierhenry, B. *Published by UC Davis*, 1-24.

Fikru, A., Tadese, A. & Gebreegziabher, Z. (2015). Prevalence of equine wound and associated risk factors in and around Kombolcha Town, North Ethiopia. *Veterinary Science & Technology*, 6:1-4.

Gasser, R.B. (2006). Molecular tools—advances, opportunities and prospects. *Veterinary Parasitology*, 136:69–89.

Gizaw, Y., Megersa, M. & Fayera, T. (2017). Dourine: a neglected disease of equids. *Tropical Animal Health Production*, 49:887–897.

Hagos, A., Abebe, G., Philip, B., Bruno, M.G & Claes, F. (2010). Serological and parasitological survey of dourine in the Arsi–Bale highlands of Ethiopia. *Tropical Animal Health and Production*, 42:769–776.

Hagos, A., Degefa, G., Yacob, H.B., Fikru, R., Alemu, T., Feseha, G., Claes, F. & Goddeeris, B.M. (2010b). Seroepidemiological survey of *Trypanozoon* infection in horses in the suspected dourine–infected Bale highlands of the Oromia region, Ethiopia. *Revue Scientifique Et Technique De L`Office International Des Epizooties*, 29:649–654.

Hagos, A., Goddeeris, B.M., Yilkal, K., Alemu, T., Fikru, R., Yacoba, H.T., Feseha, G. & Claes, F. (2010). Efficacy of Cymelarsan® and Diminasan® against *Trypanosoma equiperdum* infections in mice and horses. *Veterinary Parasitology*, 171:200–206.

Khurana, S.K., Dhama, K., Minakshi, P., Gulati, B., Malik, Y. S. & Karthik, K. (2016). Leptospirosis in horses: special reference to equine recurrent uveitis. *Journal of Experimental Biology and Agricultural Sciences*, 4(Spl 4-EHIDZ).

Konnai, S., Mekata, H., Mingala, C.N., Abes, N.S., Gutierrez, C.A., Herrera, J.R.V., Dargantes, A.P., Witola, W.H., Cruz, L.C., Inoue, N., Onuma, M. & Ohashi, K. (2009). Development and application of a quantitative real-time PCR for the diagnosis of Surra in water buffaloes. *Infection, Genetics and Evolution*, 9:449–452.

Kumba, F.F., Claasen, B. & Petrus, P. (2002). Apparent prevalence of dourine in the Khomas region of Namibia. *Onderstepoort Journal of Veterinary Research*, 69:295-298.

- Liu, Y-T. (2009). A technological update of molecular diagnostics for infectious diseases. *Infectious Disorder Drug Targets*, 8:83–188.
- Lu, K.G. & Morresey, P.R. (2007). Infectious diseases in breeding stallions. *Clinical Techniques in Equine Practice*, 6:285-290.
- Luciani M., Di Pancrazio C., Di Febo Y., Tittarelli M., Vulpiana M.P., Puglielli M.O., Naessens J. & Sacchini, F. (2013). IgG antibodies from dourine infected horses identify a distinctive *Trypanosoma equiperdum* antigenic pattern of low molecular weight molecules. *Veterinary Immunology and Immunopathology*, 151:140-146.
- Luckins, A.G., Barrowman, P.R., Stoltz, W.H. & Van der Lugt, J.J. (2004). Dourine. In: Infectious diseases of livestock, (eds) Coetzer J.A.W & Tustin R.C. Oxford University Press 3:297–302.
- Marlow, C.H.B. (2010). A brief history of equine private practice in South Africa. *South African Veterinary Association*, 81:190–200.
- Mlangeni, M.A. (2016). Molecular epidemiology of dourine, equine piroplasmiasis and ehrlichiosis in equines in South Africa. (MSc Dissertation)
- Ndao, M. (2009). Diagnosis of parasitic diseases: old and new approaches. *Interdisciplinary Perspectives on Infectious Diseases*, 1-15.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28: e63-e63.
- OIE, (2013). Chapter 2.5.3. Dourine. *Terrestrial Manual*, 1–10.
- OIE, (2015). Dourine. *The Center for Food Security and Public Health, Iowa State University, College of Veterinary Medicine*, 1-4.
- Prasad, M., Brar, B., Iqbal, Ranjan, K., Lalme, U., Manimegalai, J., Vashisht, B., Khurana, S.K. & Prasad, G. (2016). Biotechnological tools for diagnosis of equine infectious diseases. *Journal of Experimental Biology and Agricultural Sciences*, 4:161-181.

Ricketts, S. & McGladdery, A. (2011). Dourine – an emerging venereal threat to European horses. *Equine Quarterly Disease Surveillance report* 6, 7:15-18.

Samper, J.C. & Tibary, A. (2006). Disease transmission in horses. *Theriogenology*, 66:551–559.

Starkey, P. & Starkey, M. (in press). Regional and world trends in donkey populations. *Regional and World Trends in Donkey Populations*, 10-21.

Suganuma, K., Yamasaki, S., Molefe, N.I., Musinguzi, P.S., Davaasuren, B., Mossaad, E., Narantsatsral, S., Battur, B., Battsetseg, B. & Inoue, N. (2017). The establishment of *in vitro* culture and drug screening systems for a newly isolated strain of *Trypanosoma equiperdum*. *International Journal for Parasitology: Drugs and Drug Resistance*, 7:200 – 205.

Thekisoe, O.M.M., Kuboki, N., Nambota, A., Fujisaki, K., Sugimoto, C., Igarashi, I., Yasuda, J. & Inoue, N. (2007). Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomosis. *Acta Tropica*, 102:182–189.

Voller, A., Bartlett, A., & Bidwell, D.E. (1978). Enzyme immunoassays with special reference to ELISA techniques. *Journal of Clinical Pathology*, 31:507-520.

Wei, Y., Wen, Y-Z., Desquesnes, M. & Lun, Z-R. (2011). Molecular epidemiology of *Trypanosoma evansi* and *T. equiperdum* and atypical human infection by animal trypanosomes. *Madame Curie Report*, 1-14.

CHAPTER 2 LITERATURE REVIEW

2.1 General overview of dourine

Dourine is a serious chronic contagious disease of equines (horses, donkeys, mules and zebras), caused by a protozoan parasite *Trypanosoma equiperdum* (Luckins *et al.*, 2004; OIE, 2015). This tissue parasite, is the only trypanosome species that does not require an invertebrate vector for transmission (Bonfini *et al.*, 2018), unlike other trypanosomes (Hagos *et al.*, 2010, Pascucci *et al.*, 2013). Therefore, dourine is a truly venereal disease which is transmitted almost exclusively by coitus (Gizaw *et al.*, 2017). This disease can develop neurological signs from either asymptomatic or intestinal form (DARD dourine report, 2018). Based on the kinetoplast (kDNA) components, *T. equiperdum* is classified along with *T. brucei* subspecies and *T. evansi* in the sub-genus *Trypanozoon*. The maxicircle kDNA of *T. equiperdum* differs in each strain, while *T. evansi* lacks maxicircle kDNA totally and *T. brucei* have a complete maxicircle kDNA (Suganuma *et al.*, 2016). There is no known natural reservoir of the parasite other than infected equids (OIE, 2013). For centuries the disease was known to the Arabs and horsemen of North Africa before it was reported in Europe in the late 18th century (Luckins *et al.*, 2004).

2.2 Distribution of dourine

(i) Globally

Due to the strict implementation of control measures, dourine cases declined rapidly in most parts of the world during the 20th Century, predominantly from the 1950s onwards. Zablotskij *et al.*, (2003) reported that the disease is still prevalent in some countries such as Botswana, Lesotho, Namibia, Russia and South Africa. However, the officially recommended diagnostic test for the international trade in equines, the complement fixation test (CFT), generates false positive results (Zablotskij *et al.*, 2003, Samper & Tibary, 2006). Despite, *T. equiperdum* having a wide geographical distribution, it is still believed that cases of dourine are rarely reported possibly due to the difficulty of diagnosis (Brun *et al.*, 1998; Gizaw *et al.*, 2017). Small numbers of new cases of dourine have been reported from countries such as, China, Kazakhstan, Kyrgyzstan, Pakistan, Ethiopia, Botswana, Namibia, South Africa, Brazil, Italy, Germany and in Mongolia recently (Li *et*

al., 2007; Taylor *et al.*, 2007; Gizaw *et al.*, 2017). In South America, *T. equiperdum* is also reported to exist, although there is a little or no information from this region (OIE, 2015; Bonfini *et al.* 2018).

(ii) Africa

Dourine positive cases are recorded in Botswana, Ethiopia, Lesotho, South Africa, Swaziland and Rhodesia (Barrowman & van Vuuren, 1976).

(iii) South Africa

A total of 105 cases of dourine are confirmed in South Africa. Eastern Cape with 40% reported cases and the most severely affected province, followed by 19% in KwaZulu-Natal, 16% in Northern Cape whilst Mpumalanga, Free State and other provinces have a comparatively low report cases (Epidemiology report, 2012; DARD dourine report, 2018).

2.3 Pathogenesis

According to Luckins *et al.*, (2004), most pathological effects of *T. equiperdum* infection in the host are characteristic of trypanosome infections in general, particularly those of the *T. brucei* complex. The disease is marked by phases of exacerbation, tolerance or relapse, which varies in duration and which may occur once or several times before death or recovery (Taylor *et al.*, 2007). Natural transmission of the pathogen happens when the parasite is placed in the mucous membranes of the genitalia during sexual intercourse. *Trypanosoma equiperdum* also has the ability to enter mucous membranes entirely and lymphatic vessels like the other members of the *T. brucei* complex but it also has a preference for connective tissue (OIE, 2015). It multiplies mostly in extracellular tissue spaces and it is rarely found in peripheral blood and probably only uses the bloodstream as a means of transport from one site to another. Nutrient content of extracellular fluid may favour the development of the parasites. To some degree this site may also aid their evasion of the activity of antibodies present in circulating blood. The onset of the nervous form of the disease appears to coincide with the presence of parasites in the cerebrospinal fluid (Luckins *et al.*, 2004).

2.4 Transmission of *Trypanosoma equiperdum*

Unlike other trypanosomal infections, dourine is transmitted almost completely during mating (OIE, 2015). The agents of disease are transmitted during sexual intercourse between stallion and mare (Mehlhorn, 2017). The trypanosomes which are present in the seminal fluid and mucous membranes of the genitalia of the infected donor animal are transferred to the recipient during sexual intercourse (Luckins, 1994; Brun *et al.*, 1998; Gizaw *et al.*, 2017). Then the transferred trypanosomes penetrate the intact mucous membranes and initiate an infection in the recipient animal (Brun *et al.*, 1998). In the disease process, transmission is most likely early as non-infectious periods are more common at later stages (Ricketts *et al.*, 2011). Male donkeys can be asymptomatic carriers and sexually immature animals that become infected can transmit the organisms when they mature. There is currently no evidence that arthropod vectors play any role in *T. equiperdum* transmission (OIE, 2015; Gizaw *et al.*, 2017). However, a few trypanosomes occasionally appear in the peripheral blood of animals with chronic infection of which it could provide an opportunity for bloodsucking insects to mechanically transmit this parasite, but this is considered very rare (Gizaw *et al.*, 2017). The fact that foals have been found to be infected with *T. equiperdum* may be an indication that this parasite can also be directly transmitted through the milk or from udder lesions (Brun *et al.*, 1998; Pascussi *et al.*, 2013) though it is considered rare (Gizaw *et al.*, 2017).

2.5 Clinical signs of dourine

According to Gizaw *et al.*, (2017), dourine has been broken in three stages, whereby genital lesions is regarded as stage 1, cutaneous signs as stage 2 and lastly nervous signs as stage 3. Stage 1, in mares there is a discharge from the vagina, slight fever, edema, swelling and loss of appetite manifesting 1 to 2 weeks after infection. In stage 2, silver dollar plaques “cutaneous plaques” and round rash appears (Gizaw *et al.*, 2017; Bonfini *et al.*, 2018), with thickening of the skin, considered pathognomonic (Gizaw *et al.*, 2017). The third stage involves stiffness, weakness of the limbs with lack of coordination, anemia, neurological disorders, often ending in death. In the stallion, the first clinical sign is a variable swelling involving the glans penis and prepuce. Clinical signs of dourine frequently develop over weeks to months, often waxing and waning with relapses probably precipitated by stress. This can occur several times before the animal either dies or experiences an apparent recovery (OIE, 2015; Gizaw *et al.*, 2017).

2.6 Treatment of dourine

Pharmaceutical treatment is not prescribed because animals may improve clinically but continue to be carriers of the parasite. There are no formally accepted drugs to treat horses suffering from dourine although some older publications mentioned experimental treatment of horses with suramin and neoarsphenamine or quinapyramine sulfate (Gizaw *et al.*, 2017). These are the same drugs used for infections of *Trypanosoma evansi* and are similarly used for treatment of *T. equiperdum* infections, although there are no published reports on their clinical efficacy (Brun *et al.*, 1998; Gizaw *et al.*, 2017). Treatment is not recommended because it may result in unapparent carriers that can spread the disease (Ferraro *et al.*, 2006). Infected animals should be humanely slaughtered or castrated to prevent further transmission of the disease (Claes *et al.*, 2005; Gizaw *et al.*, 2017).

2.7 Economic importance of dourine

Trypanosomes are protozoan parasites that are pathogenic to humans, livestock, and are commercially important (Shapiro, 1993). Other than *T. equiperdum*, animal trypanosomiasis is caused by *Trypanosoma brucei brucei*, *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma simiae* and *Trypanosoma evansi*, which have significant socio-economic impact as they limit animal protein productivity throughout the world (Sanchez *et al.*, 2015). Dourine is a contagious disease of great economic importance and well documented as a trade barrier for the movement of horses (Chin *et al.*, 2013, Gizaw *et al.*, 2017). Ferraro *et al.*, (2006); stated that, this contagious disease has the potential for causing significant losses to the economy of equine-based businesses and unfavourably affect the health and welfare of horses. The mortality rate in untreated cases is high 50-70% and no vaccine is available (OIE, 2015).

2.8 Diagnosis of trypanosomes

In practice, diagnosis of dourine is based on clinical evidence supported by serology (Clausen *et al.*, 2003; Gizaw *et al.*, 2017). Even though in the developed disease clinical

signs may be pathognomonic, the disease cannot constantly be identified with certainty, especially in the early stages or in latent cases (Clausen *et al.*, 2003). Complement fixation test (CFT) is the official and international method for serological examination for dourine, conferring to the World Organisation for Animal Health (Clausen *et al.*, 2003; Claes *et al.*, 2005; Lu *et al.*, 2007; Cencek *et al.*, 2008; Chin *et al.*, 2013). Nevertheless, recent studies have revealed that this test cannot differentiate between *T. brucei*, *T. evansi* and *T. equiperdum* (Samper & Tibary, 2006; Luciana *et al.*, 2013). According to Brun *et al.*, (1998), Polymerase chain reaction (PCR) using species specific primers have been used to distinguish *T. equiperdum* from *T. evansi*. However, since *T. equiperdum* is rarely found in the blood, the indirect methods may have a greater potential to detect infections (Brun *et al.*, 1998, OIE 2013).

Different approaches have been employed to detect *T. brucei*, *T. equiperdum* and *T. evansi* infections. These include parasitological, serological or immunological and molecular based techniques (Li *et al.*, 2007). Diagnosis of *T. equiperdum* by means of standard parasitological techniques is difficult, due to the low numbers of parasites present in the blood or tissue fluids (Hagos *et al.*, 2010; Gizaw *et al.*, 2017) and the frequent absence of clinical signs of disease (Hagos *et al.*, 2010). Parasitological techniques are also known to lack sensitivity, especially for the detection of *T. equiperdum*, which is considered to be a tissue parasite rather than a blood parasite (Claes *et al.*, 2005; Li *et al.*, 2007). Therefore, the serological techniques, which detect the anti-Trypanosoma circulating antibodies, are more effective for the diagnosis of the disease (Ryena-Bello *et al.*, 1998). However, there is currently a lot of focus on using the molecular methods as alternative tools for the diagnosis of diseases. These methods can provide sensitive, specific, rapid and reliable detection of parasites. Currently, these require highly experienced personnel and well-equipped laboratories; however, efforts are being made to simplify the use of these tools (Adams *et al.*, 2014). Nevertheless, the diagnosis of *T. equiperdum* infection is still strongly based on serological evidence as recommended by OIE (Gizaw *et al.*, 2017).

2.9 Serological techniques

According to Hagos *et al.*, (2010), the purpose of the use of serological tests in the diagnosis of trypanosomiasis is to overcome the low sensitivity of parasitological tests in detecting chronic infection. Serological methods such as enzyme linked immunosorbent assays (ELISA), the falcon assay screening test–ELISA, indirect or direct immunofluorescent antibody tests, immunoblotting dot-ELISA, peptide based-ELISA, the complement fixation test, the card agglutination test (CATT), agar gel immunodiffusion and neutralization tests are widely accepted for the diagnosis of blood pathogens (Umezawa *et al.*, 2001; Ahmed *et al.*, 2013; Prasad *et al.*, 2016). These tests can be used to detect antigens or antibodies (Verloo *et al.*, 2000). Immunoassays may generate results in only a few hours by measuring antibody or antigen (Ag specific for the microbe (Buxton *et al.*, 1975). Detection of antibodies is used in many clinical or epidemiological situations and importantly, for certification purposes required for international horse-trading (Monzon *et al.*, 2003). The demonstration of trypanosomal antibodies in the serum has become the most important parameter in determining the disease status of individual animals. *Trypanozoon* group-specific trypanosomal antigen can be used in an antibody assay for the diagnosis of *T. equiperdum* infections. The complement fixation test is the most commonly OIE-prescribed sero-diagnostic test developed for *T. equiperdum* (Claes *et al.*, 2005; Gizaw *et al.*, 2017).

2.9.1 Complement fixation test

The complement-fixation (CF) test is an indirect, two phases assay (Cencek *et al.*, 2008) and one of the most appropriate serological tests available, because it can be applied to the diagnosis of various kinds of infectious diseases just by changing the antigen (Bannai *et al.*, 2013). This test may be done by tube (macro) method or microplate method.

Complement fixation test (CFT) detects antibodies against *T. equiperdum* in the serum of the host (Claes *et al.*, 2005). The benefit of this technique is a small consumption of expensive reagents during analysis. The standard of this test is binding the complement with specific antigen-antibody complex. When there is a lack of specific antibodies in the examined serum, the free complement is bound to the indicatory compound causing haemolysis, which is easy to perceive (Cencek *et al.*, 2008; Bannai *et al.*, 2013).

Although the CFT is the only commonly appreciated method for the examination of soliped serum for dourine, it is very time-consuming and complicated. The CFT has also been reported to have a main disadvantage of, often giving false positive results (Clausen *et al.*, 2003; Cencek *et al.*, 2008; Potts *et al.*, 2013) due to cross reactions or anti-complementary activity in equid serum. It also requires good laboratory equipment and well trained staff, to precisely titrate and maintain the reagents (Clausen *et al.*, 2003; Potts *et al.*, 2013). Since isolation of the parasite is difficult to do, the CFT consequently is used to confirm dourine with clinical symptoms or in latent carriers (Calistri *et al.*, 2013; Potts *et al.*, 2013).

According to Gizaw *et al.*, (2017), the complement fixation test is still used for international trade in monitoring horses for export/import. Despite the usefulness and universal acceptance of the CFT for diagnosing dourine, some inconsistencies have been recorded. The CFT, in general lacks the sensitivity to detect low infection intensities, the specificity to make species descriptions and are laborious, time consuming and often inaccurate. Hence, there is a need for more sensitive and specific techniques not to replace CFT but to supplement existing methods of diagnosis (Cencek *et al.*, 2008; Gizaw *et al.*, 2017). Uninfected equids, predominantly donkeys and mules, often give unpredictable or non-specific reactions with CFT because of the anti-complementary effects of their sera (Clausen *et al.*, 2003; OIE, 2009; Calistri *et al.*, 2013; Gizaw *et al.*, 2017). The CFT is not a species specific, but only specific for the subgenus *Trypanozoon*. Therefore, the test is most useful in areas where these parasites do not yet occur (Zablotskij *et al.*, 2003; OIE, 2013). The disadvantage of the test is lower specificity where it cannot differentiate *T. equiperdum* from other related *Trypanozoon* trypanosomes i.e. *T. brucei* and *T. evansi* (Bishop *et al.*, 1995; Luckins *et al.*, 2004; Samper & Tibary, 2006; Cauchard *et al.*, 2014; Gizaw *et al.*, 2017). Therefore, the diagnostic importance of CFT is consequently uncertain in countries where both *T. equiperdum* and *T. evansi* infections occur in equines. Though the CFT has been used for many years for identification of dourine, it is considered to be less sensitive than ELISA and the indirect fluorescent antibody (IFA) test for the detection of serum antibodies against *T. equiperdum* (Bishop *et al.*, 1995; Luckins *et al.*, 2004; Cauchard *et al.*, 2014; Gizaw *et al.*, 2017).

2.9.1.1 Dourine complement fixation test

Antigen and serum are mixed with complement (normal guinea-pig), in the first stage of the CFT. The indicator or haemolytic system is added, which consists of sheep red blood cells (SRBC) that have been sensitized with anti-sheep red blood cell antibody (amboceptor or haemolysin). If the test serum contains antibodies to *T. equiperdum* (positive reaction), complement will be used up or fixed so that it cannot react in the haemolytic system. Therefore, no lysis of SRBC will take place and the SRBC will remain intact. If the test serum does not contain antibodies of *T. equiperdum* (negative reaction) complement will not be fixed and lysis of the SRBC will take place (figure 2.1).

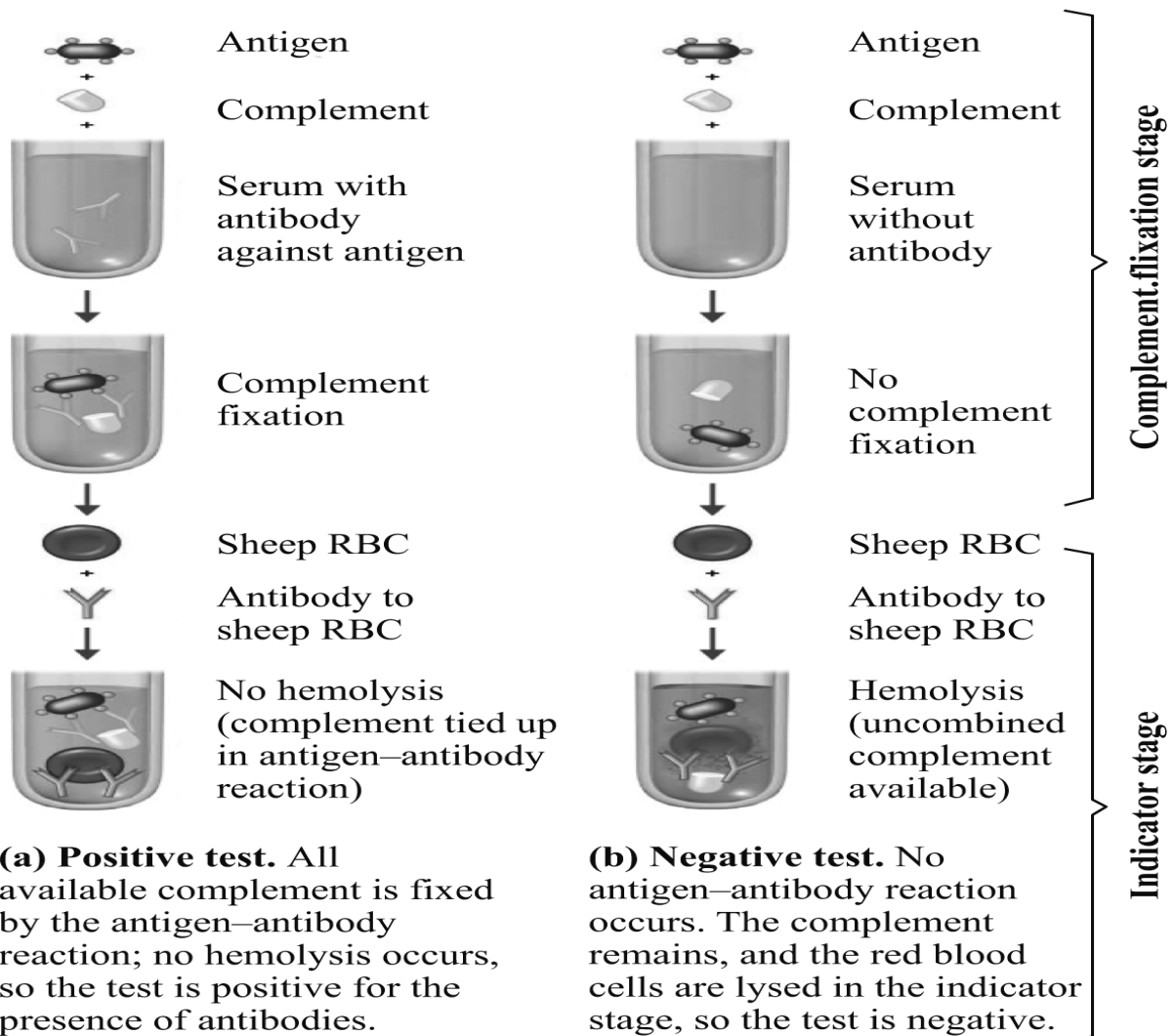


Figure 2.1: Illustration of the Complement fixation test (Microbiology module)

The introduction of primary binding assays for the detection of trypanosomal antibodies came as a breakthrough in immunological diagnosis. These tests specifically measure the interaction between antigen and antibody rather than depending on a secondary reaction consequential upon the initial binding (Luckins *et al.*, 2004). In spite of the development of an indirect fluorescent antibody (IFA) test and enzyme-linked immunosorbent assays for *T. equiperdum*, the CFT remains the only recognised test for international trade purposes, and is widely used in disease eradication programs (Clausen *et al.*, 2003; Luciana *et al.*, 2013).

2.9.2 Immunofluorescence antibody test for trypanosomes

The immunofluorescence antibody test (IFAT) is a serological test which uses IgG antibodies conjugated to fluorescein isothiocyanate for both serum and dried whole blood on filter papers (Mitashi *et al.*, 2012; Mule & Okwaro, 2016). The IFAT has been used extensively in the detection of trypanosomal antibodies in animals and humans. The IFAT test has the disadvantages that it requires skilled operators, expensive equipment and the interpretation of the results is subjective although it has a significant practical value (Williamson *et al.*, 1988; Luckins *et al.*, 2004). Blood smears which are fixed in acetone are used to prepare antigens and then stored at a low temperature. The IFA test in infected cattle and camels have proven to be both specific and sensitive in detecting trypanosomal antibodies (Luckins *et al.*, 2004). The IFAT for dourine can be used as a confirmatory test to resolve unsatisfying results attained by the CF test (OIE, 2013, Calistri *et al.*, 2013; Cauchard *et al.*, 2014). According to OIE (2013), in order to perform IFAT, the antigen must be prepared and a careful attention has to be paid to which *T. equiperdum* strain is used for the antigen preparation. The antigen is then standardised by titration against a 1/5 dilution of a standard low-titre antiserum and the test is performed as described below.

2.9.2.1 Immunofluorescence antibody test for dourine (OIE, 2013)

The antigen slides are allowed to reach room temperature in a glass container. Another method is to remove slides directly from the freezer and fix them in acetone for 15 minutes. Then slides are marked out. Separate spots of test sera diluted in phosphate-

buffered saline (PBS) are applied, and the slides are incubated in a humid chamber at ambient temperature for 30 minutes. The slides are washed in PBS, pH 7.2, three times for 5 minutes each, and air-dried. Fluorescence-labelled conjugate is added at the correct dilution. Individual batches of antigen and conjugate should be titrated against each other using control sera to optimise the conjugate dilution. The slides are then incubated in a humid chamber at ambient temperature for 30 minutes. The slides are again washed in PBS, three times for 5 minutes each, and air-dried. An alternative method, to reduce background fluorescence, is to counter-stain, using Evans Blue (0.01% in distilled water) for 1 minute, rinse in PBS and then air dry. The slides are mounted in glycerol/PBS (50/50), immersion oil (commercially available, non-fluorescing grade), or mounting reagent for fluorescent staining (commercially available). The slides are then examined under UV illumination. Incident light illumination is used with a suitable filter set. Slides may be stored at 4°C for 4–5 days. Sera diluted at 1/80 and above showing strong fluorescence of the parasites are usually considered to be positive. Estimating the intensity of fluorescence demands experience on the part of the observer. Standard positive and negative control sera should be included in each batch of tests, and due consideration should be given to the pattern of fluorescence in these controls when assessing the results of test sera.

Immunofluorescence antibody test has been successfully used in diagnosing dourine in Italy (Calistri *et al.*, 2013). It was therefore concluded that this test can be used for the purpose of assessing prevalence of infection as well as declaring a population free from the infection (Ahmed *et al.*, 2018). Furthermore, uninfected equids, particularly donkeys and mules, because of the anti-complementary effects of their sera, often give inconsistent or nonspecific reactions (Clausen *et al.*, 2003; OIE, 2013). In such cases, the indirect fluorescent antibody (IFA) test has several benefits (OIE, 2013). However, this test is costly and only amenable to laboratory settings (Mule & Okwaro, 2016). Complement fixation test is considered to be less sensitive than ELISA and IFAT for the detection of the serum antibodies against *T. equiperdum*, even though the CFT has been used for many years in the diagnosis of dourine (Gizaw *et al.*, 2017).

No serological test is specific for dourine. Cross-reactions can occur with old world trypanosomes, especially *T. brucei* and *T. evansi* (OIE, 2009). Although, serological tests can be a method of choice for mass screening of populations, their main restriction will remain a failure to determine the parasite (Luckins *et al.*, 2004). The utilization of ELISA

for routine analysis of dourine would give a substantial advantage over current serological tests if a characterized antigen was utilized, since it will allow test standardization and more promptly permit correlation of tests among research centres (Gizaw *et al.*, 2017). The ELISA is considered to be the method of choice as the antigen is used in minute quantities and appears to be more stable than the CF test antigen (Luckins *et al.*, 2004). Description of the group-specific antigen would permit identification of the peptide sequence in the epitope which could be manufactured and used in a standardized ELISA for serological testing for dourine (Bishop *et al.*, 1995). The detection of antibodies by ELISA assays against trypanosomes is often based on crude antigens which have shown variability and cross reactions between species of trypanosomes (Madruga *et al.*, 2006). Most of the enzyme-immunoassays are comparable to fluorescence or radioimmunoassay's because they involve at least one separation step in which the 'bound' enzyme labelled reagent is detached from the unbound enzyme, allowing measurement of either bound or free activity (Voller *et al.*, 1978). The application of ELISAs assays for the detection of *T. equiperdum* infections could improve the efficiency of control measures against this parasite (Alemu *et al.*, 1997).

2.9.3 Enzyme-linked immune sorbent assay

The enzyme-linked immunosorbent assay (ELISA) is an immunological test that indirectly demonstrates the presence of an infecting parasite in body fluids (Mule & Okwaro, 2016). Enzyme-linked immune-sorbent assays, are progressively being used for detection of parasitic-specific antibodies, antigens and immune complexes (Salih *et al.*, 2014). As compared to other serological tests that have been used for detection of antibodies, ELISA appears to offer a combination of the best qualities of all (Walls *et al.*, 1977). Enzyme-linked immune-sorbent assay involves antigen, antibody and enzymes for the detection of specific immune responses (Prasad *et al.*, 2016) (figure 2.2). According to Nguyen *et al.*, (2015) antibody detection using ELISA for trypanosome crude antigen is regarded as a conventional and standard method for the diagnosis of animal trypanosomiasis. The uniqueness of this method is that specific trypanosome antibodies can be identified by enzyme-linked anti-immunoglobulins using solid-phase polystyrene plates coated with soluble antigen (OIE, 2013). The ELISAs based on *Trypanozoon* group-specific antigen revealed capacity for detecting antibodies to pathogenic

trypanosomes, including *T. equiperdum*. Tests based on defined antigens could more readily enable standardization than tests based on the use of crude, sonicated antigen preparations (Luckins *et al.*, 2004).

There has been a remarkable growth in the number and variety of immunodiagnostic tests performed over the last two decades (Voller *et al.*, 1978). One of the motives for this has been the improvement and excellence of methods which uses labelled antigens or antibodies, resulting in tests with very high levels of sensitivity and specificity. Enzymes linked to antibodies or antigens results in complexes where both will have immunological as well as enzymatic activity (Voller *et al.*, 1978; Aydin *et al.*, 2015). An amplification factor produced during degradation by the enzymes of a chromogenic or fluorogenic substrate, enables accurate and sensitive detection of the presence of the enzyme (Voller *et al.*, 1978). If defined antigens were used, then the benefit of ELISAs for routine diagnostic serology for dourine might provide a substantial advantage over current serological tests, since it would permit test standardization and more readily allow comparison of tests between laboratories (Bishop *et al.*, 1995).

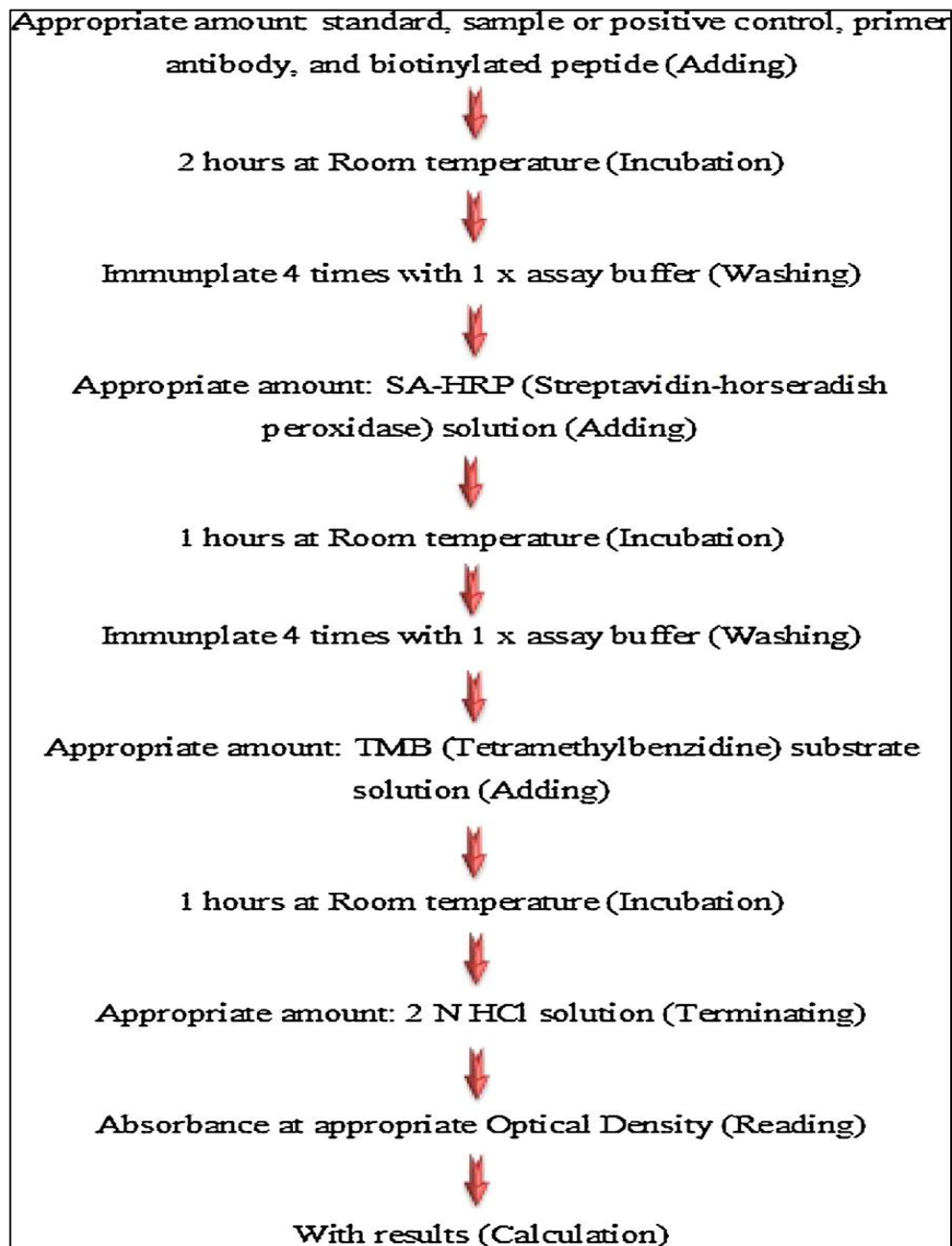


Figure 2.2: General steps for performing ELISA (For interpretation of the references to color in this text, the reader is referred to the web version of the article). Adapted and modified from resource number (Aydin, 2015).

Members of the genus *Trypanozoon* all share conserved cytoskeletal elements that result in a strong and cross-reactive serological reaction. All diagnostic antigens and antisera currently available for use in sero-diagnostic testing contain the conserved elements or antibodies to them, and, as a result, none of the serological procedures is specific for dourine (OIE, 2013). According to Chisi *et al.*, (2017), ELISA tests were developed to be more sensitive and specific alternatives to conventional serological tests (CFT). However, ELISA does not differentiate among *T. equiperdum*, *T. evansi* and *T. brucei* (Luciani *et al.*, 2013). Although serological tests working with antibody detection are more sensitive, they fail to distinguish between an active (present) infection and a cured (past) one (Clausen *et al.*, 1999; Thekiso *et al.*, 2007). Development of more *T. equiperdum*-specific subunit antigens and antibodies will be required as a significant improvement in dourine sero-diagnosis (OIE, 2013).

Serological tests are also limited by a high number of false-positive results (Becker *et al.*, 2004). These tests are also of limited value when investigating individuals (Eze & Eze, 2015). Based on circumstantial evidence, it appears that *T. equiperdum*-infected laboratory animals and horses suspected of dourine also positively react to the Card Agglutination test trypanosomiasis (CATT)/*T. evansi* and Enzyme-linked Immunosorbent Assay (ELISA)/*T. evansi* prepared with fixed whole trypanosomes of the RoTat 1.2 VAT (Gizaw *et al.*, 2017). However, these methods may lack specificity and it cannot differentiate between current infections and previous exposure to infection (Solano *et al.*, 1999; Ahmed *et al.*, 2013; Takeet *et al.*, 2013). Furthermore, the cost of producing specific purified antigens is generally very high and as a result, crude antigen preparations are frequently used, resulting in reduced specificity and sensitivity (Eze & Eze, 2015). Serological tests can be the method of choice for mass screening of populations, but their main limitation will remain the failure to demonstrate the parasite (Claes *et al.*, 2005).

2.10 Molecular techniques

Serology remains the most practical method available to screen and confirm diagnosis of dourine. As such, it is important to identify diagnostic tests that are reliable, specific, cost effective and easy to perform, which will ensure that no uninfected animals are affected (Chisi *et al.*, 2017) and also that can differentiate between various *Trypanozoon* species, i.e. *T. equiperdum*/*T. brucei* from *T. evansi* (Li *et al.*, 2007). While serological tests can

be the method of choice for mass screening of populations, their main limitation will remain the failure to demonstrate the presence of the parasite (Gizaw *et al.*, 2017). Therefore, the development of rapid, accurate, and sensitive diagnostic methods for the identification of pathogens is fundamental for treating and controlling, or even eradicating infectious diseases (Mori *et al.*, 2009). Recent developments in nucleic acid diagnostic techniques (e.g. PCR, restriction fragment length polymorphism, random amplification of polymorphic DNA, real-time PCR, LAMP, etc.) have made it possible to reliably identify trypanosomes from among the subgenus *Trypanozoon* (Zablotskij *et al.*, 2003). Utilizing nucleic acid technologies such as PCR, real-time PCR and LAMP may also offer higher sensitivity and might be of use in the diagnosis of *T. equiperdum*. Signal amplification and target amplification are there two broad categories used to describe nucleic acid amplification (Muldrew, 2009). Nucleic amplification is one of the most important tools for many investigators, including molecular biologists and it is used for monitoring infectious diseases as well as in genetic disorders and genetic traits in application fields such as clinical medicine and genetic diagnosis (Nagamine *et al.*, 2002).

Molecular diagnosis based on nucleic acids which are generally more sensitive and specific, has become the method of choice to identify certain pathogens (Liu, 2009) over the existing diagnostic tests (Ndao, 2009). Diagnosis of nucleic acids permits the detection of infections from very low parasitized samples including those from asymptomatic samples (Ndao, 2009). Accurate analyses are key in effectively treating, preventing and achieving excellent prognosis (Abdullahi *et al.*, 2015). Molecular assays have effectively assisted in the diagnosis, treatment and epidemiological studies of parasitic diseases that affect people worldwide, helping to control mortality ascribed to parasitic diseases (Eze & Eze, 2015). Since in the last century the development of molecular biology was one of the greatest achievements in biological science (Valones *et al.*, 2009). Molecular or DNA-based methods are established to address some of the problems encountered when using conventional methods such as microscopy for parasite identification (Eze & Eze, 2015). There are several techniques in molecular biology that have been developed since the advent of the PCR: Nested PCR, Multiplex PCR, Real-time PCR (qPCR) and LAMP assays are few that can be mentioned, and by applying these molecular diagnostics different questions can be answered (Muldrew, 2009; Mitashi *et al.*, 2012). The PCR and other related DNA amplification methods have been used to

examine exudates or tissue samples, taking into account their failure on blood samples after the initial phase of the disease infection (Gizaw *et al.*, 2017).

According to Li *et al.*, (2007), a wide range of molecular techniques have been applied for the diagnosis of trypanosomiasis and/or the characterization of the causative agents. These diagnostics evolved successively through DNA probing, PCR associated to DNA probing, and currently to PCR alone (Desquesnes & Davila, 2002). Some conventional DNA techniques such as, restriction fragment length polymorphism (RFLP) analysis, genome fingerprinting, analysis of repetitive DNA and kinetoplasts DNA (kDNA) have been employed. Polymerase chain reaction based approaches also have been utilized for the analysis of genetic variation within and among species of *Trypanosoma*. In spite of the application of these techniques (amplified length fragment polymorphism (ALFP), multiplex-endonuclease genotyping (MEGA), mobile genetic elements (MGE-PCR), simple sequence repeats (SSR-PCR), random amplification of polymorphic DNA (RAPD), fluorescence in situ hybridization (FISH) with peptide nucleic acid (PNA) and loop-mediated isothermal amplification (LAMP), it has not yet been possible to clearly differentiate genetically between *T. brucei*, *T. evansi* and *T. equiperdum* (Li *et al.*, 2007). Diagnostic accuracy of molecular assays can differ depending on the sample used and method of DNA recapture as well as the molecular target and protocol employed (Adams *et al.*, 2014).

2.10.1 Conventional polymerase chain reaction (conPCR)

According to Fraga *et al.*, (2008), quantitative endpoint or conventional PCR analysis depends on quantifying the end point of a PCR reaction by ethidium bromide visualization of the DNA product size separated by gel electrophoresis. Amplified fragments from samples are examined by electrophoresis for length differences, restriction site variation using restriction enzymes, sequencing or HRM curve analysis (Akhoundi *et al.*, 2017). End-point PCR discloses nothing about the initial amounts of target molecules that were present in the samples and they only distinguish a positive from a negative sample (Kubista *et al.*, 2006). Conventional PCR tests are however technically inconvenient in detection and liable to carry over contamination of PCR products (Becker *et al.*, 2004; Piron *et al.*, 2007). Since PCR does not measure the initial target sequence quantity, it was important to design proper controls for the quantitation of the initial target sequences

(Heid *et al.*, 1996). Many detection methods and equipment have been developed and amongst those commonly used is real-time PCR (Sue *et al.*, 2014), which merges the amplification of DNA and detection steps into one homogeneous assay and hinders the need for gel electrophoresis to detect amplification products (Bustin, 2005).

2.10.2 Real-time polymerase chain reaction (qPCR)

Fluorescent real-time monitoring of the amplicon accumulation was developed which was known to overcome the limitations, because post-PCR end point analysis is not necessary (Becker *et al.*, 2004). This method carries out the reaction in the presence of a fluorescent intercalated and observing the fluorescence of the reaction solution (Tomita *et al.*, 2008). These real-time assays have many advantages over conventional PCR methods, including rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity and easy standardisation (Parida *et al.*, 2008). This method (Real time PCR), has become widely used in many research applications such as, the quantitative analyses of mRNA expression and single nucleotide polymorphisms (Mori *et al.*, 2004). The monitoring of parasitemia levels in some protozoan infections, is fundamental for the determination of disease stages and risk of transmission (Konnai *et al.*, 2009). The development of new technologies such as the TaqMan probe and the Molecular Beacon methods, are known to overcome the shortcoming of low specificity in PCR and because of that, real-time PCR is considered as a vital technique in quantitative genetic tests for infectious diseases (Mori *et al.*, 2004). The fluorescent chemistry coupled with advanced optical detectors makes it more sensitive than conventional gel-based PCR (Parida *et al.*, 2008).

2.10.3 Loop-mediated isothermal amplification (LAMP)

The LAMP method is a highly efficient amplification method that allows the synthesis of large amounts of DNA in a short time (Parida *et al.*, 2004; Mori *et al.*, 2004). Since this innovation is practical and broadly accessible it is increasingly being utilized for rapid detection of disease infections (Cai *et al.*, 2013). The most important advantage is, under isothermal conditions in only 15-60 min, it has the ability to amplify specific targeted DNA sequences (Nagamine *et al.*, 2008; Cai *et al.*, 2013). As a screening tool, it has been used

in numerous nucleic acid researches and in clinical application and promises to fill those gaps in molecular diagnostics (Abdullahi *et al.*, 2015). Loop-mediated isothermal amplification assays have been widely developed for detection of various pathogens of medical and veterinary importance (Thekiso *et al.*, 2009; Njiru, 2012), including viral, bacterial, fungal and protozoan diseases as well as application in embryo sex identification (Thekiso *et al.*, 2009; Nakao *et al.*, 2010). According to Thekiso *et al.*, (2009), LAMP has the potential to be used under field conditions for diagnosis of trypanosome infections without being affected by temperature of the surrounding environment in tropical and subtropical countries where trypanosomiasis is prevalent.

REFERENCES

- Abdullahi, U.F., Naim, R., Taib, W.R.W., Saleh, A., Muazu, A., Aliyu, S. & Baig, A.A. (2015). Loop-Mediated Isothermal Amplification (LAMP), an Innovation in Gene Amplification: Bridging the Gap in Molecular Diagnostics; A Review. *Indian Journal of Science and Technology*, 8:1–14.
- Adams, E.R., Gomez, M.A., Scheske, L., Rios, R., Marquez, R., Cossio, A., Albertini, A., Schallig, H. & Saravia, N.G. (2014). Sensitive diagnosis of cutaneous leishmaniasis by lesion swab sampling coupled to qPCR. *Parasitology*, 141:1891–1897.
- Ahmed, H.A., Picozzi, K., Welburn, S.C. & MacLeod, E.T. (2013). A comparative evaluation of PCR- based methods for species- specific determination of African animal trypanosomes in Ugandan cattle. *Parasites and Vectors*, 6:1–7.
- Ahmed, Y., Hagos, A., Merga, B., Van Soom, A., Duchateau, L., Goddeeris, B.M. & Govaere, J. (2018). *Trypanosoma equiperdum* in the horse – a neglected threat? *Vlaams Diergeneeskundig Tijdschrift*, 87:66–75.
- Akhoundi, M., Downing, T., Votýpka, J., Kuhls, K., Lukeš, J., Cannet, A., Ravel, C., Marty, P., Delaunay, P., Kasbari, M. & Granouillac, B. (2017). Leishmania infections: molecular targets and diagnosis. *Molecular Aspects of Medicine*, 57:1–29.
- Alemu, T., Luckins, A.G., Phipps, L.P., Reid, S.W.J. & Holmes, P.H. (1997). The use of enzyme linked immunosorbent assays to investigate the prevalence of *Trypanosoma equiperdum* in Ethiopian horses. *Veterinary Parasitology*, 71:239–250.
- Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*, 72:4–15.
- Bannai, H., Nemoto, M., Tsujimura, K., Yamanaka, T., Kondo, T., & Matsumura, T. (2013). Improving a complement-fixation test for equine herpesvirus Type-1 by pretreating sera with potassium periodate to reduce non-specific hemolysis. *Journal of Equine Science*, 24:71–74.
- Barrowman, P.R. & van Vuuren, M. (1976). The prevalence of dourine in southern Africa. *Journal of South African Veterinary Association*, 47:83–85

- Becker, S., Franco, J.R., Simarro, P.P., Stich, A., Abele, P.M. & Steverding, D. (2004). Real-time PCR for detection of *Trypanosoma brucei* in human blood samples. *Diagnostic Microbiology and Infectious Disease*, 50:193–199.
- Bishop, S., Rae, P.F., Phipps, L.P., Boid, R. & Luckins, A.G. (1995). *Trypanosoma equiperdum*: detection of trypanosomal antibodies and antigen by enzyme-linked immunosorbent assay. *The British Veterinary Journal*, 151:715–720.
- Bonfini, B., Tittarelli, M., Luciani, M., Di Pancrazio, C., Rodomonti, D., Iannetti, L., Vulpiani, M.P. & Di Febo, T. (2018). Development of an indirect ELISA for the serological diagnosis of dourine. *Veterinary Parasitology*, 261:86–90.
- Brun, R., Hecker, H. & Lun, Z-R. (1998). *Trypanosoma evansi* and *T. equiperdum*, distribution, biology, treatment and phylogenetic relationship. *Veterinary Parasitology*, 95–107.
- Bustin, S.A. (2005). Real-time, fluorescence-based quantitative PCR: a snapshot of current procedures and preferences. *Expert Review of Molecular Diagnostics*, 5:493–498.
- Buxton, T.B., Crockett, J.K. & Rissing, J. P. (1979). Enzyme-linked immunosorbent assay: Available for infectious disease surveillance. *Laboratory Medicine*, 10:630–634.
- Cai, X., Lei, Z., Fu, W., Zhu, Z., Zou, M., Gao, J., Wang, Y., Hong, M., Wang, J., Li, W. & Xu, D. (2013). Optimisation of reverse transcription loop-mediated isothermal amplification assay for the rapid detection of pandemic (H1N1) 2009 virus. *African Journal of Microbiology Research*, 7:3919–3925.
- Calistri, P., Narcisi, V., Atzeni, M., DE Massis, F., Tittarelli, M., Mercante, M.T., Ruggieri, E. & Scacchia, M. (2013). Dourine reemergence in Italy. *Journal of Equine Veterinary Science*, 33:83–89.
- Cauchard, J., Soldan, A., Madeline, A., Johnson, P., Büscher, P. & Petry, S. (2014). Inter-laboratory ring trials to evaluate serological methods for dourine diagnosis. *Veterinary Parasitology*, 205:70–76.
- Cencek, T., Karamon, J., Ziomko, I. & Sroka, J. (2008). Application of ELISA microplate reader in complement fixation test used for dourine diagnosis. A preliminary study. *Bulletin of the Veterinary Institute Pulawy*, 52:363–368.

- Chin S.W., Uppal P.K., Premaalatha B., Chandrawathani P., Norazura A.H. & Ramlan M. (2013). Freedom status of dourine (*Trypanosoma equiperdum*) in Malaysia. *Malaysian Journal of Veterinary Research*, 4:61–64.
- Chisi, S. L., Marageni, Y., Naidoo, P., Zulu, G., Akol, G.W. & Van Heerden, H. (2017). An evaluation of serological tests in the diagnosis of bovine brucellosis in naturally infected cattle in KwaZulu-Natal province in South Africa. *Journal of the South African Veterinary Association*, 88:1–7.
- Claes F., Ilgekabayeva G.D., Verloo D., Saidouldin T.S., Geerts S., Buscher, P. & Goddeeris, B.M. (2005). Comparison of serological tests for equine trypanosomiasis in naturally infected horses from Kazakhstan. *Veterinary Parasitology*, 131:221–225.
- Claes, F. & Büscher, P. (2007). Molecular markers for the different (sub)-species of the *Trypanozoon* subgenus. *Developing methodologies for the use of polymerase chain reaction in the diagnosis and monitoring of trypanosomosis*, 15–26.
- Claes, F., Büscher, P., Touratier, L. & Goddeeris B.M. (2005). *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends in Parasitology*, 21:316–321.
- Clausen, P-H., Chuluun, S., Sodnomdarjaa, R., Greiner, M., Noeckler, K., Staak, C., Zessin, K-H. & Schein, E. (2003). A field study to estimate the prevalence of *Trypanosoma equiperdum* in Mongolian horses. *Veterinary Parasitology*, 9–18.
- Clausen, P-H., Gerbeselassie, G., Abditcho, S., Mehlitz., D. & Staak, C. (1999). Detection of trypanosome DNA in serologically positive but aparasitaemic horses suspected of dourine in Ethiopia. *Tokai Journal of Experimental and Clinical Medicine*, 23:303–308.
- Dard report. (2018). Dourine surveillance in the KwaZulu-Natal rural horse population. <https://www.kzndard.gov.za>, Accessed date: 20 August 2018.
- Desquesnes, M. & Davila, A.M.R. (2002). Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. *Veterinary Parasitology*, 109:213–231.
- Epidemiology report. (2012). Dourine. *Western Cape Government Agriculture, Veterinary Services South Africa* 4:1–5.

- Eze, U.A. & Eze, N.M. (2015). Emerging molecular methods for the diagnosis and epidemiological study of parasitic infections. *American Association for Science and Technology Journal of Health*, 2:32–43.
- Ferraro, G.L. (2016). Global health and the sport horse. Edited by Meierhenry, B. *Published by UC Davis*, 1–24.
- Fraga, D., Meulia, T. & Fenster, S. (2008). Real-time PCR. *Current Protocols Essential Laboratory Techniques*, 1–3.
- Gizaw, Y., Megersa, M. & Fayera, T. (2017). Dourine: a neglected disease of equids. *Tropical Animal and Health Production*, 49:887–897.
- Hagos, A., Abebe, G., Philip, B., Bruno, M.G. & Claes, F. (2010). Serological and parasitological survey of dourine in the Arsi–Bale highlands of Ethiopia. *Tropical Animal Health and Production* 42:769–776.
- Hagos, A., Degefa, G., Yacob, H.B., Fikru, R., Alemu, T., Feseha, G., Claes, F. & Goddeeris, B.M. (2010b). Seroepidemiological survey of *Trypanozoon* infection in horses in the suspected dourine–infected Bale highlands of the Oromia region, Ethiopia. *Revue Scientifique Et Technique De L`Office International Des Epizooties*, 29:649–654.
- Hagos, A., Goddeeris, B.M., Yilkal, K., Alemu, T., Fikru, R., Yacoba, H.T., Feseha, G. & Claes, F. (2010). Efficacy of Cymelarsan® and Diminasan® against *Trypanosoma equiperdum* infections in mice and horses. *Veterinary Parasitology*, 171:200–206.
- Heid, C.A., Stevens, J., Livak, K.J. & Williams, P.M. (1996). Real time quantitative PCR. *Genome Research*, 6:986–994.
- Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A. & Zoric, N. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine*, 27:95–125.
- Li, F. J., Lai, D.H., Lukeš, J., Chen, X.G. & Lun, Z.R. (2006). Doubts about *Trypanosoma equiperdum* strains classed as *Trypanosoma brucei* or *Trypanosoma evansi*. *Trends in Parasitology*, 22:55–56.
- Li, F-J., Gasser, R.B., Lai, D-H., Claes, F., Zhu, X-Q. & Lun, Z-R. (2007). PCR approach for the detection of *Trypanosoma brucei* and *T. equiperdum* and their differentiation from *T. evansi* based on maxucircle kinetoplast DNA. *Molecular and Cellular Probes*, 21:1–7.

- Liu, Y-T. (2009). A technological update of molecular diagnostics for infectious diseases. *Infectious Disorder Drug Targets*, 8:83–188.
- Lu, K.G. & Morresey, P.R. (2007). Infectious diseases in breeding stallions. *Clinical Techniques in Equine Practice*, 6:285-290.
- Luciani M., Di Pancrazio C., Di Febo Y., Tittarelli M., Vulpiana M.P., Puglielli M.O., Naessens, J. & Sacchini, F. (2013). IgG antibodies from dourine infected horses identify a distinctive *Trypanosoma equiperdum* antigenic pattern of low molecular weight molecules. *Veterinary Immunology and Immunopathology*, 151:140–146.
- Luckins, A.G., Barrowman, P.R., Stoltz, W.H. & Van der Lugt, J.J. (2004). Dourine. In: Infectious diseases of livestock, (eds) Coetzer J.A.W & Tustin R.C. Oxford University Press 3:297–302.
- Madrugá, C.R., Araújo, F.R., Cavalcante-Goes, G., Martins, C., Pfeifer, I.B., Ribeiro, L.R., Kessler, R.H., Soares, C.O., Miguita, M., Melo, E.P.S., Almeida, R.F.C. & Almeida, M.M.S.C.L. (2006). The development of an enzyme-linked immunosorbent assay for *Trypanosoma vivax* antibodies and its use in epidemiological surveys. *Memórias do Instituto Oswaldo Cruz*, 101:801–807.
- Mehlhorn, H. (2016). *Trypanosoma equiperdum*. In *Encyclopedia of Parasitology*, 2937–2939 Springer, Berlin, Heidelberg, Germany.
- Mitashi, P., Hasker, E., Lejon, V., Kande, V., Muyembe, J.J., Lutumba, P. & Boelaert, M. (2012). Human African trypanosomiasis diagnosis in first-line health services of endemic countries, a systematic review. *PLoS Neglected Tropical Diseases*, 6: e1919.
- Monzon, C.M., Mancebo, O.A. & Russo, A.M. (2003). Antibody levels by indirect ELISA test in *Trypanosoma evansi* infected horses following treatment with quinapyramine sulphate. *Veterinary Parasitology*, 111:59–63.
- Mori, Y., Kitao, M., Tomita, N. & Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods*, 59:145–157.
- Muldrew, K.L. (2009). Molecular diagnostics of infectious diseases. *Current Opinion in Pediatrics*, 21:102–111.

- Mule, S.N. & Okwaro, L.A. (2016). Antibody-antigen detection tests for sleeping sickness diagnosis: are there opportunities for advancement? *International Journal of Life Sciences Research*, 4:114–123.
- Nagamine, K., Hase, T. & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, 16:223–229.
- Nakao, R., Stromdahl, E.Y., Magona, J.W., Faburay, B., Namangala, B., Malele, I., Inoue, N., Geysen, D., Kajino, K., Jongejan, F. & Sugimoto, C. (2010). Development of loop-mediated isothermal amplification (LAMP) assays for rapid detection of *Ehrlichia ruminantium*. *BMC Microbiology*, 10:296.
- Ndao, M. (2009). Diagnosis of parasitic diseases: old and new approaches. *Interdisciplinary Perspectives on Infectious Diseases*, 1–15.
- Nguyen, T.T., Motsiri, M.S., Taioe, M.O., Mtshali, M.S., Goto, Y., Kawazu, S-I., Thekiso, O.M.M. & Inoue, N. (2015). Application of crude and recombinant ELISAs and immunochromatographic test for serodiagnosis of 114 animal trypanosomosis in the Umkhanyakude district of KwaZulu–Natal province, South Africa. *Journal of Veterinary Medical Science*, 77:217–220.
- Njiru, Z.K. (2012). Loop mediated isothermal amplification technology: Towards point of care diagnostic. *PLoS Neglected Tropical Diseases*, 6(6): e1572.
- OIE, (2009). Dourine. *The Center for Food Security and Public Health, Iowa State University, College of Veterinary Medicine*, 1–4.
- OIE, (2013). Chapter 2.5.3. Dourine. *Terrestrial Manual*, 1–10.
- OIE, (2015). Dourine. *The Center for Food Security and Public Health, Iowa State University, College of Veterinary Medicine*, 1–4.
- Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L. & Morita, K. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology*, 18:407–421.
- Pascucci, I., Di provvido, A., Cammà, C., Di Francesco, G., Calistri, P., Tittarelli, M., Ferri, N., Scacchia, M. & Caporale, N. (2013). Diagnosis of dourine in outbreaks in Italy. *Veterinary Parasitology*, 193:30–38.

- Piron, M., Fisa, R., Casamitjana, N., López-Chejade, P., Puig, L., Vergés, M., Gascón, J., i Prat, J.G., Portús, M. & Sauleda, S. (2007). Development of a real-time PCR assay for *Trypanosoma cruzi* detection in blood samples. *Acta Tropica*, 103:195–200.
- Potts, A., Human, M., Theron, R., Dreyer, M., Olivier, H., Naidoo, P. & Vermeulen, k. (2013). SAVLSF harmonised serology SOP for dourine complement fixation test (CFT). Reviewed by Kangumba, J. 1-27 <http://www.daff.gov.za/vetweb-Epidemiology>
- Prasad, M., Brar, B., Ikbal, Ranjan, K., Lalme, U., Manimegalai, J., Vashisht, B., Khurana, S.K. & Prasad, G. (2016). Biotechnological tools for diagnosis of equine infectious diseases. *Journal of Experimental Biology and Agricultural Sciences*, 4:161–181.
- Ricketts, S. & McGladdery, A. (2011). Dourine – an emerging venereal threat to European horses. *Equine Quarterly Disease Surveillance report*, 6,7:15–18.
- Ryena-Bello, A., García, F.A., Rivera, M., Sansó, B. & Aso, P.M. (1998). Enzyme-linked immunosorbent assay (ELISA) for detection of anti-*Trypanosoma evansi* equine antibodies. *Veterinary Parasitology*, 80:149–157.
- Salih, D.A., El Hussein, A.M. & Singla, L.D. (2015). Diagnostic approaches for tick-borne haemoparasitic diseases in livestock. *Journal of Veterinary Medicine and Animal Health*, 7:45–56.
- Samper, J.C. & Tibary, A. (2006). Disease transmission in horses. *Theriogenology*, 66:551–559.
- Sanchez, E., Perrone, T., Recchimuzzi, G., Cardozo, I., Biteau, N., Aso, P.M., Mijares, A., Baltz, T., Berthier, D., Balzano-Nogueira, L. & Gonzatti, M.I. (2015). Molecular characterization and classification of *Trypanosoma* spp. Venezuelan isolates based on microsatellite markers and kinetoplast maxicircle genes. *Parasites and Vectors*, 8:1–11.
- Shapiro, T.A. (1993). Kinetoplast DNA maxicircles: networks within networks (trypanosomes/topology). *Proceedings of the National Academy of Sciences. USA*, 90: 7809–7813.
- Solano, P., Michel, J.F., Lefrançois, T. de La Rocque, S., Sidibé, I., Zoungrana, A. & Cuisance, D. (1999). Polymerase chain reaction as a diagnosis tool for detecting

trypanosomes in naturally infected cattle in Burkina Faso. *Veterinary Parasitology*, 86:95–103.

Sue, M.J., Yeap, S.K., Omar, A.R. & Tan, S.W. (2014). Application of PCR-ELISA in molecular diagnosis. *BioMedical Research International*, 2014.

Suganuma, K., Narantsatsral, S., Battur, B., Yamasaki, S., Otgonsuren, D., Musinguzi, S.P., Davaasuren, B., Battsetseg, B. & Inoue, N. (2016). Isolation, cultivation and molecular characterization of a new *Trypanosoma equiperdum* strain in Mongolia. *Parasites and Vectors*, 9:1–9.

Takeet, M.I., Fagbemi, B.O., De Donato, M., Yakubu, A, Rodulfo, H.E., Peters, S.O., Wheto, M. & Imumorin, I.G (2013). Molecular survey of pathogenic trypanosomes in naturally infected Nigerian cattle. *Research in Veterinary Science*, 94:555–561.

Taylor, MA, Coop, R.L. & Wall, R.L. (2007). Parasites of the reproductive/urogenital system. In: *Veterinary Parasitology* 3rd edition. *Blackwell Publishing Ltd, State Avenue, Ames, Iowa, USA*, 738–740.

Thekiso, O. M., Bazie, R. S., Coronel-Servian, A. M., Sugimoto, C., Kawazu, S. I., & Inoue, N. (2009). Stability of loop-mediated isothermal amplification (LAMP) reagents and its amplification efficiency on crude trypanosome DNA templates. *Journal of Veterinary Medical Science*, 71:471–475.

Thekiso, O.M., Kuboki, N., Nambota, A., Fujisaki, K., Sugimoto, C., Igarashi, I. & Inoue, N. (2007). Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomiasis. *Acta Tropica*, 102:182–189.

Tomita, N., Mori, Y., Kanda, H. & Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature Protocols*, 3:877.

Umezawa, E.S., Nascimento, M.S. & Stolf, A.M.S. (2001). Enzyme-linked immunosorbent assay with *Trypanosoma cruzi* excreted-secreted antigens (TESA-ELISA) for serodiagnosis of acute and chronic Chagas' disease. *Diagnostic Microbiology and Infectious Disease*, 39:169–176.

Unknown. Complement fixation test. Microbiology module. <https://nios.ac.in/media/documents/dmlt/Microbiology/Lesson-61.pdf> , Accessed date: 10 July 2017.

Valones, M.A.A., Guimarães, R.L., Brandão, L.A.C., Souza, P.R.E.D., Carvalho, A.D.A.T. & Crovela, S. (2009). Principles and applications of polymerase chain reaction in medical diagnostic fields: a review. *Brazilian Journal of Microbiology*, 40:1–11.

Verloo, D., Holland, W., My, L.N., Thanh, N.G., Tam, P.T., Goddeeris, B., Vercruyse, J. & Büscher, P. (2000). Comparison of serological tests for *Trypanosoma evansi* natural infections in water buffaloes from North Vietnam. *Veterinary Parasitology*, 92:87–96.

Voller, A., Bartlett, A. & Bidwell, D.E. (1978). Enzyme immunoassays with special reference to ELISA techniques. *Journal of Clinical Pathology*, 31:507–520.

Walls, K.W., Bullock, S.L. & English, D.K. (1977). Use of the enzyme-linked immunosorbent assay (ELISA) and its microadaptation for the serodiagnosis of toxoplasmosis. *Journal of Clinical Microbiology*, 5:273–277.

Williamson, C.C., Stoltsz, W.H., Mattheus, A. & Schiele, G.J. (1988). An investigation into alternative methods for the serodiagnosis of dourine. *Onderstepoort Journal of Veterinary Research*, 55:117–119.

Zablotskij, V.T., Georgiu C., De Waal, T., Clausen, P.H., Claes, F. & Touratier, L (2003). The current challenges of dourine: difficulties in differentiating *Trypanosoma equiperdum* within the subgenus *Trypanozoon*. *Scientific and Technical Review of the Office International des Epizooties (Paris)*, 22:1087–1096.

CHAPTER 3 CONVENTIONAL PCR FOR DETECTION OF *TRYPANOSOMA EQUIPERDUM* INFECTIONS IN SOUTH AFRICAN EQUIDS

3.1 Introduction

3.1.1 Polymerase chain reaction

In 1987, Kary Mullis (Nobel Prize Winner in Chemistry in 1993) and acquaintances developed a Polymerase Chain Reaction (PCR) technique (Dorak, 2006; Lo & Chan, 2006; Rao, 2006) which turned out to be the most widely used technology in the molecular diagnostics of transmissible diseases (Muldrew, 2009; Joshi & Deshpande, 2011). Polymerase chain reaction may be regarded as a simplified version of the DNA replication process that occurs during cell division (Lo & Chan, 2006) and it has been considered an essential tool in molecular biology, which allows amplification of nucleic acid sequences (DNA and RNA) through repetitive cycles (Rodríguez & Ramirez, 2012). Polymerase chain reaction is regarded a powerful and superior technique for the detection of parasite antigen due to its sensitivity to diagnose the dormant and chronic phase of infections (Tehseen *et al.*, 2015). The high sensitivity of this technique has the ability to allow for early detection of diseases (Joshi & Deshpande, 2011).

Amplification is performed by an automated temperature cycling in the PCR (Ririe *et al.*, 1997; Kubista *et al.*, 2006), however, manual steps are required for product analysis (Ririe *et al.*, 1997). The only requirement is that the nucleotide sequence of short regions of DNA flanking the region of interest is known (Peake 1989; Kubista *et al.*, 2006). The PCR technique involves three major steps namely, denaturation, annealing, and extension (Joshi & Deshpande, 2011) in order to achieve synthesis (Hill & Stewart, 1992) from minute quantities of DNA material. Even when that source DNA is of relatively poor quality this technique can amplify the desired specific DNA fragments (Fraga *et al.*, 2008). The technique utilizes high temperatures to amplify DNA and it is aided by two primers that span the sequence selected for amplification which results in an exponential increase in DNA from repeated cycles of synthesis (Arakawa *et al.*, 1990). The PCR relies on the ability of DNA copying enzymes to remain stable during these high temperatures. The basic principles of PCR are straightforward and as the name implies, it is a chain reaction:

One DNA molecule is used to produce two copies, then four, then eight and so forth at an exponential growth rate (Joshi & Deshpande, 2011). According to Ahmed *et al.*, (2013), PCR has been widely applied for the identification of trypanosomes in laboratories and it appears to be highly sensitive and specific. It was further demonstrated that this technique is the most reliable and precise method available for the specific identification of natural animal infections for most trypanosome species and sub-species (Desquesnes & Davila, 2002).

3.1.2 Polymerase chain reaction mechanism

In practice PCR is performed on a single or double-stranded template DNA (Kubista *et al.*, 2006). By means of heating, the double-stranded DNA is separated into two single strands of DNA at 96°C (Joshi & Deshpande, 2011). Two particular oligonucleotide primers synthesised with the goal that they flank the region of interest when added to the DNA (Kubista *et al.*, 2006), whereby primer 1 being a duplicate of the coding strand, and primer 2 a duplicate of the non-coding strand. Once the primers have been added, binding to the DNA (annealing) is accomplished by melting the double stranded DNA at 94°C and cooling the mixture to 40-55°C. At this point the addition of the polymerase enzyme will lead to synthesis of complementary DNA strands along with both the original coding and non-coding strands from the primer binding positions. Then the area of DNA between the two primers will become amplified because newly synthesised strands become templates for the excess primers. The examination of the DNA produced is analysed by gel electrophoresis stained with ethidium bromide, where a prevalent single band, equivalent in length to the distance between the primers, is observed under ultraviolet light (Peake, 1989).

The PCR process is generally carried out using the *Taq* polymerase. A *Taq* polymerase, is an enzyme that creates or constructs two new strands of DNA, using the original strands as templates. This procedure results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. At that juncture, each of these strands can be utilized to make two new duplicates, so on and so on (Joshi & Deshpande, 2011). More than 30 cycles are used and incubation times for the denaturing stage (where the DNA is denatured at 90–97°C), the annealing stage (primers anneal to the DNA template strands to prime extension at 40–60°C), and the

extension stage (extension occurs at the end of the annealed primers to create a complimentary copy strand of DNA) are in the order of two to three minutes, giving an estimated enrichment of the selected sequence of 10^5 to 10^6 . The whole process has been automated, using microprocessor controlled heating blocks in which rapid temperature changes can be achieved. Depending on the type of experiment and research question asked, the times for the various stages within the process can be variable (Peake, 1989; Joshi & Deshpande, 2011).

3.1.3 Polymerase chain reaction technique for trypanosomes

The diagnosis of trypanosomes has been improved by DNA-based detection since the 1980's, as it was initially based on microscopic observations and the host range of the parasites (Desquesnes *et al.*, 2002). The introduction and development of new laboratory techniques have increased the capacity for characterization of the parasites. Techniques such as restriction enzymes, sequencing, and synthesis of the DNA, DNA probing and PCR contribute substantially to trypanosome identification, characterization, diagnosis accuracy, and reliability, at various taxonomic level (Desquesnes *et al.*, 2002). Additionally, proper understanding of the epidemiology of the disease requires accurate diagnosis of trypanosome infections, leading to the implementation of suitable control strategies (Moti *et al.*, 2014).

The PCR technique replicates both DNA and RNA either in a test tube or *in vitro* and it has been mentioned that is a far better parasitological technique as it can detect DNA from samples with low parasitemia (Saiki *et al.*, 1988; Akhondi *et al.*, 2017). Accurate identification of each of the pathogenic species, subspecies and subgroups of the genus *Trypanosoma* remains a challenging problem in tropical Africa which is essential in understanding the epidemiology of human and animal trypanosomiasis (Gibson, 2002). Nonetheless, in recent years, PCR has been shown to be highly sensitive, specific and because of that it has commonly been applied for the detection of trypanosomes in laboratories (Ahmed *et al.*, 2013; Djohan *et al.*, 2015). Ahmed *et al.*, (2013) noted that the use of PCR for accurate detection of animals harbouring natural infections of trypanosome species and sub-species is the most reliable and precise method available for detecting trypanosome DNA. The use of species complex or species specific primers for example in multiplex or nested PCR is another approach that can be applied to amplify

DNA fragments. In addition to diagnosis, PCR has applications in quantification of the parasites and thus also monitoring disease progression (Akhoundi *et al.*, 2017).

Members of the order Kinetoplastida consists of a DNA network (kDNA) which contains tens of maxicircles and thousands of minicircles. The major difference at molecular level among the three *Trypanozoon* species namely, *T. brucei*, *T. evansi* and *T. equiperdum* is the absence of maxicircles from some of *T. equiperdum* strains whereas *T. evansi* completely lacks maxicircles (Claes & Buscher, 2007; Suganuma *et al.*, 2016; Gizaw *et al.*, 2017). Kinetoplast sequences and microsatellite markers have been used to characterize and distinguish these trypanosome species (Sánchez *et al.*, 2015). Ahmed *et al.*, (2013) detected *Trypanozoon* species using the most widely recognized target DNA satellite repeat sequence (TBR) and NAD-5 PCR to demonstrate the presence of genomic DNA in each sample representing this sub-genus. Although there is no *T. equiperdum*-specific PCR method available, subgenus *Trypanozoon*-specific PCR can be used for detection of *T. equiperdum* DNA (Gizaw *et al.*, 2017), using those identified for *T. b. brucei* as well as *T. evansi* that have specific markers (Claes & Buscher, 2007). Studies have further indicated that *T. equiperdum* is more closely related to *T. b. brucei* than to *T. evansi* and that it might even be a particular strain of *T. b. brucei*. As for *T. evansi* specific PCR based on the RoTat 1.2 VSG was also developed (Claes & Buscher, 2007; Li *et al.*, 2007).

Molecular detection of different members from the subgenus *Trypanozoon* still remains challenging despite the most recent advances in technology (AIEA, 2007). In fact, the unequivocal genetic differentiation of *T. equiperdum* and *T. evansi* is not possible on the basis of genetic characterisation studies performed so far (Scacchia *et al.*, 2011).

3.2 Objective of the study

To develop a conventional species specific PCR assay for the detection of *Trypanosoma equiperdum* infections in horse and donkeys in South Africa.

3.3 Materials and Methods

3.3.1 Horse and donkey blood samples

Blood samples were collected from equines (34 donkeys and 252 horses) from 4 provinces in South Africa (figure 3.1). Blood samples were collected from jugular vein of each horse into EDTA vacutainers at an abattoir (Middelvlei in Randfontein). The samples were placed in a cooler box and transported to the laboratory for DNA extraction and other molecular and serological applications.

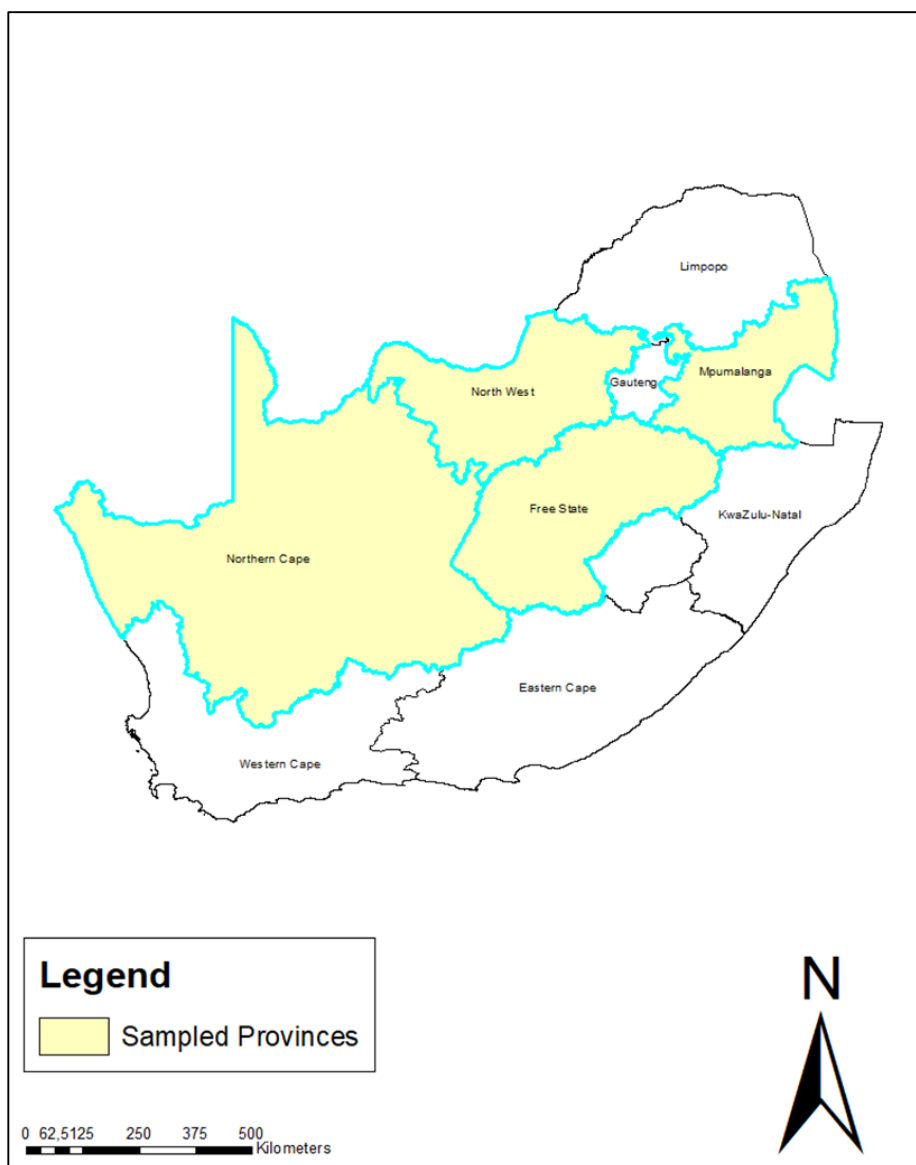


Figure 3.1: South African map indicating provinces where the equine samples were collected (Map constructed using ArgGIS)

3.4 Genomic DNA Isolation

Genomic DNA was extracted from the collected equine blood samples using Zymo DNA Kit and eluted in 50 µl elution buffer following the manufacturer's instructions. Briefly, blood samples collected at Free State, Mpumalanga, Northern Cape and North West, were extracted with a Zymo DNA blood extraction kit according to manufacturer's instructions (Zymo, USA). Beta-mercaptoethanol (250 µl) was added to the Genomic Lysis Buffer. Then 200 µl of genomic lysis buffer was added on to 50 µl of blood samples and mixed completely by vortexing for 6 seconds, then left to stand at room temperature for 10 minutes. The mixture was transferred to a Zymo-Spin IICTMColumn2 in a collection tube and centrifuged at 10 000 rpm for 1 minute. The supernatant was discarded, and 200 µl of DNA pre-wash buffer was added to the spin column and centrifuged at 10 000 rpm for 1 minute. Five hundred microliters of g-DNA Wash buffer were added to the spin column then centrifuged at 10 000 rpm for 1 minute. Spin columns were transferred to a clean micro centrifuge tubes. A 50 µl of DNA elution buffer was added onto each tube and then incubated at room temperature for 5 minutes then centrifuged at top speed (13 500 rpm) for 30 seconds to elute the DNA. The isolated DNA was stored at -20°C until the molecular analysis. DNA concentrations of the samples were measured in nano drop Jenway 73 series spectrophotometer before molecular analyses (Becker *et al.*, 2004). The DNA concentration was quantified by spectrophotometry in Nanodrop ND1000.

3.5 Conventional PCR using F3 and B3 primers

The primers were designed using Optigene LAMP primer software (Optigene Company UK website). The final volume for this PCR was adjusted to 25 µl, containing 12.5 µl of Amplitaq Gold® 360 Master Mix (Applied Biosystem, USA), 1 µl of each primer (10 µM of each primer), for amplification of 250 bp region of RIME gene (RIME 2 F3: CCT GGA CTC TCC CAA AGA and RIME B3: TCC AGT ACC CCG TAT CAT C), 2 µl of template DNA and 8.5 µl of double distilled water (DDW) to adjust the volume. *Trypanosoma equiperdum* DNA obtained from Obihiro University of Agriculture and Veterinary Medicine, Japan, was used as a positive control and DDW was used as a negative control.

PCR conditions for RIME 2 primers were as follows: initial denaturation at 95°C for 10 minutes, followed by 35 cycles at 95°C for 30 seconds, annealing at 60°C for 60 seconds, extension at 72°C for 1:30 seconds and final extension at 72°C for 7 minutes.

3.5.1 PCR specificity

The specificity of the RIME 2 primers was verified by using 2 µl of various DNA concentrations of several trypanosome species which included *Trypanosoma equiperdum* (Mongolian strain), *T. brucei brucei* (GuTat3.1) and *T. evansi* (Tansui).

3.5.2 Reaction sensitivity

To determine the detection limit of the PCR assay for *T. equiperdum*, a 10-fold dilution series from 10⁻¹ to 10⁻⁸ was prepared using *Trypanosoma equiperdum* (Mongolian strain) positive control obtained from Obihiro University of Agriculture and Veterinary Medicine, Japan, with the initial concentration of 53.2 ng/µl. The number of parasites corresponding to each DNA dilution was calculated as described by Njiru *et al.*, (2005).

3.5.3 Evaluation of the conventional PCR detection efficiency

Polymerase chain reaction using RIME 2 B3 and F3 primers were evaluated using field samples, 10 donkeys and 38 horses (N = 48) collected from Middlevelei abattoir and these samples tested positive using ELISA TeGM6-4r for a serological test in another study by (Mlangeni, 2016). Control samples were also included, and the amplified DNAs were confirmed by fragment size on gel electrophoresis and subsequently sequenced. Polymerase chain reaction was conducted as mentioned in section 3.5

3.6 Agarose gel electrophoresis

Polymerase chain reaction amplicons were visualised by a 1.0% agarose gel in 1 x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, at pH 8.0) stained with 1 µg/ml Ethidium Bromide then visualised under UV light. Five microliters of the PCR product and

1 µl of 6x Blue Loading Dye (Fermentas Life Sciences, US) were mixed, and loaded into the respective wells. A 5 µl of 100 bp molecular weight marker (O'GeneRuler, Fermentas Life Sciences, US) was used to confirm the size of the amplification products. Electrophoresis was performed for 30 minutes at 100 V using a mini-sub cell GT electrophoreses system (Bio-Rad, UK). Gel images were captured using Gene Genius Bio Imaging System (Syngene, Synoptics, UK) GeneSnap (version 6.00.22) software.

3.7 Statistical analysis

Statistical significance of sensitivity and specificity of the test were evaluated using 2x2 Chi-square and they were determined as follows (TP is true positive, TN represents true negative, FN is false negative and FP is false positive): (A) Sensitivity = $TP/(TP + FN) \times 100$; (B) Specificity = $TN/(TN + FP) \times 100$ (Chaouch *et al.*, 2013). The significance of the differences between the frequencies of positive results obtained in the tests was measured by Fisher's exact test. Analysis was performed with the aid of SPSS software and Graph Prism. Positive samples were summarized as percentages and confirmed as true positives using the basic local alignment search tool (BLASTn) on NCBI.

3.8 Results

3.8.1 Sensitivity and specificity of conPCR using F3 and B3 primers

The primers used in the study are not published, the specificity and sensitivity tests had to be conducted to determine if these primers could indeed amplify *T. equiperdum* and as such 10-fold serially diluted *T. equiperdum* DNA was used with concentrations ranging from 10ng/ μ l to 10ag. Conventional PCR had a detection limit of 100 fg which is equivalent to 1 trypanosome/ml and no reaction reactivity was recorded with non-targeted DNA's (figure 3.2) for specificity.

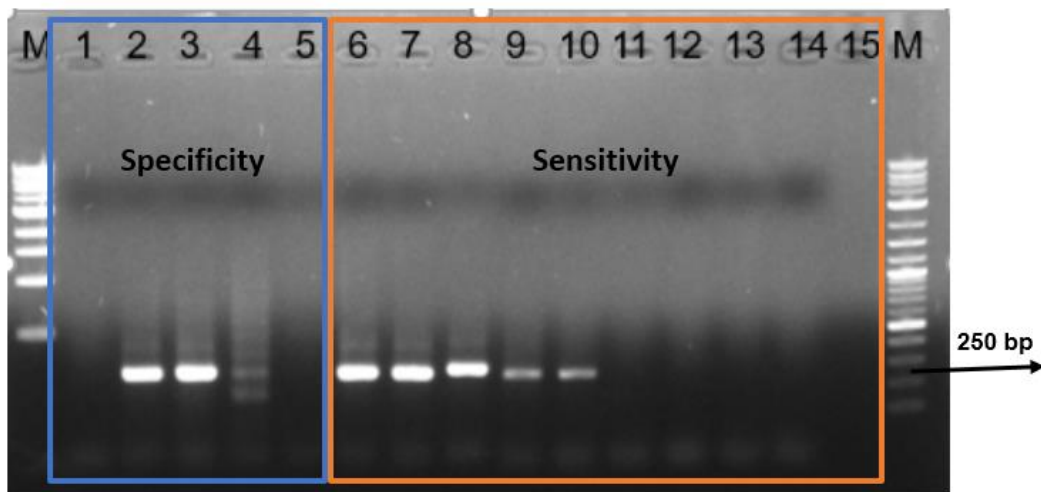


Figure 3.2: Gel image of 1% agarose gel electrophoresis showing specificity and sensitivity of *Trypanosoma equiperdum*. **M:** Ladder (1kb), 1. DDW, 2. *T. equiperdum*, 3. *T. brucei brucei*, 4. *T. evansi*, 5. H-DNA for specificity, 6. 10^{-1} , 7. 10^{-2} , 8. 10^{-3} , 9. 10^{-4} , 10. 10^{-5} , 11. 10^{-6} , 12. 10^{-7} , 13. 10^{-8} for *T. equiperdum* sensitivity, 14. DDW. PCR had a detection limit of 10^{-5} which is equivalent to 1 trypanosome/ml.

3.8.2 Conventional PCR analyses of field blood samples

A total of 48 blood samples from horses and donkeys were screened for the presence of *T. equiperdum* infections. The presence of the *T. equiperdum* parasite and the amplification revealed positive bands at 250 bp for the target gene (figure 3.3). In contrast, no amplification was observed when normal horse blood DNA was used as the template. Out of 48 samples screened, 30 (62.5%) were positive while 18 were negative. Of those 30 positive samples, 6/10 (60.0%) were donkeys while 24/38 (63.0%) were horses amplified DNA (table 3.1), [$\chi^2 = 11.73$ (df = 3) and $p < 0.05$]. Therefore, there was a significant difference observed between the groups in the sampled provinces. The DNA's amplified with *T. equiperdum* were submitted to direct sequencing using the new RIME 2 primers to confirm the positive results. The BLASTn from GenBank was used to confirm identity matches from the sequenced positive samples. As such it was revealed that 5 of 10 sequences were RIME gene sequences that matched with *Trypanosoma evansi* and *T. brucei* accession number EF567424.1, EF567426.1, and K01801.1 with 90-96% identity and an e-value of 0.0 (figure 3.4).

Table 3.1: Summary of *Trypanosoma equiperdum* overall infections in horse and donkey DNA samples tested by means of conPCR: $p < 0.0084$

Provinces	Animal Species	conPCR +ve(%+ve)	Total conPCR +ve(%+ve)
Free State (N=12)	Horses (n=10)	8(80.0)	10(83.3)
	Donkeys (n=2)	2(100.0)	
Mpumalanga (N=12)	Horses (n=12)	10(76.9)	10(83.3)
	NDSC	-	
Northern Cape (N=12)	Horses (n=8)	4(50.0)	7(58.3%)
	Donkeys (n=4)	3(75.0)	
North West (N=12)	Horses (n=8)	2(25.0)	3(25.0%)
	Donkeys (n=4)	1(25.0)	

NDSC: No donkey samples collected

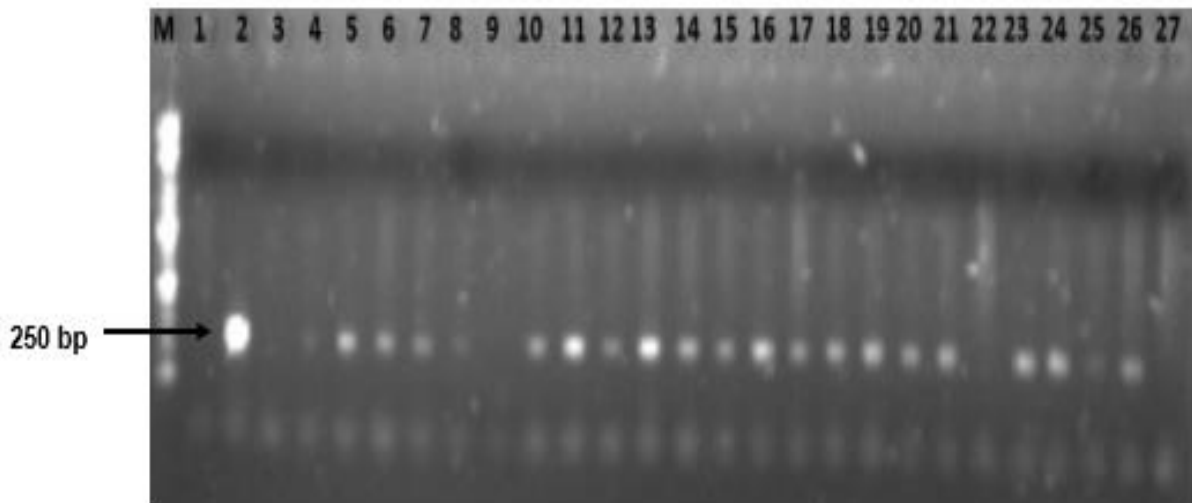


Figure 3.3: Gel image of 1% agarose gel electrophoresis of *Trypanosoma equiperdum*. **M:** Ladder (1kb), 1. DDW, 2. *T. equiperdum*, 3. P1, 4. P2, 5. P3, 6. P8, 7. H1, 8. H2, 9. H5, 10. H8, 11. H10, 12. H14, 13. H26, 14. H28, 15. MP9, 16. MP10, 17. MP12, 18. MP13, 19. MP14, 20. MP46, 21. MP47, 22. MP51, 23. MP52, 24. MP62, 25. MP77, 26. MP87 & 27. H-DNA.

Score	Expect	Identities	Gaps	Strand
331 bits(366)	7e-87	198/208(95%)	0/208(0%)	Plus/Plus
H14-FS	CACATCACCGGACAGCAGCATCTTAATGCGGATGATACTGGTGATGGCAGCTGGCACGT	★	★	64
<i>T. brucei</i> (RIME) rRNA	CACATCACCGGACAGCAGCATCTTAGTGCGGATGATACTGCTGATGGCAGCTGGCACGT			564
H14-FS	GCCCAGGGGCGGACCAATGAAAAACGGGGTCCCAGCAAGACGCTGCTGATCCCTGTGCTG	★ ★ ★		124
<i>T. brucei</i> (RIME) rRNA	GCCCAGGGACCGACAAATGAAAAACGGGTGCCAGCAAGACGCTGCTGATCCCTGTGCTG			624
H14-FS	TATGATGCCCTAACCGGAGCACAGCAGTGTTCCCCCTGAAAGGAACGCTGCTGGGCTTTG	★ ★ ★	★	184
<i>T. brucei</i> (RIME) rRNA	TATGATGCCGTAGCCGGAGCAAAGCAGTGTTCCCCATGAAAGGAACGCTGCTGGCTTTG			684
H14-FS	GCTTCCCCCGATGATACGGGGTACTGGA			212
<i>T. brucei</i> (RIME) rRNA	GCTTCCCCCGATGATACGGGGTACTGGA			712

Figure 3.4: BLASTn results showing the alignment of *T. brucei* and one of the sequences from this study which was from a horse sample from Free State Province. The subject sequence (*Trypanosoma brucei* (RIME) in rRNA gene: Accession no. K01801.1, it had a match of 95% on the query sequence (FS_H14 Equine) The black stars indicate trans-versions as well as transitions that occurred between sequences.

3.9 Discussion

Dourine, a disease caused by *Trypanosoma equiperdum* in equines, is known to be present in South Africa as one of the controlled disease under the Animal Diseases Act 35 of 1984. There were, however, reported cases of dourine in South Africa between 2005 and 2015 of which the prevalence was determined based on the CFT. No molecular techniques that have been conducted to detect dourine in South Africa, despite the fact that different approaches have been employed to detect other *Trypanozoon* parasites (Li *et al.*, 2007). Therefore, there is a need for tools that can assist in a rapid assessment of dourine in addition to parasitological or serological methods.

The sensitivity and specificity of the RIME 2 PCR test recorded in the study and its ability to detect parasite DNA in the equine samples could prove useful in confirming the presence or absence of *T. equiperdum* infections. The PCR technique is sensitive to a point whereby a parasitaemia as low as 10 parasites per millilitre of blood can be detected (Takeet *et al.*, 2013). In many infectious diseases, PCR technology has been commonly used for the diagnosis and monitoring of disease progression and therapy outcome (Schijman *et al.*, 2001). Therefore, in this study, we report on the newly designed RIME 2 F3 and B3 primers for detection of *Trypanosoma equiperdum*. The 100 fg analytical sensitivity of the current assay was lower than the sensitivity obtained in the study conducted by Njiru *et al.*, (2011), using TgsGP gene to detect Group 1 *Trypanosoma brucei gambiense*, and similar to the findings of Njiru *et al.*, (2008a) where the sensitivity of conPCR test ranged from 0.1 to 1000 trypanosomes/ml. The conventional PCR technique developed was specific, no false positives were observed.

The DNA samples amplified were confirmed positive by sequencing. The sequenced DNA samples matched with the strains of HAT trypanosomes available in Genbank with accession number K01801.1 (*T. brucei*), EF567426.1 (*T. b. brucei*) and *T. b. gambiense* (FN554964), which belong to subgenus *Trypanozoon* together with *T. equiperdum*. Sequence analysis revealed that the partial RIME gene sequences amplified from these samples tested in this study were 80-99% identical and varied from all known *T. brucei* sequences in GenBank, with the e-value of 0.0. There are currently no *T. equiperdum* sequences available in the GenBank because of the lack of information regarding the parasite. According to Njiru *et al.*, (2008a), the RIME gene is universal, specific to the subgenus *Trypanozoon* and forms the most common mobile element in the *T. brucei*

genome. Furthermore, all trypanosome species in this subgenus are genetically closely related (Hide *et al.*, 1990) and currently it is difficult to differentiate between them (Claes *et al.*, 2003). However, there are molecular techniques that can be used to distinguish the genetic differences between *Trypanozoon* parasites (Musaya *et al.*, 2017). Serum resistance associated gene is present in *Trypanosoma brucei rhodesiense* but absent in *T. b. gambiense* and is a technique used to differentiate the genetic difference between the two members of the *Trypanozoon*. Furthermore, a recognised PCR that is specific to *T. evansi* was developed based on the RoTat 1.2 VSG (Claes *et al.*, 2005). In addition, it has been shown that RoTat 1.2 VSG is not present in *T. equiperdum* recently (Ahmed *et al.*, 2018). Despite the application of other molecular techniques, it has not yet been possible to clearly distinguish genetically between *T. brucei*, *T. evansi* and *T. equiperdum* (Li *et al.*, 2007). Furthermore, it is difficult to differentiate *T. equiperdum* and *T. evansi* based on parasite morphology, especially in areas where both organisms are found (Ahmed *et al.*, 2018). Although South Africa and parts of Russia are regions where dourine is considered to be endemic and these regions lie outside the distribution area of *T. evansi* (Claes *et al.*, 2005b). Due to the absence of tsetse fly vectors in the sampled provinces in South Africa, this conventional PCR can be used as supplementary or alternative tool to confirm sero-positive animals. Acknowledging that the newly RIME 2 F3 and B3 primers designed cannot differentiate the three species of the subgenus *Trypanozoon*: *T. equiperdum*, *T. brucei* subspecies and *T. evansi*. In this study, the samples used were from the areas which are known to be free from *T. evansi* and *T. b. brucei* infections. Thus, the positive samples revealed are *T. equiperdum* infections.

As a result of comparison on PCR tests developed to detect *T. equiperdum* with previous reports, the prevalence obtained using conPCR from the current study detected more samples than the study conducted by Njiru *et al.*, (2008a), where TBR primers were employed and detected 5/18 (27.8%) while the SRA gene-specific PCR test and TgsGP PCR gave negative results. Clausen *et al.*, (2003) obtained 6.2% prevalence in Mongolia and 16.7% in Kazakhstan (Suganuma *et al.*, 2016) which is less than what is reported in the current study. This could be due to the samples screened and geographical distribution. Horse samples were more susceptible than donkeys with a higher infection rate of 63.2% and 60% respectively. This could be associated with the anti-complementary activity present in donkeys since they are generally more resistant and often remain carriers without obvious clinical signs (Brun *et al.*, 1998; Vulpiani *et al.*,

2013). The overall rate of infection of all the tested equine populations from the sampled provinces showed a significant difference. Polymerase chain reaction is generally used to improve the sensitivity of the detection regardless of being relatively expensive and technical (Desquesnes *et al.*, 2013). The use of the PCR to assess trypanosome prevalence in animals seems a valuable tool in most of epidemiological studies. In addition, PCR is still the most widely used method for the detection of *Trypanosoma* DNA in clinical specimens (Deborggraeve & Büscher, 2012). Therefore, the results of the study, suggest that the conPCR method can be a useful tool to screen equine samples in the areas where *T. equiperdum* is suspected to occur and also be used as an addition to other serological and parasitological methods.

REFERENCES

- Ahmed, H.A., Picozzi, K., Welburn, S.C. & MacLeod, E.T. (2013). A comparative evaluation of PC-based methods for species-specific determination of African animal trypanosomes in Ugandan cattle. *Parasites and Vectors*, 6:1–7.
- Ahmed, Y., Hagos, A., Merga, B., Van Soom, A., Duchateau, L., Goddeeris, B.M. & Govaere, J. (2018). *Trypanosoma equiperdum* in the horse – a neglected threat? *Vlaams Diergeneeskundig Tijdschrift*, 87:66–75.
- Akhoundi, M., Downing, T., Votýpka, J., Kuhls, K., Lukeš, J., Cannet, A., Ravel, C., Marty, P., Delaunay, P., Kasbari, M. & Granouillac, B. (2017). Leishmania infections: molecular targets and diagnosis. *Molecular Aspects of Medicine*, 57:1–29.
- Arakawa, C.K., Deering, R.E., Higman, K.H., Oshima, K.H., O'Hara, P.J. & Winton, J.R. (1990). Polymerase chain reaction (PCR) amplification of a nucleoprotein gene sequence of infectious hematopoietic necrosis virus. *Disease of Aquatic Organisms*, 8:165–170.
- Brun, R., Hecker, H. & Lun, Z-R. (1998). *Trypanosoma evansi* and *T. equiperdum*, distribution, biology, treatment and phylogenetic relationship. *Veterinary Parasitology*, 95–107.
- Chaouch, M., Mhadhbi, M., Adams, E.R., Schoone, G.J., Limam, S., Gharbi Z, Darghouth, M.A., Guizania, I. & BenAbderrazaka, S. (2013). Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Leishmania infantum* in canine leishmaniasis based on cysteine protease B genes. *Veterinary Parasitology*, 198:74–78.
- Claes F., Ilgekbayeva G.D., Verloo D., Saidouldin T.S., Geerts S., Buscher, P. & Goddeeris, B.M. (2005). Comparison of serological tests for equine trypanosomiasis in naturally infected horses from Kazakhstan. *Veterinary Parasitology*, 131:221–225.
- Claes, F. & Büscher, P. (2007). Molecular markers for the different (sub)-species of the *Trypanozoon* subgenus. *Developing Methodologies for the use of Polymerase Chain Reaction in the Diagnosis and Monitoring of Trypanosomosis*, 15–26.

Claes, F., Agbo, E.C., Radwanska, M., Te Pas, M.F.W., Baltz, T., De Waal, D.T., Goddeeris, B.M., Claassen, E. & Büscher, P. (2003). How does *Trypanosoma equiperdum* fit into the *Trypanozoon* group? A cluster analysis by RAPD and multiplex-endonuclease genotyping approach. *Parasitology*, 126:425–431.

Claes, F., Büscher, P., Touratier, L. & Goddeeris B.M. (2005). *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends in Parasitology*, 21:316–321.

Claes, F., Verloo, D., De Waal, D.T., Majiwa, P.A.O., Baltz, T., Goddeeris, B.M. & Büscher, P. (2003). The expression of RoTat 1.2 variable surface glycoprotein (VSG) in *Trypanosoma evansi* and *T. equiperdum*. *Veterinary Parasitology*, 116:209–216.

Clausen, P-H., Chuluun, S., Sodnomdarjaa, R., Greiner, M., Noeckler, K., Staak, C., Zessin, K-H. & Schein, E. (2003). A field study to estimate the prevalence of *Trypanosoma equiperdum* in Mongolian horses. *Veterinary Parasitology*, 9–18.

Deborggraeve, S. & Büscher, P. (2012). Recent progress in molecular diagnosis of sleeping sickness. *Expert Review of Molecular Diagnostics*, 12:719–730.

Desquesnes, M. & Davila, A.M.R. (2002). Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. *Veterinary Parasitology*, 109:213–231.

Desquesnes, M., Holzmüller, P., Lai, D.H., Dargantes, A., Lun, Z.R. & Jittaplapong, S. (2013). *Trypanosoma evansi* and surra: a review and perspectives on origin, history, distribution, taxonomy, morphology, hosts, and pathogenic effects. *BioMedical Research International*, 2013:1–20.

Djohan, V., Kaba, D., Rayaissé, J-B., Dayo, G-K., Coulibaly, B., Salou, E., Dofini, F., Kouadio, A.D.M.K., Menan, H. & Solano, P. (2015). Detection and identification of pathogenic trypanosome species in tsetse flies along the Comoé River in Côte d'Ivoire. *Parasite*, 22:1–7.

Dorak, M.T. (2006). Real-time PCR. In: Bios Advanced Methods Series. Taylor & Francis, Oxford, UK.

Fraga, D., Meulia, T. & Fenster, S. (2008). Real-time PCR. *Current Protocols Essential Laboratory Techniques*, 10–3.

- Gibson, W., Backhouse, T. & Griffiths, A. (2002). The human serum resistance associated gene is ubiquitous and conserved in *Trypanosoma brucei rhodesiense* throughout East Africa. *Infectious Genetics Evolution*, 25:1–8
- Gizaw, Y., Megersa, M. & Fayera, T. (2017). Dourine: a neglected disease of equids. *Tropical Animal Health Production*, 49:887–897.
- Hasan, G., Turner, M.J. & Cordingley, J.S. (1984). Complete nucleotide sequence of an unusual mobile element from *Trypanosoma brucei*. *Cell* 37:333–341.
- Hernández-Rodríguez, P. & Ramirez, A.G. (2012). Polymerase chain reaction: types, utilities and limitations. In *Polymerase Chain Reaction*. InTech. 157–172.
- Hide, G., Cattand, P., Leray, D., Barry, J.D. & Tait, A. (1990). The identification of *Trypanosoma brucei* subspecies using repetitive DNA sequences. *Molecular and Biochemical Parasitology*, 39:213–225.
- Hill, P.J. & Stewart, G.S.A.B (1992). The polymerase chain reaction in molecular and microbiology. *Biotechnology and Genetic Engineering Reviews*, 10:343–377
- Joshi, M. & Deshpande, J.D. (2011). Polymerase Chain Reaction: Methods, Principles and Application. *International Journal of Biomedical Research*, 2:81–97.
- Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A. & Zoric, N. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine*, 27:95–125.
- Li, F-J., Gasser, R.B., Lai, D-H., Claes, F., Zhu, X-Q. & Lun, Z-R. (2007). PCR approach for the detection of *Trypanosoma brucei* and *T. equiperdum* and their differentiation from *T. evansi* based on maxucircle kinetoplast DNA. *Molecular and Cellular Probes*, 21:1–7.
- Lo, Y.M.D & Chan, K.C.A. (2006). Introduction to the Polymerase Chain Reaction. In: *Clinical Applications of PCR* 2nd edition, (eds) Lo Y.M.D., Chiu R.W.K. & Chan K.C.A. Humana Press Inc. 336
- Mlangeni, M.A. (2016). Molecular epidemiology of dourine, equine piroplasmiasis and ehrlichiosis in equines in South Africa. (MSc Dissertation)

Moti, Y, Fikru, R., Büscher, P., Van Den Abbeele, J., Duchateau, L. & Delespaux, V. (2014). Detection of African animal trypanosomes: The haematocrit centrifugation technique compared to PCR with samples stored on filter paper or in DNA protecting buffer. *Veterinary Parasitology*, 203:253–258.

Muldrew, K.L. (2009). Molecular diagnostics of infectious diseases. *Current Opinion in Pediatrics*, 21:102–111.

Musaya, J., Chisi, J., Senga, E., Nambala, P., Maganga, E., Matovu, E. & Enyaru, J. (2017). Polymerase chain reaction identification of *Trypanosoma brucei rhodesiense* in wild tsetse flies from Nkhotakota Wildlife Reserve, Malawi. *Malawi Medical Journal*, 29:11–15.

Njiru, Z.K., Constantine, C.C., Guya, S., Crowther, J., Kiragu, J.M., Thompson, R.C.A. & Dávila A.M.R. (2005). The use of ITS rDNA PCR in detecting pathogenic African trypanosomes. *Parasitology Research*, 95:186–192.

Njiru, Z.K., Mikosza, A.S.J., Matovu, E., Enyaru, J.C.K., Ouma, J.O., Kibona, S.N., Thompson, R.C.A. & Ndung'u, J.M. (2008a). African trypanosomiasis: Sensitive and rapid detection of the sub-genus *Trypanozoon* by loop-mediated isothermal amplification (LAMP) of parasite DNA. *International Journal for Parasitology*, 38:589–599.

Njiru, Z.K., Traub R., Ouma, J.O., Enyaru, J.C & Matovu, E. (2011). Detection of Group 1 *Trypanosoma brucei gambiense* by Loop-Mediated Isothermal Amplification. *Journal of Clinical Microbiology*, 49:1530–1536.

Peake, I. (1989). The polymerase chain reaction. *Journal of Clinical Pathology*, 42:673–676.

Rao, P.N.S. (2006). Polymerase Chain Reaction. www.microrao.com

Ririe, K.M., Rasmussen, R.P. & Wittwer C.T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry*, 245:145–160.

Saiki, R.K., Gelfand, D.H., Stofel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988). Amplification of a short DNA stretch by repeated cycles of *in vitro* DNA polymerization. *Science*, 239:487–491.

- Sánchez, E., Perrone, T., Recchimuzzi, G., Cardozo, I., Biteau, N., Aso, P.M., Mijares, A., Baltz, T., Berthier, D., Balzano-Nogueira, L. & Gonzatti, M.I. (2015). Molecular characterization and classification of *Trypanosoma* spp. Venezuelan isolates based on microsatellite markers and kinetoplast maxicircle genes. *Parasites and Vectors*, 8:1–11.
- Scacchia, M., Cammà, C., Di Francesco, G., Di Provido, A., Giunta, R., Luciani, M., Marino, A.M.F., Pascucci, I. & Caporale, V. (2011). A clinical case of dourine in an outbreak in Italy. *Veterinary Italy*, 47:473–475.
- Schijman, A.G., Bisio, M., Orellana, L., Sued, M., Duffy, T., Jaramillo, A.M.M., Cura, C., Auter, F., Veron, V., Qvarnstrom, Y. & Deborggraeve, S. (2011). International study to evaluate PCR methods for detection of *Trypanosoma cruzi* DNA in blood samples from Chagas disease patients. *PLoS Neglected Tropical Diseases*, 5: p.e931.
- Sue, M.J., Yeap, S.K., Omar, A.R. & Tan, S.W. (2014). Application of PCR-ELISA in molecular diagnosis. *BioMedical Research International*, 2014
- Suganuma, K., Narantsatsral, S., Battur, B., Yamasaki, S., Otgonsuren, D., Musinguzi, S.P., Davaasuren, B., Battsetseg, B. & Inoue, N. (2016). Isolation, cultivation and molecular characterization of a new *Trypanosoma equiperdum* strain in Mongolia. *Parasites and Vectors*, 9:1–9.
- Takeet, M.I., Fagbemi, B.O., De Donato, M., Yakubu, A., Rodulfo, H.E., Peters, S.O., Wheto, M. & Imumorin, I.G (2013). Molecular survey of pathogenic trypanosomes in naturally infected Nigerian cattle. *Research in Veterinary Science*, 94:555–561.
- Tehseen, S., Jahan, N., Qamar, M.F., Desquesnes, M., Shahzad, M.I., Deborggraeve, S. & Büscher P. (2015). Parasitological, serological and molecular survey of *Trypanosoma evansi* infection in dromedary camels from Cholistan Desert, Pakistan. *Parasites & Vectors*, 8:1–11.
- Vulpiani, M.P., Carvelli, A., Biologist, D.G., Iannino, F., Paganico, D., & Ferri, N. (2013). Reemergence of Dourine in Italy: Clinical cases in some positive horses. *Journal of Equine Veterinary Science*, 33:468–474.

CHAPTER 4 REAL-TIME PCR ASSAY FOR DETECTION OF *TRYPANOSOMA EQUIPERDUM* INFECTIONS IN SOUTH AFRICAN EQUIDS

4.1 Introduction

4.1.1 Real time PCR (qPCR) assay

Real-time PCR, is a method for amplification in which fluorescent dyes are used to detect the amount of PCR product after each PCR cycle (Becker *et al.*, 2004; Bustin, 2005; Piron *et al.*, 2007; Fraga *et al.*, 2008; Kim *et al.*, 2008). This technique was established in the early 1990s (Fraga *et al.*, 2008; Bustin, 2010; Tavares *et al.*, 2011). The fluorescence signal is observed during the reaction and its intensity relate to the quantity of product formed (Kubista *et al.*, 2006; Tavares *et al.*, 2011). The detection of a fluorescent signal produced is directly proportional to the amount of the targeted PCR amplicon produced during each cycle of amplification (Bustin, 2005; Kubista *et al.*, 2006; Nolan *et al.*, 2006). Real-time assays determine the point in time when amplification of a PCR product is first detected during cycling (Bustin, 2005).

According to Bustin *et al.*, (2009), quantitative real-time PCR (qPCR) is a recent modification of PCR (Fraga *et al.*, 2008) with its capacity to identify and measure small amounts of nucleic acids in a wide range of samples from abundant sources, is therefore regarded as an ideal technique in molecular diagnosis, life sciences, agriculture, and medicine (Heid *et al.*, 1996; Piron *et al.*, 2007; Bustin *et al.*, 2009). Several detection systems have been developed in the analysis of quantitative PCR and Real-time PCR such as agarose gels and fluorescent labelling of PCR products as well as detection with laser-induced fluorescence using capillary electrophoresis (Heid *et al.*, 1996; Becker *et al.*, 2004). Most of the screening with qPCR methods developed in recent time are qualitative and use SYBR Green I or TaqMan chemistry. They aim at detecting one element (singleplex) or multiple sequences simultaneously (multiplex) and can target a taxon-specific sequence or a GM element (generic and trait) present in the transgenic constructs (Broeders *et al.*, 2014). To cleave a non-extendible hybridization probe during the extension phase of PCR the method uses the 5' nuclease activity of *Taq* polymerase. This approach uses dual-labelled fluorogenic hybridization probes namely: a reporter

(FAM 6-carboxylfluorescein) and a quencher (TAMRA 6-carboxy-tetra-methyl-rhodamine) are the mostly commonly used with a TaqMan probes. (Heid *et al.*, 1996; Dorak, 2006). The assay also uses fluorescent TaqMan (hydrolysis probe) methodology and the instrument is capable of determining fluorescence in real time. When the probe is intact, fluorescent energy transfer occurs and the reporter dye fluorescent emission is absorbed by the quenching dye (TAMRA) (Heid *et al.*, 1996).

One recognition strategy for nucleic acid detection with real-time PCR is that, it utilizes SYBR Green I to detect the accumulation of any double stranded DNA product. Although SYBR Green I is not specific, it gives sensitive detection and is often used in screening assays where further analysis of specimens is performed to confirm the results (Epsy *et al.*, 2006). For methods developed using the SYBR Green I chemistry, the melting temperature (T_m) forms beside the C_t value, is an additional parameter that needs to be taken into account (Broeders *et al.*, 2014). A melt-curve analysis can be used to identify different reaction products, including nonspecific products (Epsy *et al.*, 2006). By using melting curve analysis, real-time PCR assays also have the potential to detect low levels of parasitemia, identify mixed infections, and allow for precise differentiation of species (Magold *et al.*, 2005). A melting curve analysis is used to determine the T_m , permits detection of different amplification products based upon the %GC content and length of the amplification product (Epsy *et al.*, 2006). Fluorescent Resonance Energy Transfer (FRET) is one of the three types of nucleic acid detection methods that have been used most often in real-time PCR testing platforms which is a distance-dependent interaction between the excited states of two dye molecules. Consequently, the emission of the reporter fluorophore is quenched, in which excitation is transferred from a donor molecule to an acceptor molecule (Bustin, 2005; Dorak, 2006; Epsy *et al.*, 2006). Other two nucleic acid detections are known as 5' nuclease (TaqMan or hydrolysis probes), and molecular beacons (Dorak, 2006). These detection techniques all depend on a process referred to as fluorescence resonance energy transfer, a transfer of light energy between two adjacent dye molecules. FRET hybridization probes consist of two separate probes, a dual FRET hybridization probes and TaqMan (hydrolysis) probes or molecular beacons, the two dye molecules are attached to a single probe (Epsy *et al.*, 2006). A TaqMan probe is a short oligonucleotide (DNA) that contains a 5' fluorescent dye and 3' quenching dye, which is designed to hybridize within the target sequence (Arya *et al.*, 2005).

4.1.2 The mechanism and expected reaction steps in real-time PCR

Quantitative PCR (qPCR) is used to amplify, detect and measure nucleic acids simultaneously. Evaluation can be achieved either by absolute or relative quantification. Sequentially, relative quantification is acquired either by the comparative C_t method or the standard curve method (Arya *et al.*, 2005; Dhanasekaran *et al.*, 2010; Pabinger *et al.*, 2014). Both these methods quantify the target and reference gene expression only in comparative terms by using a calibrator, and may therefore miss a significant biological information (Dhanasekaran *et al.*, 2010).

According to Becker *et al.*, (2004), in real-time PCR, there are three measures for the determination of the amplification efficacy: (1) using the slope of a standard curve generated by plotting serially diluted control template versus the C_t values of the assays, (2) fitting single product curves with a least squares fit when TaqMan chemistry is used, and (3) analysing the linear part of an exponential product curve by linear regression when SYBR Green I is used as a detector. There are three data points (figure 4.1) in amplification plot (Heid *et al.*, 1996), whereby the first phase in a PCR reaction is the baseline which is defined as the PCR cycles in which a reporter fluorescent signal is accumulating but is below the parameters of detection of the instrument (Arya *et al.*, 2005; Bustin, 2005), the number of cycle is referred to as threshold cycle (C_t). The C_t is determined at the exponential phase (Bustin, 2005; Fraga *et al.*, 2008). The latter is a phase that shows results in a quantitative relationship between the amount of starting target sequence and amount of PCR product accumulated at any particular cycle (Arya *et al.*, 2005; Chen *et al.*, 2009). The more the targets in the starting material, the lower the C_t (Nolan *et al.*, 2006) and also the sooner the product amplification is detected (Chen *et al.*, 2009). The C_t is inversely proportional to the copy number of the target (Bustin, 2005). Ultimately, at the plateau phase, the PCR reaction ceases to generate a template, due to inhibitors of the polymerase reaction found with the template, reagent limitation or accumulation of pyrophosphate molecules (Arya *et al.*, 2005; Fraga *et al.*, 2008).

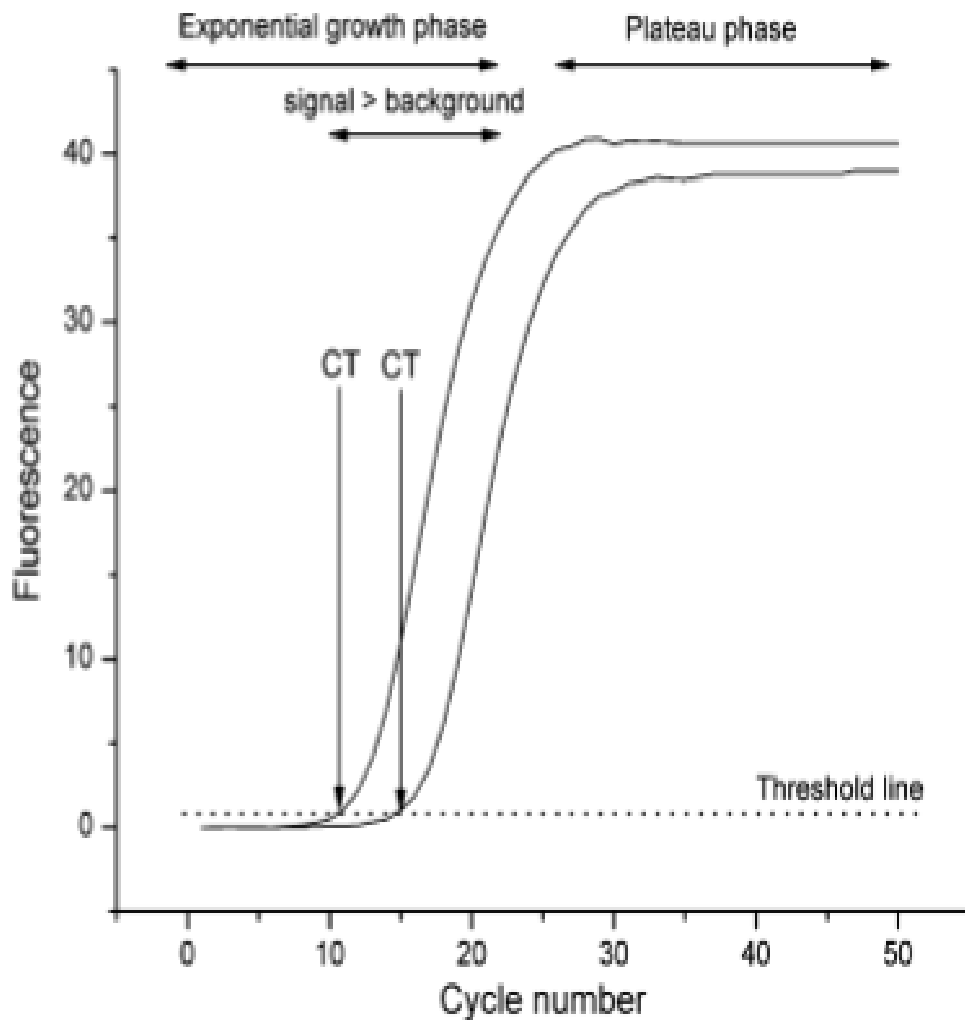


Figure 4.1: Real-time PCR response curves. A threshold level is set sufficiently above background and the number of cycles required to reach threshold (C_t) are registered (Kubista *et al.*, 2006).

To evaluate the fitness of a method and its performance, various characteristics such as PCR efficiency, linear dynamic range, LOD, and specificity needs to be established so that the method can be used as a routine analysis and also to be considered fully for further validation (Bustin *et al.*, 2009; Broeders *et al.*, 2014).

4.1.2.1 Amplification efficiency (e)

Robust and precise qPCR assays are usually correlated with high PCR efficiency (Bustin *et al.*, 2009). The amplification efficiency is calculated from standard curves (Zhao & Fernald, 2005). According to Kubista *et al.*, (2006), standard curve approach gives a good estimate of the efficiency of the PCR assay. Absolute quantification can be achieved using a standard curve constructed by amplifying known amounts of target DNA (Rutledge *et al.*, 2003; Shahsiah *et al.*, 2010). The efficiency of the reaction may be determined by running PCR on serial dilutions of the sample (Rebrikov & Trofimov, 2006) which are prepared from a specific positive material (Broeders *et al.*, 2014). The absolute quantification method depends on a standard plot made from known concentrations of standards to quantify the actual copy numbers of a specific target, hence is believed to be more useful and reliable for comparisons (Dhanasekaran *et al.*, 2010).

The concentration is measured through evaluation on standard curves. This eliminates the need to visualize the amplicons by gel electrophoresis in that way it greatly reduces the risk of contamination and the introduction of false-positives (Ndao, 2009). The development of real-time PCR has brought true quantitation of target nucleic acids out of the pure research laboratory and into the diagnostic laboratory, by combining PCR amplification with fluorescent-labelled virus specific probes able to detect amplified DNA during the amplification reaction (Parida *et al.*, 2008).

4.1.2.2 Linearity (R^2)

According to Bustin *et al.*, (2009), the dynamic range over which a reaction is linear must be described. Depending on the template used on standard curve, the dynamic range should cover at least 3 orders of magnitude and ideally should extend to 5 concentrations. The R^2 for each target should be >0.98 (Broeders *et al.*, 2014). According to Nolan *et al.*, (2006), the relationship between fluorescence and the amount of amplified product allows precise quantification of target molecules over a wide unique range, while retaining the sensitivity and specificity of conventional end-point PCR assay. The closed-tube (homogenous) designed removes the requirement for post-amplification manipulation and significantly decreases hands-on time and the risk of contamination (Nolan *et al.*, 2006; Qvarnstrom *et al.*, 2012). As an alternative, detection and quantification of a certain

PCR product is based on determining the cycle threshold (C_t), the number of PCR cycles at which the amplicon product curve exceeds a pre-set fluorescence value at an early stage of PCR (Becker *et al.*, 2004).

4.1.2.3 Sensitivity

According to Broeders *et al.*, (2014), sensitivity is an important parameter to evaluate for the qPCR method and can be expressed as the limit of detection (LOD). The LOD is defined as the lowest concentration at which 95% of the positive samples are detected (Bustin *et al.*, 2009; Forootan *et al.*, 2017).

4.1.2.4 Specificity

During the development of a method, the specificity of a set of oligonucleotides (primers and/or probe) should be checked to guarantee that the method will only react with the targeted sequence (Broeders *et al.*, 2014). Specificity must be validated with direct experimental evidence such as electrophoresis gel, melting profile, DNA sequencing, amplicon size, and/or restriction enzyme digestion (Bustin *et al.*, 2009).

4.1.3 Real-time PCR for trypanosomes

Real-time PCR technology uses fluorescent labels for continuous monitoring of amplification throughout the reaction. The main advantages are the rapid results of amplification, detection in one step, reduced time and risk of carry-over contamination (Kubista *et al.*, 2006; Piron *et al.*, 2007; Cavalcanti *et al.*, 2009, Chaisi *et al.*, 2013). Reports on trypanosomes detection based on real-time PCR technology have been described for various animal trypanosomes. However, very few studies of a real-time PCR assay with a TaqMan probe for the detection of *T. equiperdum* exist. Some quantitative real-time PCR methods for *T. brucei*, *T. cruzi* and *T. evansi* have been developed (Konnai *et al.*, 2009). Recently, a highly sensitive real-time PCR for *Trypanozoon* subgenus was applied on tissues and fluid samples from a naturally dourine-infected horse, enabling the detection of low numbers of the parasites (Gizaw *et al.*, 2017). Real-Time PCR for *Trypanosoma* sp. DNA performed on the amplification of a 135 bp portion of the 177 bp sequence of the highly repeated region within the *Trypanozoon* subgenus enabled even

low concentrations of DNA from the parasites to be detected in organs and matrices other than blood, namely vaginal and conjunctival swabs, joint fluid and mammary secretions (Pascucci *et al.*, 2013). It is possible with real-time PCR to measure the number of cycles necessary to detect a signal (Threshold cycle) and use this to determine the starting level of a certain nucleic acid present (Deprez *et al.*, 2002). However, these approaches might not be suitable in primary clinical settings in developing countries or for field use, because of the sophisticated instrumentation required, elaborate and complicated assay procedures and expensive reagents (Cai *et al.*, 2013). Nonetheless, it has been applied in variety of area to develop simpler and more cost effective methods for quantitative analysis of nucleic acid (Mori *et al.*, 2004).

Dourine in equids is characterized by short-lasting parasitaemia culminating in low number of parasite making it difficult to recover the parasite in infected tissue (Pascucci *et al.*, 2013). Real-time PCR technology uses fluorescent labels for continuous monitoring of amplification throughout the reaction and can be optimised both as a qualitative and quantitative assay (Piron *et al.*, 2007). This technique has been applied for the accurate quantification of *T. cruzi* loads in peripheral blood samples from infected patients (Moreira *et al.*, 2013). Therefore, the application of quantitative PCR (qPCR) assays for the detection of *T. equiperdum* infections could be useful to determine the bloodstream parasite load and to track a responses of the parasite to the therapeutic treatment.

4.2 Aim of the study

To develop a real-time PCR (qPCR) for the detection of *Trypanosoma equiperdum* infections in South African equids.

4.3 Materials and Methods

4.3.1 Real-time PCR assay

A repetitive insertion mobile element rRNA gene (accession no: K01801.1) for the subgenus *Trypanozoon* was identified from published literature (Hasan *et al.*, 1984). The gene sequence was downloaded and the qPCR primers and probes were designed using the online GenScript Real-time PCR Primer Design software (www.genscript.com/tools/real-time-pcr-taqma-primer-design-tool). Optimization reactions were conducted to determine the optimal reaction temperature and optimal number of cycles. Thereafter specificity and detection limit of qPCR assays were determined as well as standardization reactions. The assay was validated by use in epidemiological studies of dourine from field derived samples.



Figure 4.2: Forward primer, probe and reverse primer design based on 1402 bp RIME genomic sequence, GenBank accession number K01801.1 (Hasan *et al.*, 1984).

4.3.1.1 Optimization Real time reaction

Real-time PCR was performed with a QuantStudio™ 3 & 5 Real-Time PCR system (Thermo Scientific, Applied Biosystems, Invitrogen, Fisher Scientific and Unity Lab Services, USA). The amplification mixture contained 2µl of template DNA, 1µl of each primer (RIME F1: CGG ACA GCA GCA TCT TAG TG) and (RIME R1: ATA CAG CAC AGG GAT CAG CA), primers designed for amplification of a 115 bp region of the RIME gene for subgenus *Typanozoon* species. A 10µl of 2X Power UP SYBR Green and PrimeTime Gene expression Master Mix together with 1µl of probe (5'-/ 56-FAM/ CAC GCT ACG TGC CCA GG/36-TAMSp/-3') in a final volume of 20 µl. The following PCR program was used for SYBR Green I: 50°C for 2 min, 95°C for 2 min, then 45 cycles at 95°C for 15s, and 58°C for 1 min. and melting curve at 95°C for 30s and 60°C for 15s. For the standardization of real-time PCR, SYBR Green I and a probe-based systems were employed. The threshold (C_t) was determined as the number of cycle at which the product curve exceeded a normalized fluorescence of 0.5. Standard calibration curves were constructed by 5 serial dilution of *Trypanosoma equiperdum* DNA obtained from Obihiro University Japan, ranging from 10^0 to 10^{-5} , and reactions were done in triplicates. All the reactions (sensitivity, specificity and evaluation) were conducted using these conditions. Melting curves were used to determine the specific PCR products.

Thermal standard cycling conditions for a probe-based assay were as follows: Polymerase activation: 1 cycle at 95°C for 3 min, followed by 35 cycles at 95°C for 15 seconds, then 60°C for 1 min.

4.3.1.2 Reaction sensitivity using both dyes (SYBR and PrimeTime)

To determine the lower limit of detection of the real-time PCR assay for *T. equiperdum*, a 10-fold dilution series from 10^0 to 10^{-6} with concentrations ranging between 10ng/ul and 100fg were prepared using positive control obtained from Obihiro University of Agriculture and Veterinary Medicine Japan, then sensitivity of the primers and probe was assessed. The dilutions were subjected to both qPCR and PCR reactions.

PrimeTime probe-based assay was used to verify the results of SYBR Green I such as standard curve, sensitivity and specificity of RIME1 qPCR primers.

4.3.1.3 Reaction specificity and evaluation of the assays

The specificity of these reactions was determined using 2µl of different DNA concentrations of several trypanosome species including *Trypanosoma equiperdum*, *T. brucei brucei*, *T. evansi* and *T. congolense* were subjected to the assay. In addition, different concentrations from other blood parasites such as *Babesia caballi* and *Theileria equi* DNAs which are known to infect equines were also used. Combinations of infectious agent were also assessed, to review the influence of concurrent infections on the efficiency of the species specific reaction.

Forty-eight samples that previously tested positive with rELISA, a serology test, were used in the study to evaluate the technique. Double distilled water (DDW) and H-DNA were included in all reactions as negative controls.

4.4 Conventional PCR using RIME 1 primers

Polymerase chain reaction was conducted using a total volume of 25 µl, containing 12.5 µl of Amplitaq Gold® 360 Master Mix (Applied Biosystem, USA), 1 µl of each primer (10 µM of each primer), using the primers (10 µM each) for amplification of 115bp region of RIME gene (RIME F1: CGG ACA GCA GCA TCT TAG TG) and (RIME R1: ATA CAG CAC AGG GAT CAG CA), 2 µl of template DNA and 8.5 µl of double distilled water (DDW) to adjust the volume. *Trypanosoma equiperdum* DNA obtained from Obihiro University of Agriculture and Veterinary Medicine Japan was used as a positive control and DDW was used as a negative control.

Polymerase chain reaction conditions for RIME 1 primers were as follows: activation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, annealing at 60°C for 50 seconds, extension at 72°C for 2 minutes and final extension at 72°C for 10 minutes.

4.4.1 Analytical sensitivity and specificity of RIME1 primers

The sensitivity of the PCR reactions was tested by using serial dilutions of *Trypanosoma equiperdum* DNA ranging from 10⁰ to 10⁻⁶. In order to verify the accuracy of the assay, PCR experiments were performed with negative horse DNA. Specificity of the assay was

assessed as mentioned in section 4.3.1.3. Forty-eight samples were used to evaluate the assay. The sensitivity of the assays was also compared.

4.4.2 Comparison of real-time PCR assay with conPCR techniques

Conventional PCR and real-time PCR assays were conducted using field samples (n = 48) collected from an abattoir as mentioned in chapters 2 and 3. These samples were tested positive using ELISA TeGM6-4r for serology in the previous studies (Mlangeni, 2016). The sensitivities of the techniques were compared. Amplification for both of the assays were conducted as mentioned in section 4.4 and 4.3.1.1 respectively.

The 115-nucleotide amplicon was separated by electrophoresis on 1% agarose gel and visualised by ultraviolet trans-illumination after staining with ethidium bromide and for qPCR the results were viewed in real-time.

4.5 Statistical analysis

To perform absolute quantification, a target template solution of known concentration was diluted over several orders of multitude, amplified by real-time PCR, and data was used to generate a standard curve in which each target concentration is plotted against the resulting C_t value. The qPCR amplification efficiency (E) determined by linear regression of the standard curve was calculated from the slope (s) of the standard curve using the equation: $E = 10^{-1/s}$. The acceptable efficiency of the qPCR assay was between 90–110%. Inter assay precision was calculated using the following formula: Inter-assay precision = (Standard deviation of the mean C_t of the triplicates/ Grand mean C_t of the triplicates) x 100 (Ahmed *et al.*, 2015). Samples were considered positive when amplification curves occurred between the number of cycles that were set from the standard curve. The number of the parasites was calculated by plotting the average C_t values versus the log (10) of the parasite concentration/ml. For PCR, significance of the differences between the frequencies of positive results obtained in the tests was measured by Fisher's exact test. The level of agreement between the two diagnostic tests was performed using the kappa index as described by Kramer and Feinstein (1981).

Analysis was performed with the aid of SPSS software version 19 and Graph Prism. Positive samples were summarized as percentages and confirmed as true positives using BLASTn search. The number of parasites corresponding to each DNA dilution was calculated as described in section 3.3.4.1.

4.6 Results

4.6.1 Real-time PCR optimization and standardization

The procedure was optimized with regards to the concentration of primers and denature/extension temperature. Different temperatures and cycles were selected to find the optimum reaction (figure 4.3). SYBR Green I chemistry, the melt-curve analyses were used to identify the non-specific PCR products, primer dimers as well as to differentiate true positives from false positive (figure 4.4). The efficiencies of the qPCR assays were determined from linear regression equations, generated from tenfold serial dilutions of genomic DNA extracted from *Trypanosoma equiperdum* infected equine erythrocytes (figures 4.6 and 4.9). The qPCR assays were further tested for their ability to detect parasite DNA in field samples previously identified in South Africa using rELISA. Optimum reaction temperature and cycles was at 58°C for 35 cycles (figure 4.3). Melt peaks of *T. equiperdum* occurred at ~85.0°C (figure 4.4).

Furthermore, a probe-based real-time PCR assay together with a non DNA binding dye (SYBR Green I) were developed for *T. equiperdum*. To develop these fluorescent labels, the following principles were used from MIQE guidelines: The efficiency of the reaction was calculated using this formula: $E = 10(-1/\text{slope}) - 1$ and of which it should be 90-100%, meaning doubling of the amplicon at each cycle and that should correspond with a slope of -3.1 to -3.6 amount of the standard curve. In order to obtain accurate and reproducible results, efficiency should be similar for both target and reference and it should be as close to 100% as possible (Dorack *et al.*, 2006). To evaluate the performance of qPCR assays, the curves were generated by using serially diluted standards of known concentrations and produces a linear relationship between C_t and the logarithm of the initial amount of total template DNA showed the slope of -3.51, $R^2 = 0.99$ with the efficiency of 92.7% using SYBR Green I dye (figure 4.5, 4.6 and 4.7), -3.45, $R^2 = 0.99$ with the efficiency of 94.9% when a probe-based was used (figure 4.8 and 4.9). The standard curve maintained linearity in all assays. The reproducibility of the assay was very good, as the variability of T_m among assays was < 0.85%. The p value was <0.05 between different DNA concentrations and as such a significant difference was observed between the DNA concentrations.

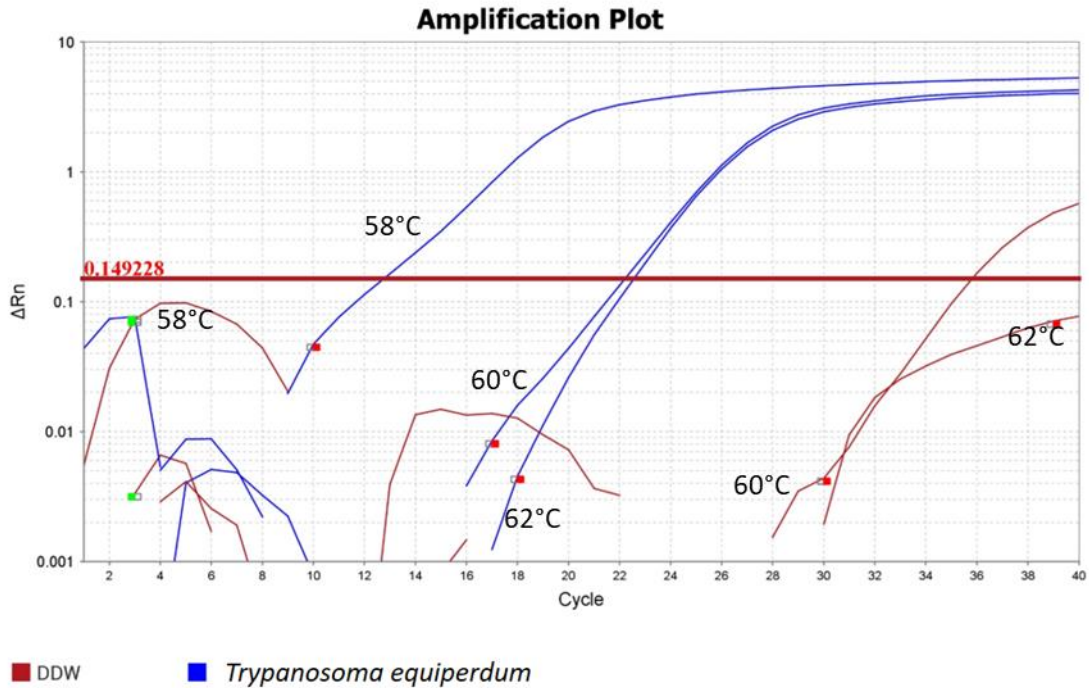


Figure 4.3: Optimization amplification plot of RIME 1 real-time PCR primers using SYBR Green I. *T. equiperdum* DNA was used and different temperatures were selected (58, 60, & 62°C) no reaction was detected at 58°C in DDW.

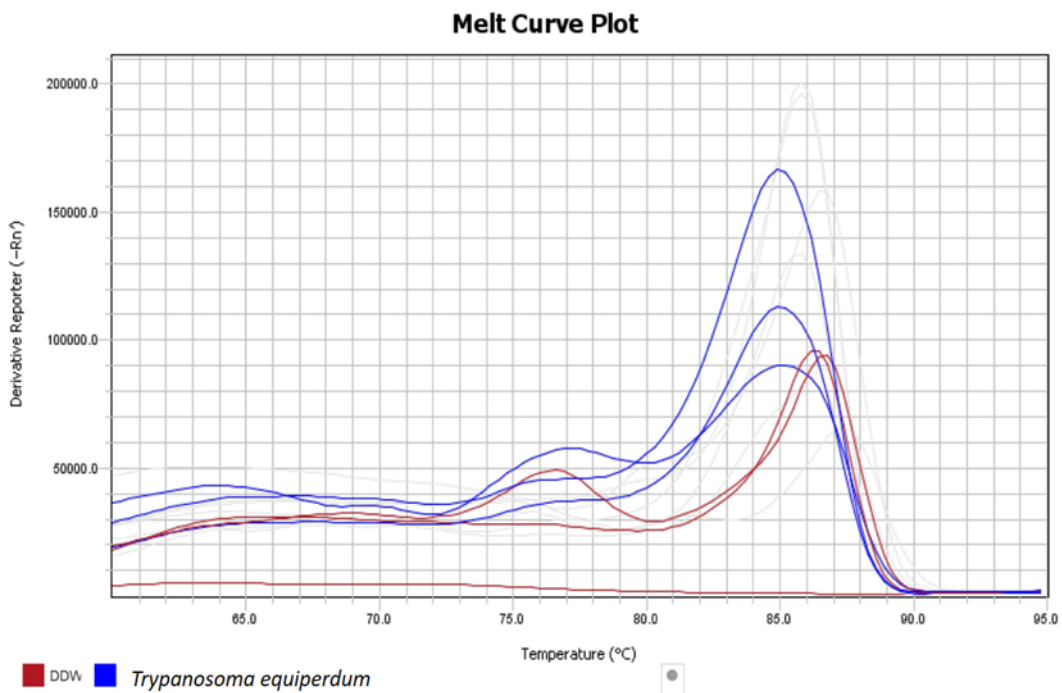


Figure 4.4: Optimization melt-curve of RIME 1 real-time PCR primers using SYBR Green I. *T. equiperdum* DNA was used and different temperatures were selected (58, 60, & 62°C) no reaction was detected at 58°C in DDW.

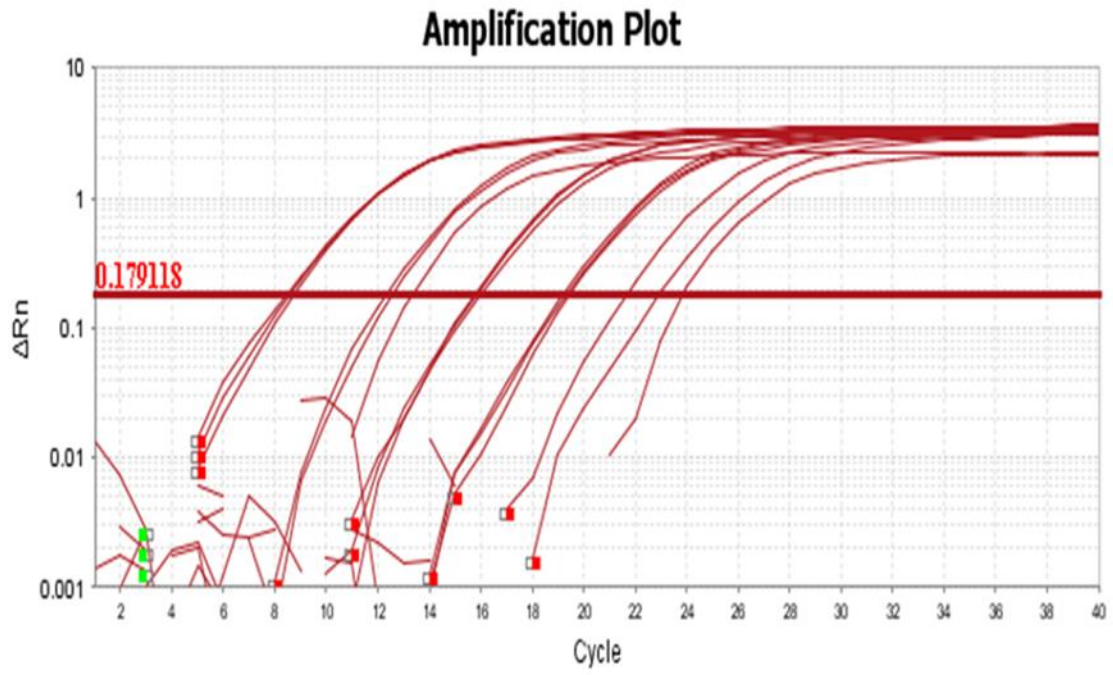
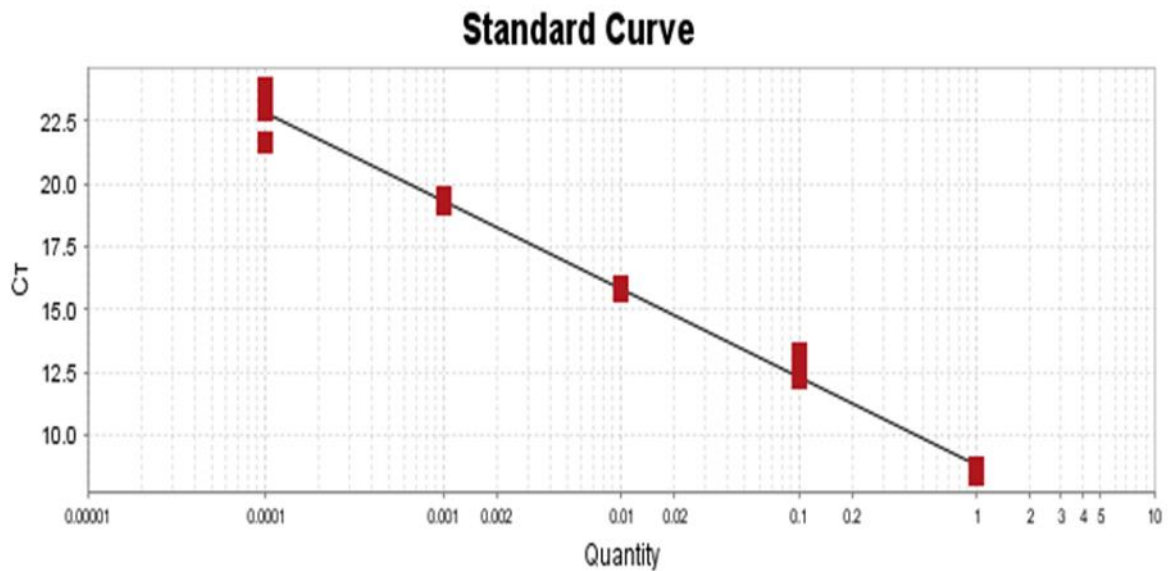


Figure 4.5: SYBR Green I amplification plot of RIME 1 primers for *T. equiperdum* parasites.



[Target:](#) Target 1 [Slope:](#) -3.508 [Y-Inter:](#) 8.847 [R²:](#) 0.99 [Eff%:](#) 92.773 [Error:](#) 0.096

Figure 4.6: SYBR Green I qPCR standard curve generated from linear region of each amplification curve of five 10-fold serially dilutions of *T. equiperdum* for RIME 1 primers.

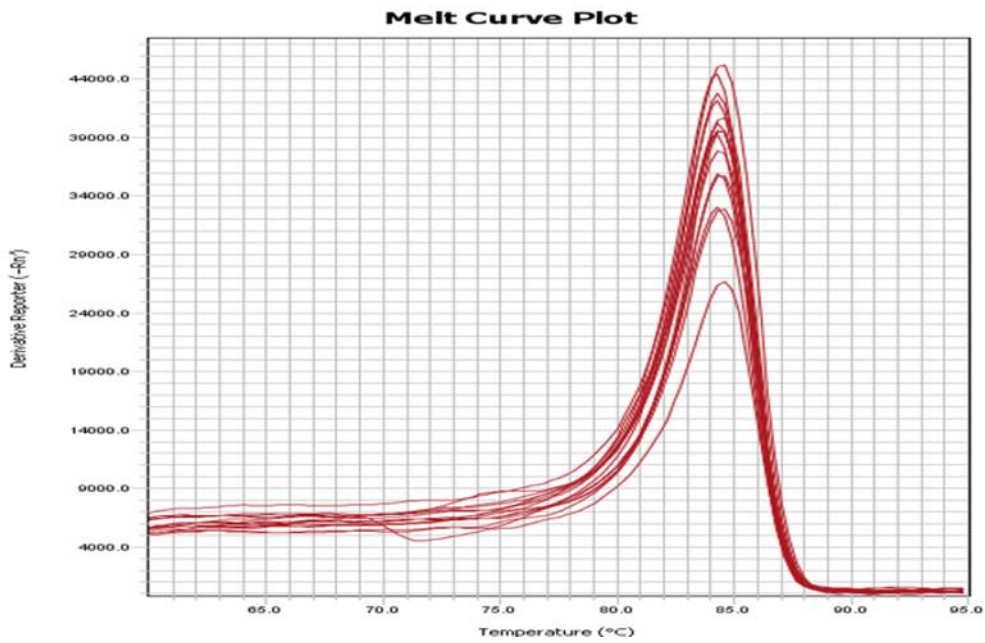


Figure 4.7: SYBR Green I qPCR melt-curve analysis of RIME 1 primers for *T. equiperdum* parasites.

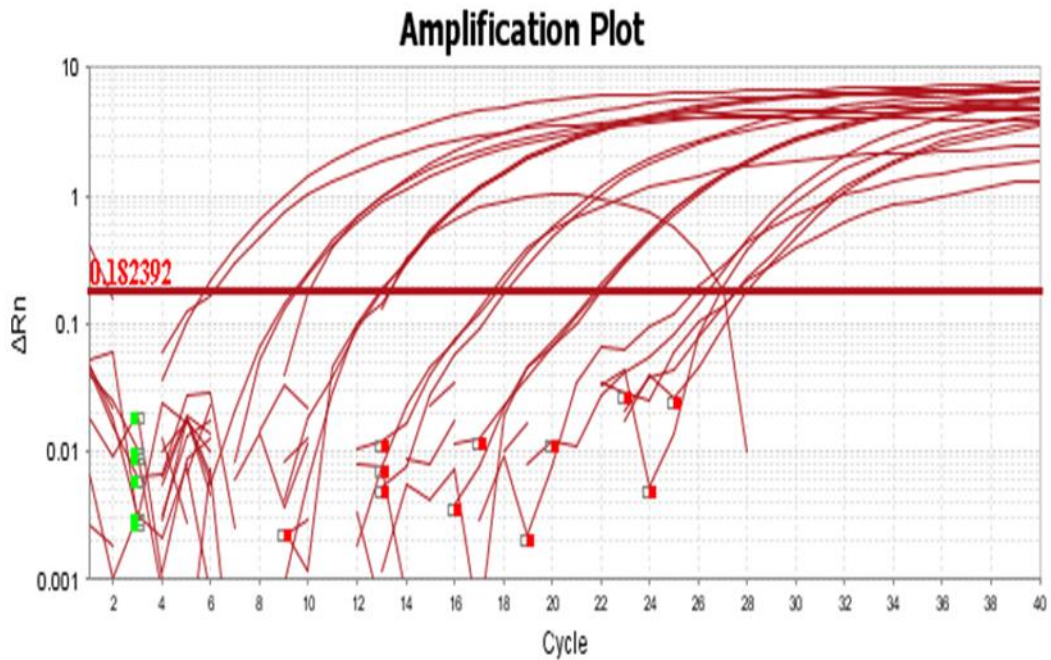


Figure 4.8: PrimeTime probe-based qPCR amplification plot of RIME 1 for *T. equiperdum* parasites.

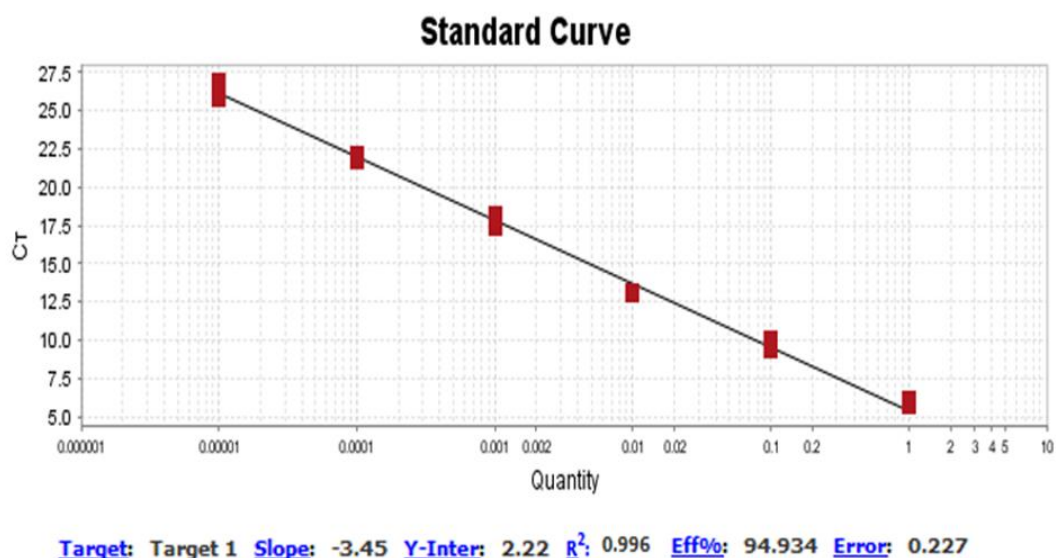


Figure 4.9: Standard curve generated from five 10-fold serially dilutions of *T. equiperdum* for RIME 1 real-time PCR primers using PrimeTime a probe-based.

4.6.2 Analytical sensitivity and specificity of RIME 1 qPCR assay

The set of dilutions prepared from *T. equiperdum*, positive DNA was detected in all replicates of the reactions. As the dilutions decreases, more cycles were required. The fewer PCR cycles are required to make enough material for detection because, more copies of a DNA template are present at the beginning of an experiment (Pabinger *et al.*, 2014). The assay had a detection limit of 1×10^{-5} which is equivalent to 100fg trypanosome/ml (figure 4.10 and 4.11). No signal (C_t value) was obtained with no template controls that contained no trypanosomes.

All assays were 100% specific and accurate for detection of *T. equiperdum*. None of non-targeted DNA's were detected for specificity of qPCR assays. In comparison between the dyes, the results were still the same (figure 4.12). For SYBR Green I dye, identity of the amplifications was verified by melting temperature determination and melt-curve analyses (figure 4.13). Finally, the potential of the real-time PCR assay as a diagnostic tool was investigated with blood samples of horses and donkeys from four provinces of South Africa with serologically (rELISA) confirmed *T. equiperdum* infections. Of the 48 equine samples, 23 (47.9%) samples were negative and 25 (52.1%) were positive indicating a

test specificity of 92.0%. In real-time PCR, 19/38 (50.0%) samples were positive from horse samples and 6/10 (60.0%) were positive donkey samples. Statistical analysis showed no significant difference between the groups ($p > 0.05$)

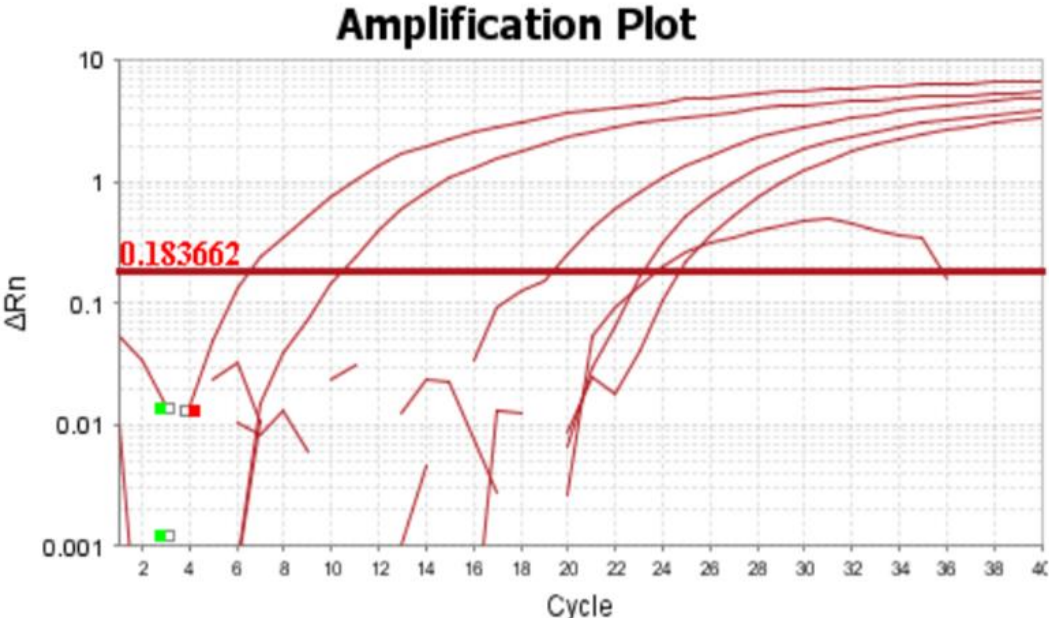


Figure 4.10: SYBR Green I qPCR sensitivity of RIME 1 primers for *T. equiperdum* parasites.

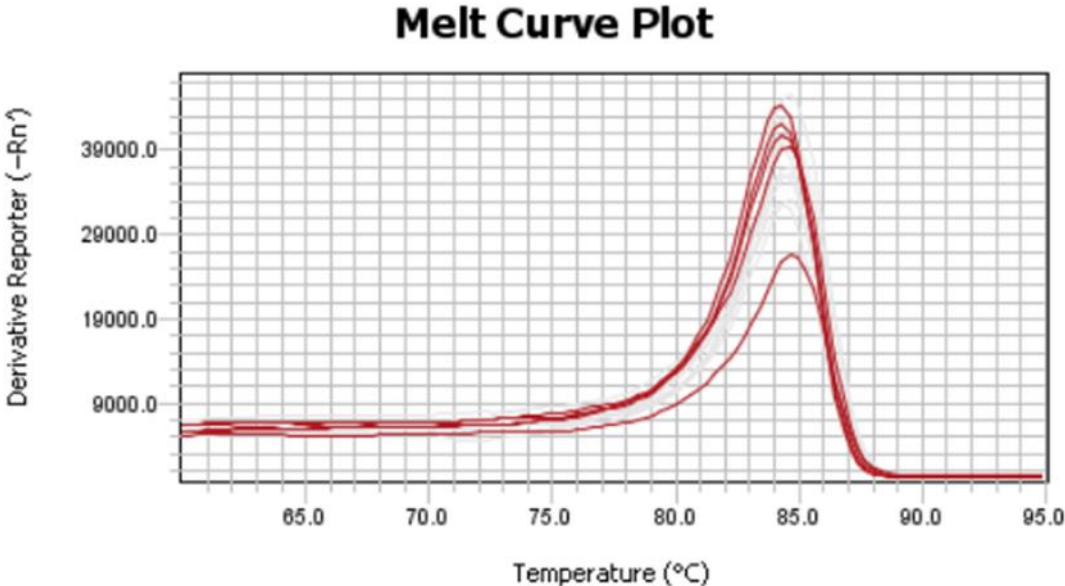


Figure 4.11: Melt-curve analysis for sensitivity of RIME 1 primers for *T. equiperdum* parasites.

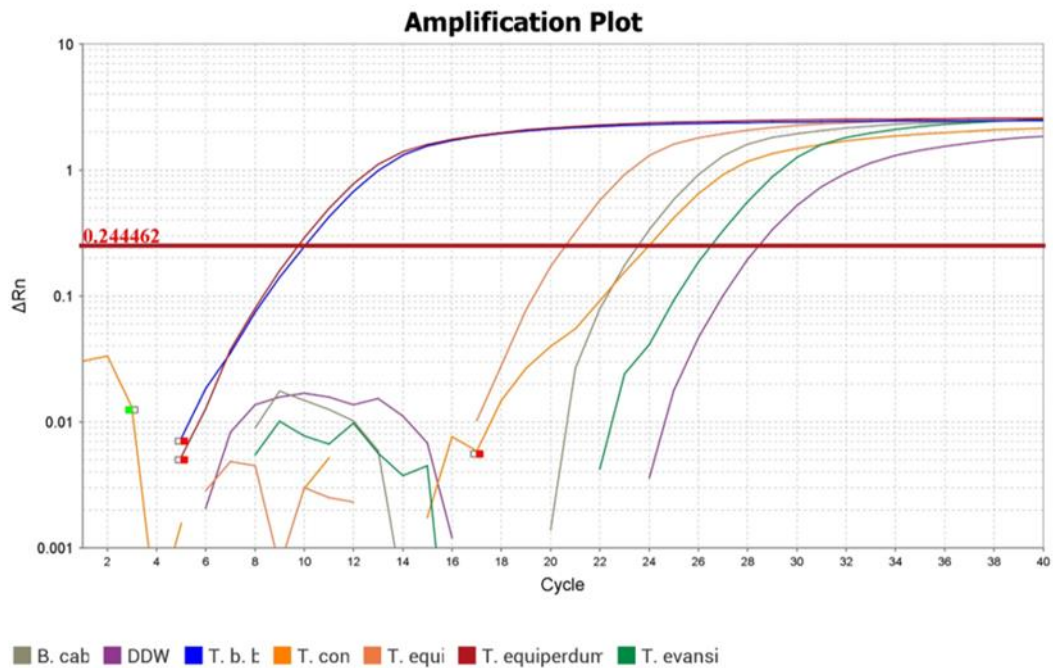


Figure 4.12: Results of specificity for RIME 1 primers using SYBR Green I. DNA's from different pathogens were used together with *T. equiperdum* DNA as a positive control. No amplification was observed in non-target DNA and DDW.

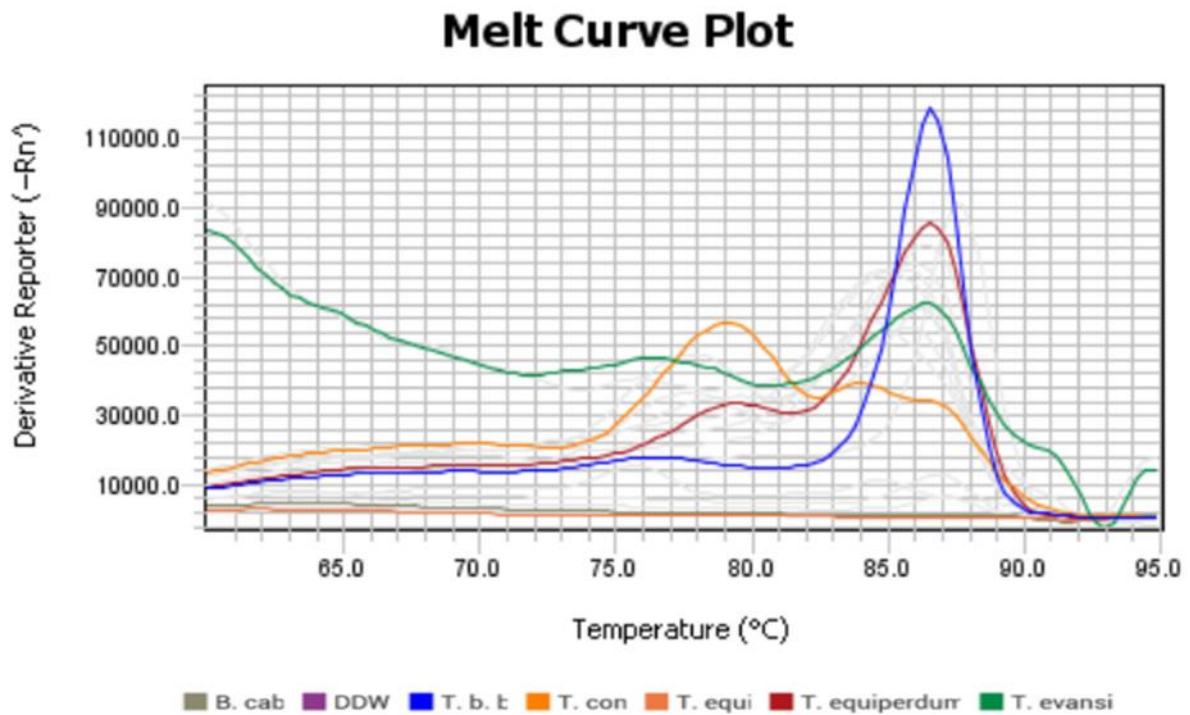


Figure 4.13: Melt-curve analysis for specificity of RIME 1 primers. Different pathogens were used together with *T. equiperdum* DNA as a positive control. No amplification was observed in non-target DNA and negative control (DDW).

4.6.3 Conventional PCR using RIME 1 primers

The RIME 1 primers used in this study are not published yet and therefore their specificity and sensitivity was checked. The analytical sensitivity of RIME 1 was tested using 10-fold serially diluted *T. equiperdum* DNA. Conventional PCR had a detection limit of 100fg which is equivalent to 1 trypanosome/ml (figure 4.14) and for specificity none of the non-targeted DNA's were amplified (figure 4.15).

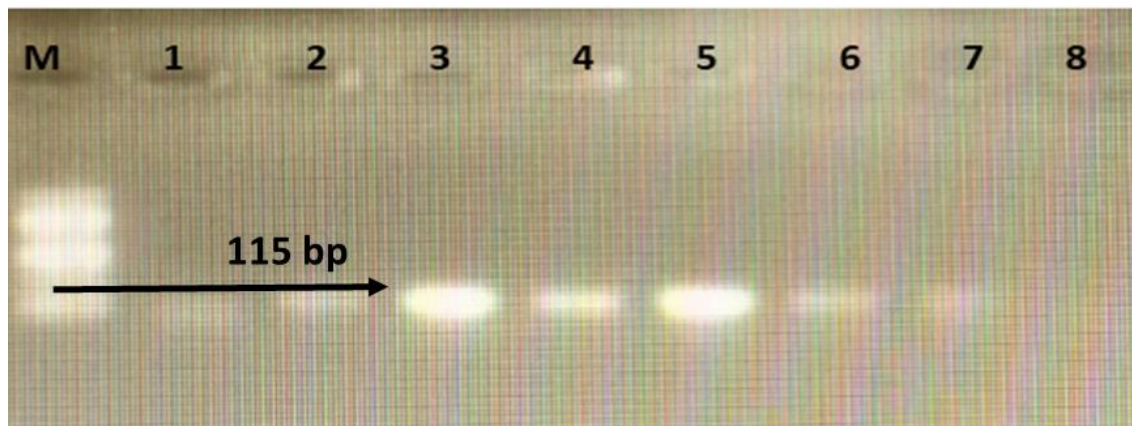


Figure 4.14: RIME1 primers sensitivity gel image of 1% agarose gel electrophoresis. **M:** Ladder (1kb), 1. DDW, 2. *Trypanosoma equiperdum*, 3. 10^{-1} , 4. 10^{-2} , 5. 10^{-3} , 6. 10^{-4} , 7. 10^{-5} , 8. 10^{-6} , PCR had a detection limit of 10^{-5} which is equivalent to 1 trypanosome/ml.

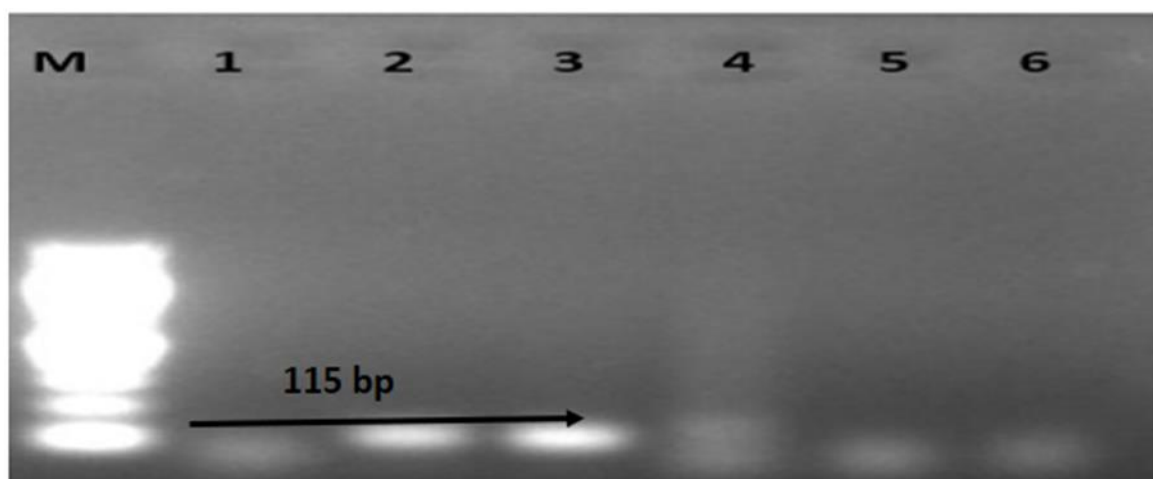


Figure 4.15: RIME 1 primers specificity gel image of 1% agarose gel electrophoresis. **M:** Ladder (1kb), 1. DDW, 2. *Trypanosoma equiperdum*, 3. *T. brucei brucei*, 4. *T. evansi*, 5. *T. congolense* & 6. H-DNA.

4.6.4 RIME 1 ConPCR amplification on field blood samples

Out of the 48 blood samples from horses and donkeys that were screened for the presence of *T. equiperdum* infections. The presence of *T. equiperdum* was revealed by positive bands at 115 bp for target gene fragment (figure 4.16). In contrast, no amplification was observed when normal horse blood DNA was used as the template. Out of 48 samples from horses and donkeys screened, 23/48 (47.9%) were positive while 25/48 (52.1%) was negative. Of these 23 positive samples, 5/10 (50.0%) were donkeys while 18/38 (47.4%) were horse amplified DNA (table 4.1) [$\chi^2 = 0.02197$ (df = 1) and $p > 0.05$]. There was no significant difference observed between the groups. Mpumalanga obtained the highest prevalence of 66.7%, followed by Free State with 58.3%, then Northern Cape with 41.7% and North West with 25.0%. The DNA's amplified with *T. equiperdum* were submitted for direct sequencing using the new RIME 1 primers to confirm the positive results. The GenBank was used to identify the amplified sequences by using BLASTn searches (<http://www.ncbi.nlm.nih.gov/BLAST>). The BLASTn searches revealed that 3 of 5 (60%) sequences were RIME gene sequences matched with *Trypanosoma evansi* and *T. brucei* accession number EF567424.1, EF567426.1 and K01801.1 with 90-97% identity and e-value of 0.0 (figure 4.17).

Table 4.1. Results of donkey and horse DNA samples screened for *T. equiperdum* using RIME1 primers by conPCR ($p > 0.05$)

Province	Animal Species	conPCR +ve(%+ve)	Total +ve(%+ve)	conPCR
Free State (N=12)	Horses (n=10)	5(50.0)		
	Donkeys (n=2)	2(100.0)	7(58.3)	
Mpumalanga (N=12)	Horses (n=12)	8(66.7%)		
	NDSC	-	8(66.7)	
Northern Cape (N=12)	Horses (n=8)	3(37.5)		
	Donkeys (n=4)	2(50.0)	5(41.7)	
North West (N =12)	Horses (n=8)	2(25.0)		
	Donkeys (n=4)	1(25.0)	3(25.0)	

NDSC: No donkey samples collected

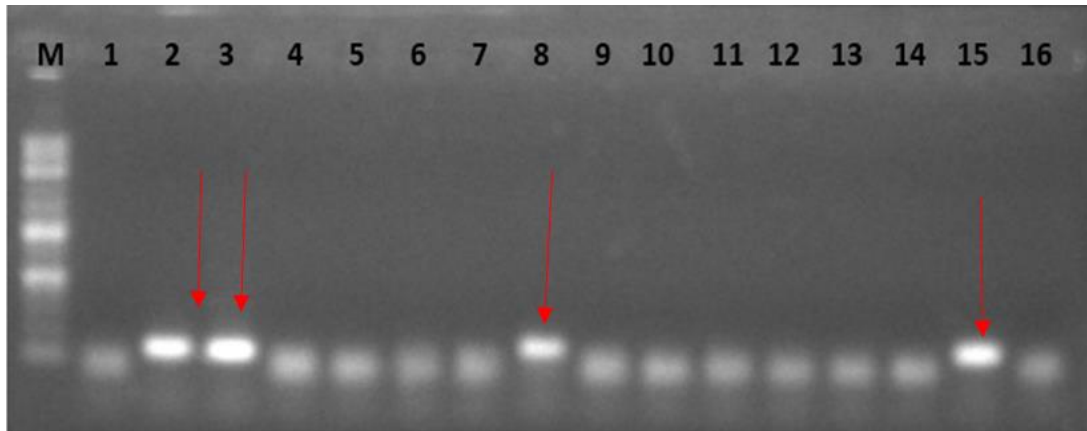


Figure 4.16: Gel image of 1% agarose electrophoresis showing amplified PCR products with amplicon size of 115 bp. **M:** Ladder (1kb), 1. DDW, 2. *Trypanosoma equiperdum*, 3. P1, 4. P2, 5. P3, 6. P8, 7. H1, 8. H2, 9. H5, 10. H8, 11. H10, 12. H14, 13. H26, 14. H28, 15. MP9 & 16. H-DNA. Red arrows indicating positive samples.

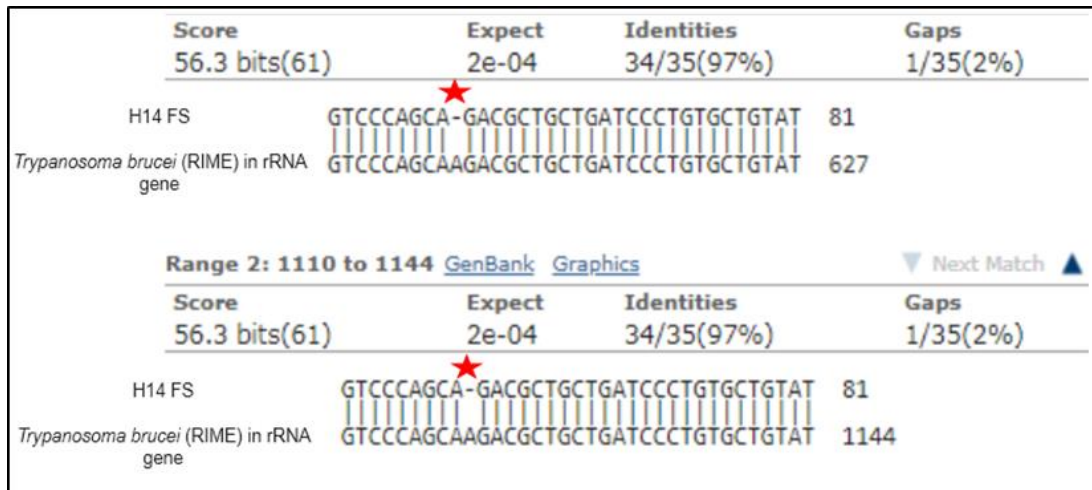


Figure 4.17: BLASTn results showing the alignment of *T. brucei* and one of the sequences from this study which was from a horse sample from Free State Province. The subject sequence (*Trypanosoma brucei* RIME in rRNA gene: Accession no.: K01801.1, it had a match of 95% on the query sequence (FS_H14 Equine). The red stars show gaps between sequences.

4.6.5 Comparison between qPCR and conPCR assays

Out of 48 samples that were screened from equines from the four sampled provinces, 25(52.1%) samples were positive by qPCR assay while 23(47.9%) samples were negative. In contrast, 23(47.9%) were positive by conPCR while 25(52.1%) samples were negative (tables 4.2 and 4.3). There was no significance difference observed among the different provinces. The level of agreement was 0.917 between the assays (table 4.4). For real-time PCR, Mpumalanga obtained the higher prevalence of 75.0% followed by Free State (58.3%), then Northern Cape (41.7%) and lastly North West (33.3%) as the lowest amongst the sampled provinces. For PCR, Mpumalanga was still the highest with 66.7%, followed Free State with 58.3%, then Northern Cape 41.7% and lastly North West with 25.0%.

Table 4.2. Results of the comparison between the two assays qPCR and conPCR for RIME 1 primers $p>0.05$

Province	Horses			Donkeys		
	No. of samples	qPCR +ve(%+ve)	ConPCR +ve(%+ve)	No. of samples	qPCR +ve(%+ve)	ConPCR +ve(%+ve)
Free State (N=12)	10	5(50.0)	5(50.0)	2	2(100.0)	2(100.0)
Mpumalanga (N=12)	12	9(75.0)	8(66.7)	NDSC	-	-
Northern Cape (N=12)	8	3(37.5)	3(37.5)	4	2(50.0)	2(50.0)
North West (N=12)	8	2(25.0)	2(25.0)	4	2(50.0)	1(25.0)
Total = 48	38	19(50.0)	18(47.4)	10	6(60.0)	5(50.0)

NDSC: No donkey samples collected

Table 4.3. Overall infection obtained from the two assays qPCR and RIME 1 conPCR for the detection of *T. equiperdum*

		qPCR		Total
		Negative	Positive	
PCR	Neg	23	2	25
	Pos	0	23	23
Total		23	25	48

Table 4.4. Symmetric measures between qPCR and RIME 1 conPCR

		Value	Asymp. Std. Error ^a	Approx. T ^b	Approx. Sig.
Measure of Agreement	Kappa	.917	.057	6.374	.000
Number of Valid Cases		48			

4.7 Discussion

Due to high levels of sensitivity, specificity and precision, real-time PCR (qPCR) is becoming a more widespread and applied technique in research focusing on bacterial, viral and parasitic infections in addition to clinical, forensic, food safety, and environmental applications (Sivaganesan *et al.*, 2010; Bell *et al.*, 2015; Forootan *et al.*, 2017). Nonetheless, few studies have been conducted on the evaluation of trypanosomes parasitaemia, therefore, there is a need for efficient, rapid and practical assays for *T. equiperdum* detection.

In the current study, SYBR Green I assay was successfully developed. We successfully applied qPCR assay targeting RIME rRNA gene to quantify total parasites from a variety of equine samples. To our knowledge, this is the first time that a newly real-time PCR which targets RIME rRNA gene of the sub-genus *Trypanozoon* is been used to quantify total parasites in donkeys and horses in South Africa. Specificity, sensitivity and quantitative range of qPCR were evaluated and the results were in agreement with the previous studies (Becker *et al.*, 2004; Konnai *et al.*, 2009) though different genes were used. The findings of the study showed that the assay is reliably for quantitative detection of *T. equiperdum*. The established real-time PCR assays for *T. equiperdum* DNA quantitation was linear over a 1×10^5 dilution range of template concentrations with a R^2 value (the square of the correlation coefficient) of 0.99 and a reaction efficiency of 92.7% and 94.9% (figures 4.6 & 4.9). With C_t values between 8 and 25.5, the qPCR assays detected *T. equiperdum* DNA concentrations with a linear relationship over five orders of magnitude between 1×10^0 and 1×10^{-5} reactions. The linearity of amplification products was judged based on the PCR efficiencies (slope) and the R-values (R^2) of the standard curve. Therefore, both assays yielded curves with comparably steep slopes and the expected differences in C_T values. The detection limit of the assay was established at 1×10^{-5} which is equivalent to 1 trypanosome/ml. Protozoan pathogens such as *Babesia caballi*, *Theleiria equi*, and other trypanosomes like *T. congolense* showed amplification, but the C_t values were after the standard of our assay, suggesting that they were not detected by these assays. These results indicate approximately optimal reaction conditions in our qPCR assays and it can be considered to be a potential technique for the detection within a wide range of input template DNA. The detection limit (100 fg) of the current study was lower than that reported in other studies (Becker *et al.*, 2004;

Konnai *et al.*, 2009). These levels of sensitivities were incomparable with the ones reported previously for other qPCR assays. For PCR technique 100fg detection limit of sensitivity was incomparable with the ones reported previously for other PCR assays (Njiru *et al.*, 2008; Njiru, 2011).

The RIME 1 set of primers designed for the SYBR Green I real-time PCR assay successfully amplified *T. equiperdum* DNA under the conditions optimized for this assay, and also the probe-based set which is known to more be specific detected *T. equiperdum*. Amplification and melting peaks were observed for specificity when non-target DNA were used but that does not mean samples were true positives, since our standard was set at 8 up to 22.5 cycles, this means anything below or after 22.5 is false positive. Interestingly, most samples with parasitaemia levels ranging from 1×10^{-1} to 1×10^{-5} were detected even though most samples fell outside the linear ranger of the standard curve. According to Deprez *et al.*, (2002), SYBR Green I dye, when it is only bounded to any double-stranded DNA emits light and that allows detection of DNA in a sequence-independent way. This implies that when both PCR fragments nonspecific and specific formed they will be measured. Therefore, a melting curve analysis was performed to visualize nonspecific PCR, as it is known to reveal the different fragments which usually appear as distinct separate melting peaks. Several molecular tests based on PCR and hybridisation assays such as RLB or LAMP are available for the detection and identification of *Trypanozoon* species. However, none of them allow reliable quantification of the *T. equiperdum* parasite, which could prove very useful following response to treatments, monitoring experimental infections or studying the role of carrier animals as a source of infection (Pascucci *et al.*, 2013, Ahmed *et al.*, 2018). Furthermore, our newly developed qPCR assays can be used for both the detection and quantification of *T. equiperdum*, as well as to monitor *T. equiperdum* parasitaemia in carrier equids. The SYBR Green I qPCR assay was used to screen pure genomic DNA from equine samples with very low DNA concentrations and resulted in C_t values that were outside the linear range of the standard curve. These results were similar to the study conducted by Becker *et al.*, (2004) for the detection of *T. brucei gambiense*. When compared to the previous reports on the detection of *T. equiperdum* (Alemu *et al.*, 1997; Clausen *et al.*, 1999; Clausen *et al.*, 2003; Gari *et al.*, 2010), the qPCR method used in this study yielded a more rapid and sensitive test result than conventional PCR and is less likely to produce false positives due to contamination during the sample preparation process.

The newly developed technique (qPCR) requires no post-PCR gel electrophoresis unlike the conventional PCR. This helps to prevent contamination of the amplicons and the assay is more reproducible and convenient. For precise estimation of parasite loads in equine samples, a conventional PCR was applied which can detect the targeted DNA. However, it requires an exogenous competitor as a control and post-PCR electrophoresis. Therefore, contamination with amplicons can be avoided. This assay is more reproducible and convenient than conventional PCR tests. The analytical sensitivities of the qPCR assays for the detection of *T. equiperdum* was determined as 100% at parasitemia of 100fg for the RIME1 qPCR assay, indicating that the latter assay might be slightly more sensitive than the former RIME PCR by (Njiru *et al.*, 2008; Njiru, 2011). Real-time PCR assays have previously been reported as being more sensitive than the 18S and RoTat 1.2 PCR assays in detecting *T. equiperdum* (Gari *et al.* 2010; Pascucci *et al.*, 2013). The assay was able to detect *T. equiperdum* in all samples shown to be positive by the conventional PCR. The comparison of real-time with conventional PCR proved to be useful in assessing the sensitivity of the real-time PCR assay with the TaqMan probe. The limitation of the comparison between conventional and real-time PCR is the absence of a gold standard. There were two samples that were negative based on the conventional PCR but positive based on real-time PCR (Table 2). The new assay increased the number of samples in which *T. equiperdum* infections were detected by 15% compared to the samples detected by means of conventional PCR.

The results obtained shows conformity between the results of the techniques of conPCR and qPCR using the SYBR Green I system in 100% of samples analysed. These data sets suggest that both PCR techniques were equally effective for detection of the genome of the parasite in the equines blood. However, a distinct advantage of the real-time assay is its ability to detect parasites at least half the time of the PCR method. Horses recorded higher infection rates than donkeys for conPCR but less infection rates than donkeys for qPCR assay which could be attributed to the limited number of samples of donkeys and horses. Generally, donkeys are known to be resistant due to the anti-complementary activity present in their serum (OIE, 2015). Therefore, the defined SYBR Green I qPCR assay used in this study, was shown to be rapid, sensitive and specific for the detection and quantification of *T. equiperdum* in equine samples in South Africa.

REFERENCES

- Ahmed, H.A., MacLeod, E.T., Welburn, S.C. & Picozzi, K. (2015). Development of real time pcr to study experimental mixed infections of *T. congolense* Savannah and *T. b. brucei* in *Glossina morsitans morsitans*. *PloS one*, 10: e0117147.
- Ahmed, Y., Hagos, A., Merga, B., Van Soom, A., Duchateau, L., Goddeeris, B.M. & Govaere, J. (2018). *Trypanosoma equiperdum* in the horse – a neglected threat? *Vlaams Diergeneeskundig Tijdschrift*, 87:66–75.
- Alemu, T., Luckins, A.G., Phipps, L.P., Reid, S.W.J. & Holmes, P.H. (1997). The use of enzyme linked immunosorbent assays to investigate the prevalence of *Trypanosoma equiperdum* in Ethiopian horses. *Veterinary Parasitology*, 71:239–250.
- Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N. & Patel, H.R.H. (2005). Basic principles of real-time quantitative PCR. *Expert Review Molecular Diagnosis*, 5:1–11.
- Becker, S., Franco, J.R., Simarro, P.P., Stich, A., Abele, P.M. & Steverding, D. (2004). Real-time PCR for detection of *Trypanosoma brucei* in human blood samples. *Diagnostic Microbiology and Infectious Disease*, 50:193–199.
- Bell, J.A., Weckstein, J.D., Fecchio, A. & Tkach, V.V. (2015). A new real-time PCR protocol for detection of avian haemosporidians. *Parasites & Vectors*, 8:383.
- Broeders, B.S., Huber, I., Grohmann, L., Berben, G., Taverniers, I., Mazzara, M., Roosensa, N. & Morisset, D. (2014). Guidelines for validation of qualitative real-time PCR methods. *Trends in Food Science & Technology*, 37:115–126.
- Bustin, S.A. (2005). Real-time, fluorescence-based quantitative PCR: a snapshot of current procedures and preferences. *Expert Review of Molecular Diagnostics*, 5:493–498.
- Bustin, S.S. (2005). Real-Time PCR. *Encyclopedia of Diagnostic Genomics and Proteomics*, 1117-1126.
- Bustin, S.A. (2010). Developments in real-time PCR research and molecular diagnostics. *Expert Review of Molecular Diagnostics*, 10:713–715.

- Bustin, S.A., Benes, V., Garson, J.A., Hellems, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L. & Vandesompele, J. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55:611–622.
- Cai, X., Lei, Z., Fu, W., Zhu, Z., Zou, M., Gao, J., Wang, Y., Hong, M., Wang, J., Li, W. & Xu, D. (2013). Optimisation of reverse transcription loop-mediated isothermal amplification assay for the rapid detection of pandemic (H1N1) 2009 virus. *African Journal of Microbiology Research*, 7:3919–3925.
- Chaisi, M.E., Janssens, M.E., Vermeiren, L., Oosthuizen, M.C., Collins, N.E. & Geysen, D. (2013). Evaluation of a real-time PCR test for the detection and discrimination of *Theileria* species in the African buffalo (*Syncerus caffer*). *PLOS one*, 8: e75827.
- Chen, H-Y., Li, X-K., Cuia, B-A., Wei, Z-Y., Li, X-S., Wang, Y-B., Zhao, L. & Wang, Z-Y. (2009). A TaqMan-based real-time polymerase chain reaction for the detection of porcine parvovirus. *Journal of Virological Methods*, 156:84–88.
- Clausen, P-H., Chuluun, S., Sodnomdarjaa, R., Greiner, M., Noeckler, K., Staak, C., Zessin, K-H. & Schein, E. (2003). A field study to estimate the prevalence of *Trypanosoma equiperdum* in Mongolian horses. *Veterinary Parasitology*, 9–18.
- Clausen, P-H., Gerbeselassie, G., Abditcho, S., Mehlitz., D. & Staak, C. (1999). Detection of trypanosome DNA in serologically positive but aparasitaemic horses suspected of dourine in Ethiopia. *Tokai Journal of Experimental and Clinical Medicine*, 23:303–308.
- de Paiva Cavalcanti, M., de Brito, M.E.F., de Souza, W.V., de Miranda Gomes, Y. & Abath, F.G. (2009). The development of a real-time PCR assay for the quantification of *Leishmania infantum* DNA in canine blood. *The Veterinary Journal*, 182:356–358.
- Deprez, R.H.L., Fijnvandraat, A.C., Ruijter, J.M. & Moorman, A.F. (2002). Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR Green I depends on cDNA synthesis conditions. *Analytical Biochemistry*, 307:63–69.
- Dhanasekaran, S., Doherty, T.M., Kenneth, J. & TB Trials Study Group (2010). Comparison of different standards for real-time PCR-based absolute quantification. *Journal of Immunological Methods*, 354:34–39.

- Dorak, M. T. (2006). Real-time PCR. In: Bios advanced methods series. Taylor & Francis, Oxford.
- Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D.C., Wengenack, N.L., Rosenblatt, J.E., Cockerill, F.III. & Smith, T.F. (2006). Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical Microbiology Reviews*, 19:165–256.
- Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L. & Kubista, M. (2017). Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomolecular Detection and Quantification*, 12:1–6.
- Fraga, D., Meulia, T. & Fenster, S. (2008). Real-time PCR. *Current Protocols Essential Laboratory Techniques*, 10–3.
- Gari, F. R., Ashenafi, H., Tola, A., Goddeeris, B. M. & Claes, F. (2010). Comparative diagnosis of parasitological, serological, and molecular tests in dourine-suspected horses. *Tropical Animal Health and Production*, 42:1649–1654.
- Gizaw, Y., Megersa, M. & Fayera, T. (2017). Dourine: a neglected disease of equids. *Tropical Animal Health and Production*, 49:887–897.
- Hasan, G., Turner, M.J. & Cordingley, J.S. (1984). Complete nucleotide sequence of an unusual mobile element from *Trypanosoma brucei*. *Cell*, 37:333–341.
- Heid, C.A., Stevens, J., Livak, K.J. & Williams, P.M. (1996). Real time quantitative PCR. *Genome Research*, 6(10):986-994.
- Kim, C., Iseki, H., Herbas, M.S., Yokoyama, N., Suzuki, H., Xuan, X., Fujisaki, K. & Igarashi, I. (2007). Development of Taqman-based real-time PCR assays for diagnostic detection of *Babesia bovis* and *Babesia bigemina*. *American Journal Tropical Medicine Hygiene*, 77:837–8.
- Konnai, S., Mekata, H., Mingala, C.N., Abes, N.S., Gutierrez, C.A., Herrera, J.R.V., Dargantes, A.P., Witola, W.H., Cruz, L.C., Inoue, N., Onuma, M. & Ohashi, K. (2009). Development and application of a quantitative real-time PCR for the diagnosis of Surra in water buffaloes. *Infection, Genetics and Evolution*, 9:449–452.

- Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A. & Zoric, N. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine*, 27:95–125.
- Mangold, K.A., Manson, R.U., Koay, E.S., Stephens, L., Regner, M., Thomson, R.B., Peterson, L.R. & Kaul, K.L. (2005). Real-time PCR for detection and identification of *Plasmodium* spp. *Journal of Clinical Microbiology*, 43:2435–2440.
- Mlangeni, M.A. (2016). Molecular epidemiology of dourine, equine piroplasmiasis and ehrlichiosis in equines in South Africa. (MSc Dissertation)
- Moreira, O.C., Ramírez, J.D., Velázquez, E., Melo, M.F.D., Lima-Ferreira, C., Guhl, F., Sosa-Estani, S., Marin-Neto, J.A., Morillo, C.A. & Britto, C., 2013. Towards the establishment of a consensus real-time qPCR to monitor *Trypanosoma cruzi* parasitemia in patients with chronic Chagas disease cardiomyopathy: A substudy from the BENEFIT trial. *Acta Tropica*, 125(1):23-31.
- Mori, Y., Kitao, M., Tomita, N. & Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods*, 59:145–157.
- Ndao, M. (2009). Diagnosis of Parasitic Diseases: Old and New Approaches. *Interdisciplinary Perspectives on Infectious Diseases*, 1–15.
- Njiru, Z.K. (2011). Rapid and sensitive detection of human African trypanosomiasis by loop-mediated isothermal amplification combined with a lateral-flow dipstick. *Diagnostic Microbiology and Infectious Disease*, 69:205–209
- Njiru, Z.K., Mikosza, A.S.J., Matovu, E., Enyaru, J.C.K., Ouma, J.O., Kibona, S.N., Thompson, R.C.A. & Ndung'u, J.M. (2008a). African trypanosomiasis: Sensitive and rapid detection of the sub-genus *Trypanozoon* by loop-mediated isothermal amplification (LAMP) of parasite DNA. *International Journal for Parasitology*, 38:589–599.
- Nolan, T., Hands, R.E. & Bustin, S.A. (2006). Quantification of mRNA using real-time RT-PCR. *Nature Protocols*, 1:1559.
- OIE, (2015). Dourine. *The Center for Food Security and Public Health, Iowa State University, College of Veterinary Medicine*, 1-4.

- Pabinger, S., Rödiger, S., Kriegner, A., Vierlinger, K., & Weinhäusel, A. (2014). A survey of tools for the analysis of quantitative PCR (qPCR) data. *Biomolecular Detection and Quantification*, 1:23–33.
- Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L. & Morita, K. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology*, 18:407–421.
- Pascucci, I., Di provvido, A., Cammà, C., Di Francesco, G., Calistri, P., Tittarelli, M., Ferri, N., Scacchia, M. & Caporale, N. (2013). Diagnosis of dourine in outbreaks in Italy. *Veterinary Parasitology*, 193:30–38.
- Piron, M., Fisa, R., Casamitjana, N., López-Chejade, P., Puig, L., Vergés, M., Gascón, J., i Prat, J.G., Portús, M. & Sauleda, S. (2007). Development of a real-time PCR assay for *Trypanosoma cruzi* detection in blood samples. *Acta Tropica*, 103:195–200.
- Qvarnstrom, Y., Schijman, A.G., Veron, V., Aznar, C., Steurer, F. & Da Silva, A.J. (2012). Sensitive and specific detection of *Trypanosoma cruzi* DNA in clinical specimens using a multi-target real-time PCR approach. *PLoS Neglected Tropical Diseases*, 6: e1689.
- Rebrikov, D.V. & Trofimov, D.Y. (2006). Real-time PCR: a review of approaches to data analysis. *Applied Biochemistry and Microbiology*, 42:455–463.
- Rutledge, R.G. & Côté, C. (2003). Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Research*, 31: e93–e93.
- Shahsiah, R., Abdollahi, A., Ardalan, F.A., Haghi-Ashtiani, M.T., Jahanzad, I. & Toosi, M. N. (2010). Result variation and efficiency kinetics in real-time PCR. *Acta Medica Iranica*, 279–282.
- Sivaganesan, M., Haugland, R.A., Chern, E.C. & Shanks, O.C. (2010). Improved strategies and optimization of calibration models for real-time PCR absolute quantification. *Water Research*, 44:4726–4735.
- Tavares, R.G., Staggermeier, R., Borges, A.L.P., Rodrigues, M.T., Castelan, L.A., Vasconcelos, J., Anschau, M.E. & Spalding, S.M. (2011). Molecular techniques for the study and diagnosis of parasite infection. *The Journal of Venomous Animals and Toxins including Tropical Diseases*, 37:239–248.

Zhao, S. & Fernald, R.D. (2005). Comprehensive algorithm for quantitative real-time polymerase chain reaction. *Journal of Computational Biology*, 12:1047–1064.

CHAPTER 5 LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR DETECTION OF *TRYPANOSOMA EQUIPERDUM* INFECTIONS IN SOUTH AFRICAN EQUIDS

5.1 Introduction

5.1.1 Loop-mediated isothermal amplification (LAMP) assay

Loop-mediated isothermal amplification (LAMP) was established by Notomi and colleagues which allows a whole reaction process to take place at an isothermal condition (Notomi *et al.*, 2000; Njiru *et al.*, 2008b; Cai *et al.*, 2013; Wang *et al.*, 2015) making it possible to easily detect the LAMP-amplified products due to a visual confirmation of the reacted tube (Iseki *et al.*, 2007). The technique is a simple recognized nucleic acid amplification method offering rapid, accurate, and a cost-effective diagnosis of infectious diseases (Notomi *et al.*, 2000; Iseki *et al.*, 2007; Liu *et al.*, 2008; Njiru *et al.*, 2008a; Mori & Notomi, 2009; Wang *et al.*, 2014; Mandal *et al.*, 2015). The approach is vigorous, specific and tolerant to several biological products that constrain conventional PCR (Njiru *et al.*, 2010). It amplifies DNA with high sensitivity and rapidity under isothermal conditions (Notomi *et al.*, 2000; Alhassan *et al.*, 2007; Iseki *et al.*, 2007; Njiru *et al.*, 2008b).

The technique uses four (Mori *et al.*, 2004; Thekisoie *et al.*, 2005; Adams *et al.*, 2008; Njiru *et al.*, 2008b; Tomita *et al.*, 2008; Thekisoie *et al.*, 2009; Thekisoie *et al.*, 2010; Abdullahi *et al.*, 2015; Tong *et al.*, 2015) to six primers that identify six to eight regions of the target DNA (Iwamoto *et al.*, 2003; Thekisoie *et al.*, 2005; Adams *et al.*, 2008; Njiru *et al.*, 2008b; Parida *et al.*, 2008; Abdullahi *et al.*, 2015), in combination with the enzyme *Bst* polymerase, which has strand displacement activity (Thekisoie *et al.*, 2005; Adams *et al.*, 2008; Njiru *et al.*, 2008b; Salih *et al.*, 2014; Abdullahi *et al.*, 2015). The inclusion of Loop primers produces huge amounts of DNA in short period (Dhama *et al.*, 2017) and thereby enhancing LAMP efficiency and rapidity (Njiru *et al.*, 2008a; Salih *et al.*, 2014). LAMP results can be visually inspected through colour change by naked eyes (Njiru *et al.*, 2008b; Thekisoie *et al.*, 2009; Tong *et al.*, 2015) and/or in real time turbidity (Thekisoie *et al.*, 2005; Liu *et al.*, 2008; Njiru *et al.*, 2008b), which significantly decreases the assay time (Njiru *et al.*, 2008b). Additionally, LAMP products can be visualized with the use of Loopamp fluorescent (by direct fluorescence) detection reagent at the start of reaction

(Thekiso *et al.*, 2010). Other fluorescent dyes, for example, SYBR Green I and Evagreen have additionally been utilized for the visualisation of LAMP products under UV light (Parida *et al.*, 2008; Thekiso *et al.*, 2010; Salih *et al.*, 2014; Abdullahi *et al.*, 2015; Dhama *et al.*, 2017). The auto-cycling reaction also produces large amounts of by products, pyrophosphate ions, leading to a white precipitate of magnesium pyrophosphate in the reaction mixture (Nagamine *et al.*, 2002; Parida *et al.*, 2008; Tomita *et al.*, 2008; Njiru *et al.*, 2012; Ngotho *et al.*, 2015). The presence of these precipitates confirms that DNA was amplified for the desired LAMP test (Nagamine *et al.*, 2002; Thekiso *et al.*, 2007). The main advantage of LAMP over PCR, is that it does not require complicated thermal cycles (Thekiso *et al.*, 2009) enhancing the amplification of samples within an hour in a laboratory heat block or water bath (Thekiso *et al.*, 2005; Thekiso *et al.*, 2007; Thekiso *et al.*, 2009; Cai *et al.*, 2013). The amplified products of LAMP can also be analysed by agarose gel electrophoresis, however, the procedure requires opening of the reaction tubes which could produce aerosol contamination resulting from a very large amount of amplified DNA (Cai *et al.*, 2013).

This novel procedure for gene amplification depends on the auto-cycling strand displacement synthesis of target DNA by *Bst* DNA polymerase (Kuboki *et al.*, 2003; Thekiso *et al.*, 2007; Liu *et al.*, 2008; Parida *et al.*, 2008; Thekiso *et al.*, 2009; Njiru *et al.*, 2010; Tong *et al.*, 2015) which synthesizes a new strands of DNA while simultaneously displacing the former complementary strand (Tong *et al.*, 2015), thus allowing DNA amplification under the optimum temperatures ranging from 60-65°C (Kuboki *et al.*, 2003; Liu *et al.*, 2008; Parida *et al.*, 2008; Thekiso *et al.*, 2009; Njiru *et al.*, 2010; Tong *et al.*, 2015). Most importantly, it is a one-step amplification reaction which does not require a denatured DNA template (Nagamine *et al.*, 2002). It is a one-step amplification reaction to produces large amounts of DNA (Nagamine *et al.*, 2002; Thekiso *et al.*, 2005; Njiru *et al.*, 2008b; Parida *et al.*, 2008; Mori & Notomi, 2009; Tomita *et al.*, 2008; Zende *et al.*, 2017). In this reaction, inner primers (FIP and BIP) provide amplification with a specificity that is greater than that observed in other methods while the outer primers (F3 and B3) are designed specially to recognize separate regions within a target DNA (Parida *et al.*, 2008; Mori & Notomi, 2009; Njiru, 2012; Abdullahi *et al.*, 2015; Notomi *et al.*, 2015). The use of six primer sets, which includes two loop primers increases the specificity, efficiency and speed of amplification (Njiru *et al.*, 2008a; Salih *et al.*, 2014), thus shortening the amplification time to about 30 minutes. For the amplification to begin,

all primers used in a reaction must have specific sequences to that of the target gene, this is a strict principle of LAMP (Abdullahi *et al.*, 2015).

5.1.2 Mechanism and expected reaction steps of LAMP

The mechanism of the LAMP reaction is based on the set of four to six target-specific primers (figure 5.1) that are specially designed to recognize six different sites which surround the amplified DNA (F1c, F2c, F3c) and (B1, B2 and B3) on 3' and 5' sides respectively (Tomita *et al.*, 2008). LAMP amplification involves two basic phases, non-cyclical and cyclical phase (Parida *et al.*, 2008; Abdullahi *et al.*, 2015). Non-cyclical step is the first phase of LAMP reaction, which involves all the four primers, FIP, BIP, F3 and B3, as well as the *Bst* DNA polymerase enzyme with a strand displacement activity. However, the cyclical step builds upon the product of the noncyclical which only involves the two outer primers (F3 and B3) and the *Bst* DNA polymerase enzyme. Loop primers might be involved in the cyclical step when six primers are used (Abdullahi *et al.*, 2015).

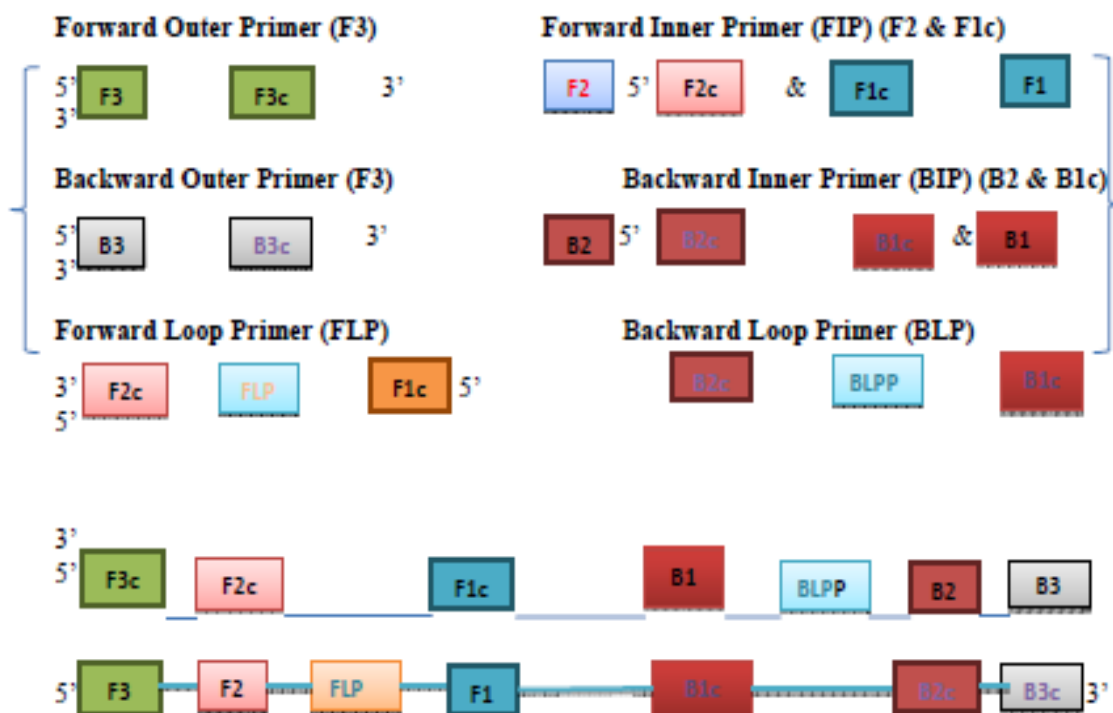


Figure 5.1: LAMP hybridizing to specific sites of target gene (Abdullahi *et al.*, 2015)

Forward inner primer hybridizes to F2c in the target DNA and this initiates the complementary strand synthesis. Outer primer (F3) hybridizes to F3c slowly in the target DNA and initiates the strand displacement DNA synthesis, releasing an FIP-linked complimentary strand, which forms a looped out structure at one end and this single-stranded DNA serves as template for BIP initiated DNA synthesis (Notomi *et al.*, 2000).

The Backward Inner Primer (BIP) now uses the displaced single-stranded DNA with a dumb bell shape at the 5' end as a template, it anneals to a complementary sequence at the B2c region of this new DNA strand and subsequently extended by the Bst DNA polymerase as seen in the first amplification involving the FIP. Following elongation of the DNA strand with the aid of the enzyme, the backward outer primer (B3) anneals to the B3c outside the BIP to initiate displacement of the DNA, resulting in the formation of a dumb bell shaped DNA at the 3' end. The non-cycling step ends with formation of DNA with stem-loops at both ends ready to go into cyclical step (Abdullahi *et al.*, 2015) the second stage of LAMP reaction (Notomi *et al.*, 2000). The cyclical stage builds upon the product of the non-cyclical step. This phase basically involves hybridization of the internal primers to the stem loop product from the non-cyclical step to initiate displacement DNA synthesis (Abdullahi *et al.*, 2015).

The FIP hybridizes to the loop in the stem-loop DNA to initiate the LAMP cycling and primer strand displacement DNA synthesis, generating as an intermediate one gapped stem-loop DNA. Subsequently self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA (Notomi *et al.*, 2000). This stage of amplification results in the production of stem looped DNA with a stem twice as long as the template stem looped DNA. Eventually the final products of this stage are stem loop DNA with several repeats of the targets and cauliflower like structure (Abdullahi *et al.*, 2015) (figure 5.2).

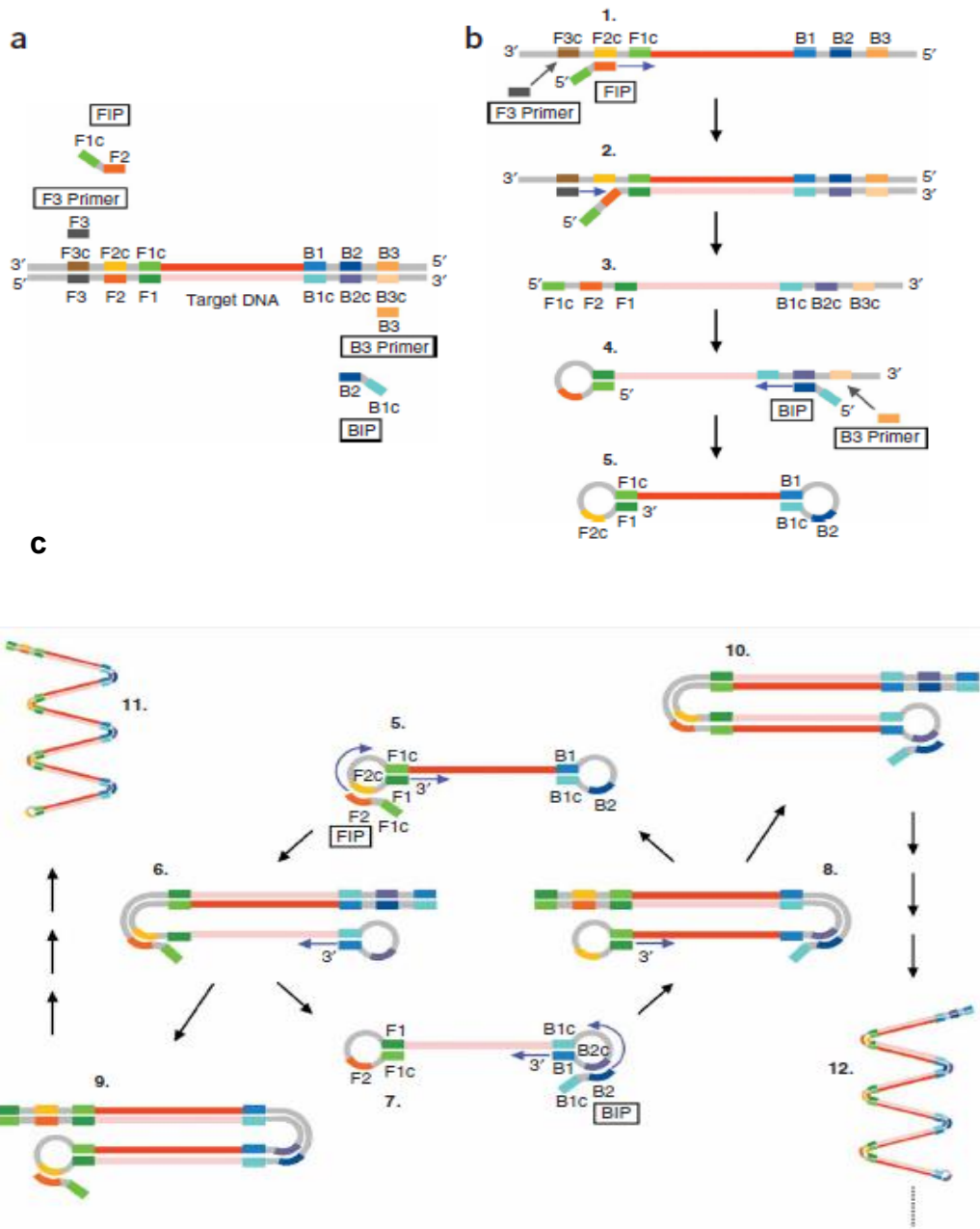


Figure 5.2: Principle of loop-mediated isothermal amplification (LAMP) method. (a) Primer design (b) starting structure producing step and (c) cycling amplification step (Parida *et al.*, 2008; Tomita *et al.*, 2008).

5.1.3 LAMP detection for trypanosomes

A simple and easy test to perform, LAMP (Thekisoie *et al.*, 2005), has effectively been established and useful in diagnosis of various pathogens including African trypanosomes (Thekisoie *et al.*, 2007). Njiru *et al.*, (2008b) stated that Kuboki *et al.*, demonstrated the potential of PFRA LAMP based, a single copy target gene for the detection of several species of *T. brucei*, Thekisoie *et al.*, (2007) have also reported LAMP tests for other species of trypanosomes such as *T. evansi*, *T. vivax*, *T. congolense* and *T. brucei gambiense*. In addition, there are also other experimental reports on the subgenus *Trypanozoon* LAMP tests (Njiru *et al.*, 2017) except for *T. equiperdum*. However, it is challenging to get a specific marker for the *Trypanozoon* species, since they are genetically indistinguishable. A conserved sequence in the repetitive insertion mobile element (RIME) gene of the subgenus *Trypanozoon*, was identified and used to develop a highly specific and sensitive LAMP amplification test for the subgenus *Trypanozoon*. This test was used to analyse *Trypanozoon* isolates and clinical samples from HAT patients excluding *T. equiperdum*. Therefore, there is a need to develop tests that will specifically detect *T. equiperdum* infections in equines.

5.2 Aim of the study

To develop loop-mediated isothermal amplification (LAMP) assay for a rapid diagnosis of *T. equiperdum* infections in South African equids.

5.3 Materials and Methods

5.3.1 Loop-mediated isothermal amplification (LAMP) assay

5.3.1.1 Design of LAMP primers

Target gene ribosomal mobile element (RIME) rRNA gene (Figure 5.3) for the subgenus *Trypanozoon* were identified from published literature on conventional PCR. The gene sequence was downloaded and LAMP primers sets were designed using Optigene LAMP primer software from Optigene Company UK website. A set of four LAMP primers (table 5.1) recognizing six specific regions of *Trypanozoon* species on RIME gene were designed. Two additional loop primers (forward and backward) were also designed (table 5.1). Optimization reactions were conducted to determine the optimal reaction temperature and optimal time for the reaction. Thereafter specificity and detection limit of LAMP assays were determined. The best assay was validated by the use in epidemiological studies of dourine from field derived samples. Samples were considered positive when the amplification and melt curves ranged within same temperature and time with the positive control and showed same dissociation curve (melt curve) similar to positive control. All reactions were conducted in triplicates and repeated three times.



Figure 5.3: Forward primer, reverse primer, FIP and BIP primers design based on 1402 bp RIME genomic sequence, GenBank accession number K01801.1 (Hasan *et al.*, 1984).

5.3.1.2 Detection of LAMP product

LAMP was carried out in total of 25µl reaction mixture containing 15 µl isothermal master mix (Optigene.co.uk) with 5µl primer mix (table 5.1) [(FIP and BIP 20µl each), (F3 and B3 5µl each) and (LF and LB 10µl)] and the specified amounts of double-stranded target DNA (2 µl). Final volume was adjusted to 25 µl with 3 µl of double distilled water (DDW). The LAMP test was carried out for 60 min at 65°C in a Genie II machine (OptiGene, Horsham, UK). Amplification of DNA in the LAMP reactions were confirmed through acquisition of melt curves and through electrophoresis in 1.0% agarose gels stained with ethidium bromide.

Table 5.1. RIME 2 LAMP primers

Name	Primer	Amplicon size
F3	CCTGGACTCTCCCAAAGA	169bp
B3	TCCAGTACCCCGTATCATC	
FIP(F1c+F2)	TTTCATTTGTCGGTCCCTGGGCGGACAGCAGCATCTTG	
BIP(B1c+B2)	AGCAAGACGCTGCTGATCCAGCAGCGTTCCTTTCATG	
LF	TGCCATCAGCAGTATCATCC	
LB	CTGTGCTGTATGATGCCGTA	

5.3.1.3 Sensitivity and specificity of LAMP

To determine the analytical sensitivity of the LAMP test, 10-fold dilutions were made from ~10 ng of DNA purified from *T. equiperdum* isolate (figure 5.4). The assay was carried out using *T. equiperdum* DNA. The LAMP test was compared with PCR tests specific for the subgenus *Trypanozoon* (Njiru *et al.*, 2008). The reactions were done in triplicates. The LAMP test was carried out using Gene II LAMP machine. The number of parasites were calculated as one trypanosome is equivalent to = 0.1 pg while ten trypanosomes were equivalent to = 1pg (Njiru *et al.*, 2005)

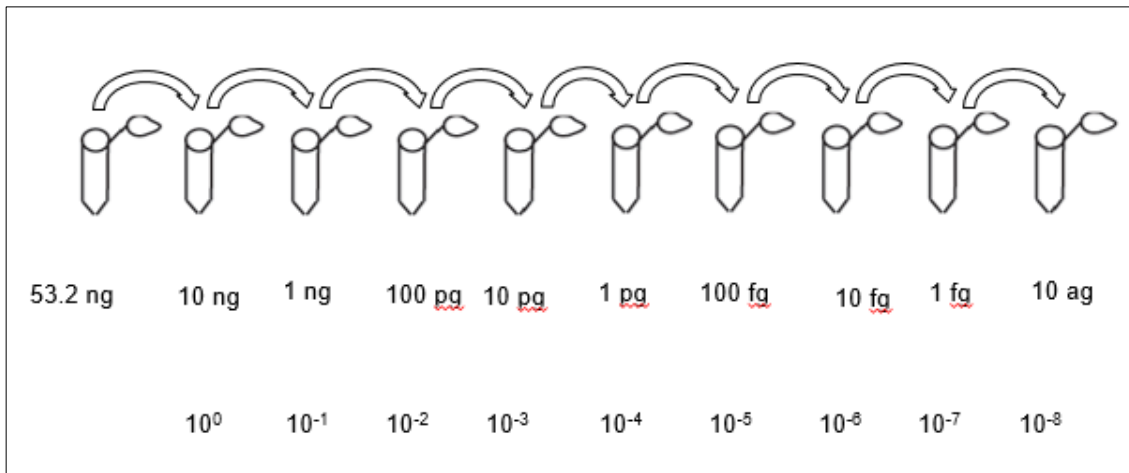


Figure 5.4: A series of ten-fold dilutions of *T. equiperdum* DNA

The specificity of the tests was assessed with DNA from trypanosomes belonging to subgenus *Trypanozoon* (*T. b. brucei*, *T. equiperdum* and *T. evansi*) and with other DNA's belonging to other species including the Horse DNA (HDNA) and double distilled water (DDW). Since the outer primer pair, designated F3 and B3, can also be used for PCR, the same target gene was amplified from serially diluted total trypanosome DNA by both LAMP and standard PCR, and the sensitivities of the two methods were compared.

The sensitivity and specificity of the tests were determined as follows (TP is true positive, TN represents true negative, FN is false negative and FP is false positive): (A) Sensitivity = $TP/(TP + FN) \times 100$; (B) Specificity = $TN/(TN + FP) \times 100$ (Chaouch *et al.*, 2013; Njiru *et al.*, 2017).

5.3.1.4 Comparison of LAMP reaction assay with PCR assay

PCR was conducted using B3 and F3 RIME primers designed in this study. Forty-eight DNA samples from the previous study were used, of which 24 were negative and 24 were

positive, with RIME2 LAMP primers. The sensitivity of the tests was compared. Samples were considered positive when the amplification of a fragment corresponds to the expected amplicon size (250 bp) visualised under UV light.

5.3.1.5 Agarose gel electrophoresis

Loop-mediated isothermal amplification reactions were confirmed using a 1.0% agarose gel in 1 x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, at pH 8.0) stained with 1 µg/ml ethidium bromide then visualised under UV light. Five microliters of the LAMP product and 1 µl of 6x blue Loading Dye (Fermentas Life Sciences, US) were mixed, and loaded into wells. A 5 µl of 100 bp molecular weight marker (O'GeneRuler, Fermentas Life Sciences, US) was used to confirm the size of the amplification products, contamination, and to differentiate between true positive and false positive. Electrophoresis was performed for 30 minutes at 100 V using a mini-sub cell GT electrophoreses system (Bio-Rad, UK). Gel images were captured using Gene Genius Bio Imaging System (Syngene, Synoptics, UK) GeneSnap (version 6.00.22) software.

5.4 Statistical analysis

Statistical significance of sensitivity and specificity of test and between the tests were evaluated using the Chi-square. The significance of the differences between the frequencies of positive results obtained in the tests was measured by Fisher's exact test. The agreement between the tests was expressed using the kappa index as described by Kramer and Feinstein (1981). Analysis was performed with the aid of SPSS software version 19 and by also using GraphPad Prism.

5.5 Results

5.5.1 Development of loop-mediated isothermal amplification (LAMP) assay

To develop a new technique (LAMP), the procedure was optimized with regard to the concentrations of primers and the denature/extension temperature. Primer sets were designed for amplification of the species of the sub-genus *Trypanozoon*, primers were then optimized using different temperatures and time ranging from 60-65°C for 30-60min, to find optimal temperature and time (Data not shown). Sixty-five (65°C) reaction temperature for 60 min was chosen as the optimal reaction temperature and time for all the RIME 2 LAMP applications (figure 5.5). The melt-curves were also used for analysis of the results (figure 5.6). Lastly the agarose gel electrophoresis analyses were in agreement with real-time LAMP and melt-curve analyses (figure 5.5-5.7). All positive RIME 2 LAMP reactions produced a characteristic ladder of multiple bands on an agarose gel (figure 5.7). Table 5.2 below shows the temperatures where the melting peaks occurred for optimization. There was also a false positive amplification in the negative control (DDW).

Table 5.2: Temperatures of melting peaks for optimization

Well	Name	Peaks
1	DDW	-
2	DDW	-
3	DDW	-
4	<i>T. equiperdum</i>	89.5°C
5	<i>T. equiperdum</i>	89.5°C
6	<i>T. equiperdum</i>	89.4°C

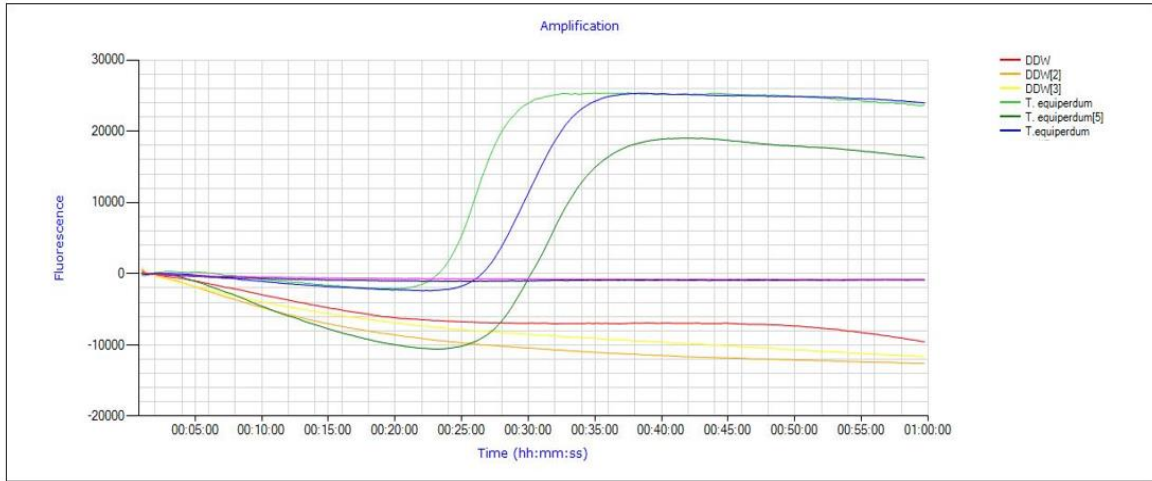


Figure 5.5: Results for optimization, when the temperature was set at 65°C for 1hr. Produced positive responses.

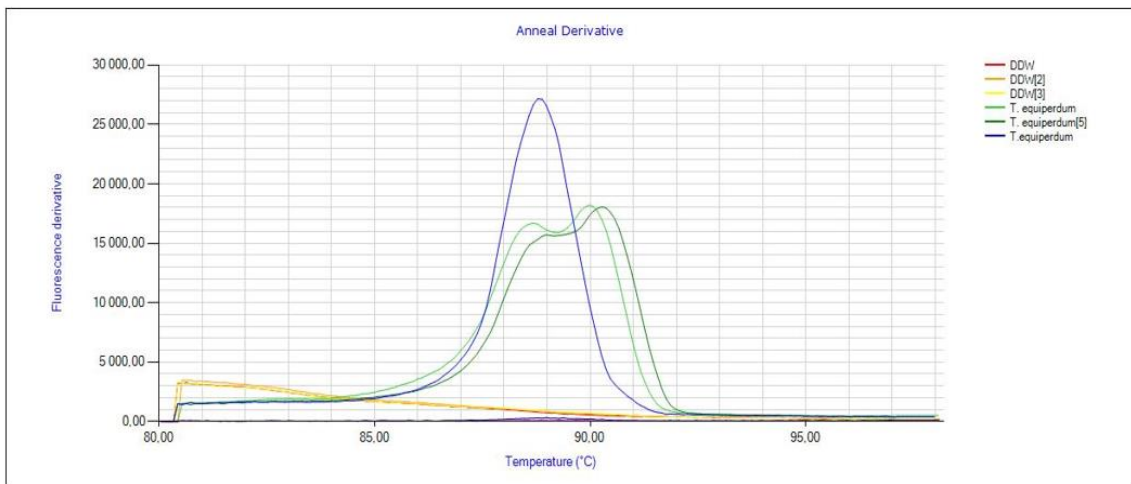


Figure 5.6: Annealing curves for the amplified *T. equiperdum* in triplicates at ~88.8°C

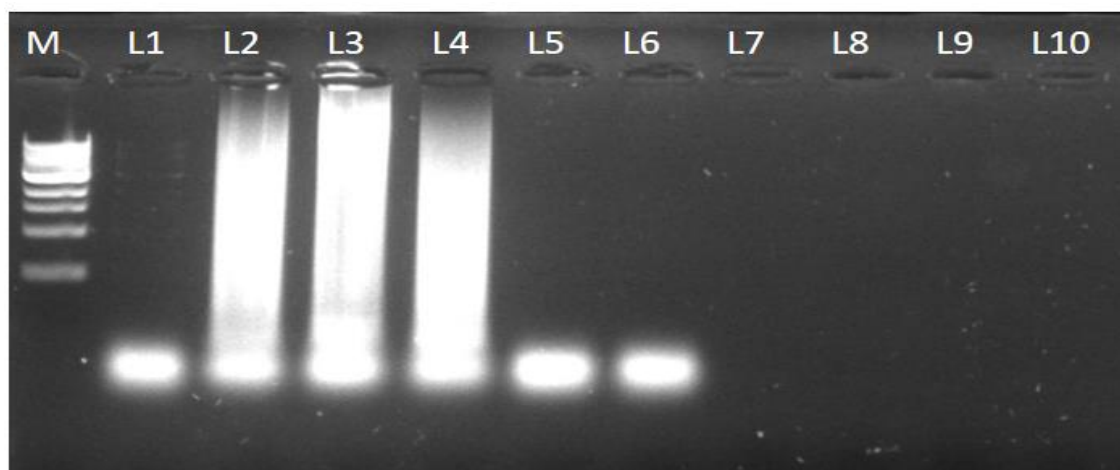


Figure 5.7: Agarose gel electrophoresis showing optimization results of RIME2 LAMP primers using *T. equiperdum* DNA. **M:** DNA 1kb; L1: DDW, L2, L3 & L4 *T. equiperdum*, L5 & L6 DDW.

5.5.1.1 The specificity of RIME2 LAMP assay

No amplification was recorded with DNA of other protozoan parasites (other than *Trypanozoon* spp) including the non-target H-DNA and DDW. The RIME2 LAMP assay was specific and the amplicons showed reproducible melt curves with a T_m ~89.5°C. All the positive LAMP reactions produced a characteristic ladder of multiple bands on an agarose gel. There was also a false positive amplification in the no template control, results are shown in figure (5.8 5.10) and in the table 5.3 below.

Table 5.3. Temperatures of melting peaks for specificity

Well	Name	Peaks (Temp °C)
1	<i>T. equiperdum</i>	89.5
4	<i>T. brucei brucei</i>	89.5
7	<i>T. evansi</i>	89.7
10	H-DNA	
14	DDW	88.3

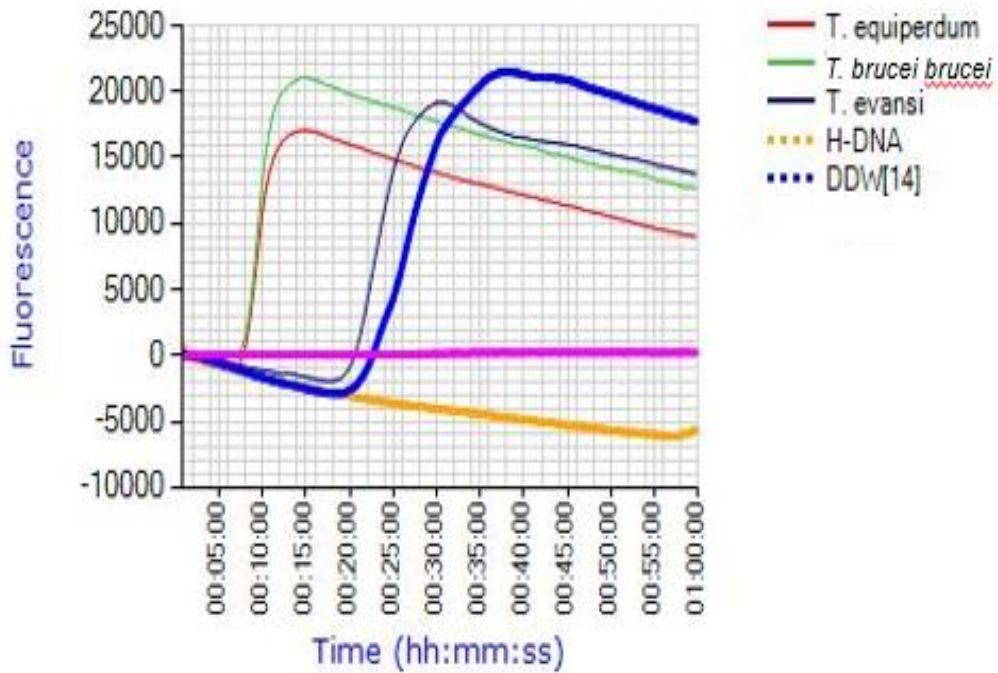


Figure 5.8: Specificity amplification at 65°C for 1 hr. The RIME2 LAMP assay was specific; no reactivity was recorded with non-target DNA (H-DNA) & DDW.

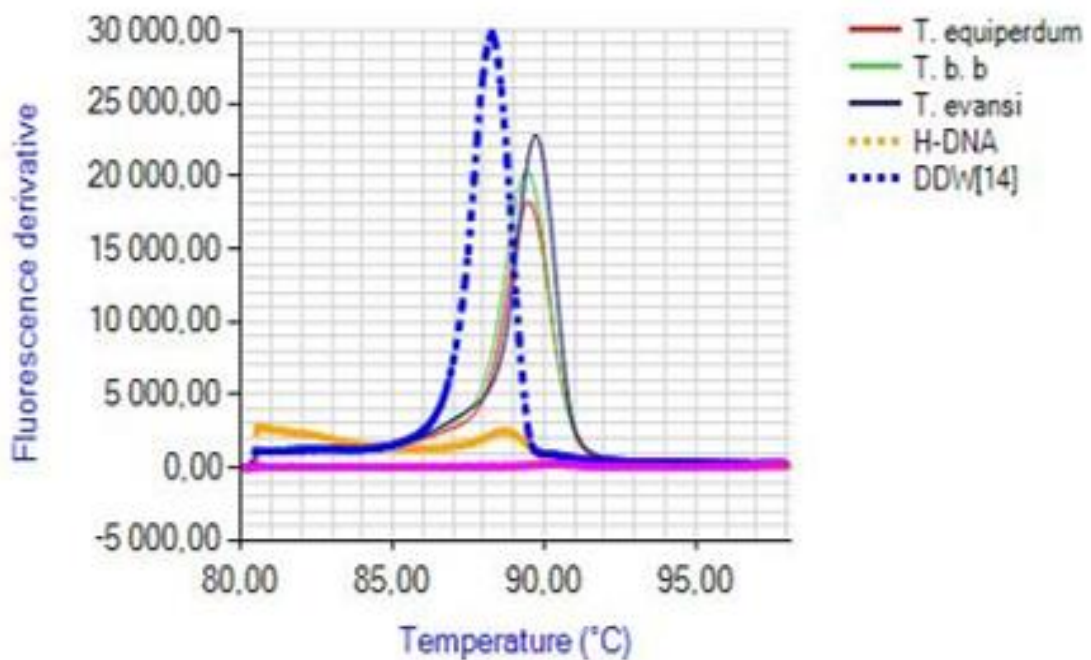


Figure 5.9: Specificity annealing curves of the *Trypanosoma equiperdum*, *T. brucei brucei* and *T. evansi* produced a single peak, at 89.5°C, indicating similar amplicons.

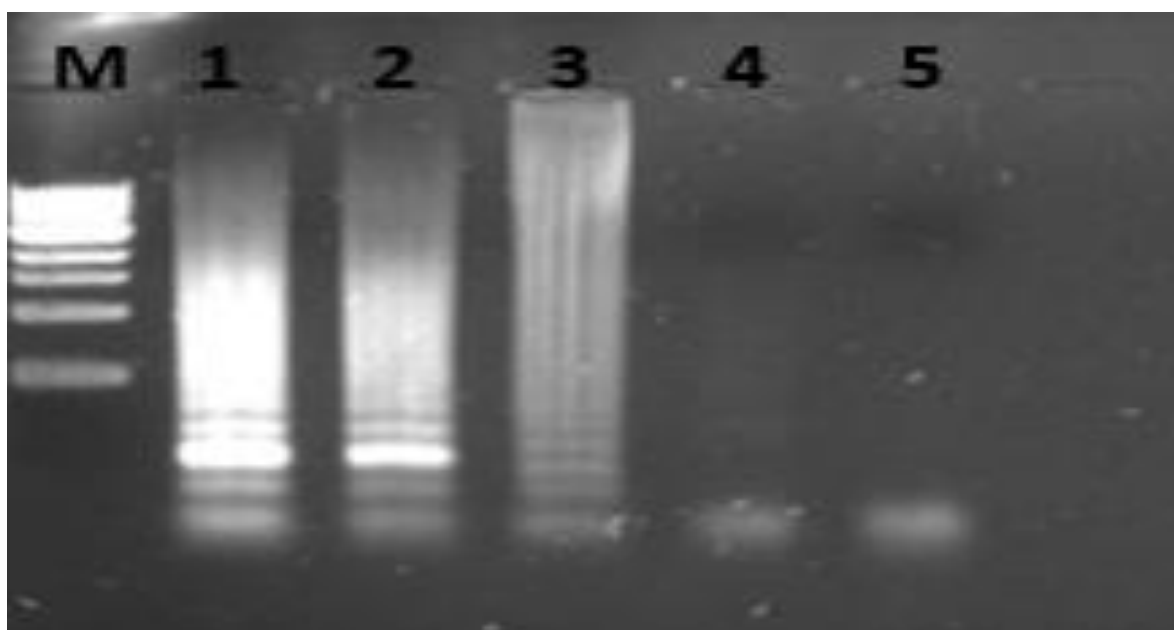


Figure 5.10: Gel agarose electrophoresis showing results of specificity of RIME2 LAMP primers **M:** DNA ladder (1kb), L1: *T. equiperdum*, L2: *T. brucei brucei*, L3: *T. evansi*, L4: H-DNA & L5: DDW.

5.5.1.2 The sensitivity of RIME2 LAMP and conventional PCR

The analytical sensitivity of RIME2 LAMP using various concentrations of *T. equiperdum* genomic DNA ranging from 10 ng to ag (0.00000001). The RIME2 LAMP had a detection limit of 1 fg genomic DNA which is equivalent to 0.001 trypanosomes/ml. Results are shown in figure (5.11– 5.13) and the table 5.4 below.

Table 5.4. Temperatures of melting peaks for RIME2 LAMP sensitivity

Well	Name	Peaks (Temp °C)
1	10 ⁻¹	89.5
2	10 ⁻²	89.5
3	10 ⁻³	89.4
4	10 ⁻⁴	89.5
5	10 ⁻⁵	89.5
6	10 ⁻⁶	89.4
7	10 ⁻⁷	89.5
8	10 ⁻⁸	89.6

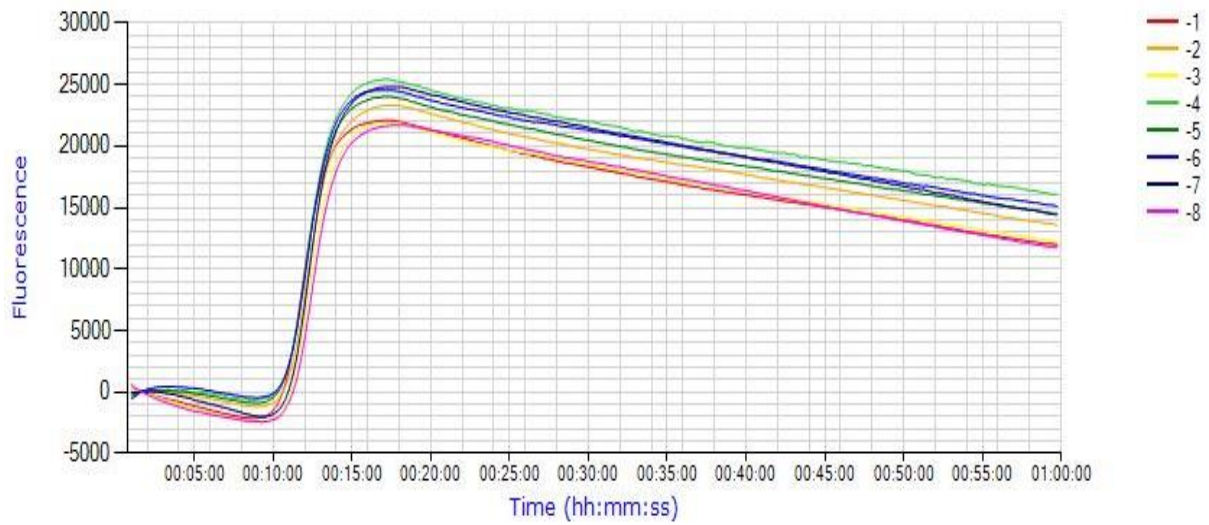


Figure 5.11: RIME 2 LAMP sensitivity 10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} at 65°C for 1 hr. Amplification had a detection limit of 10^{-7} which is equivalent to 0.001 number of trypanosomes/ml.

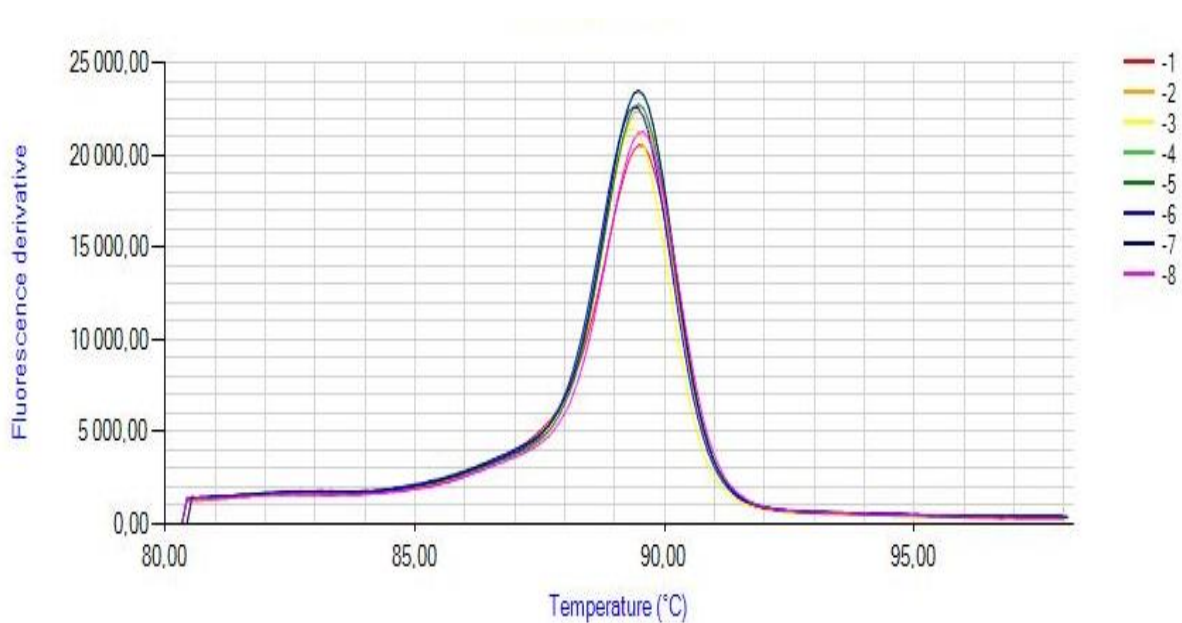


Figure 5.12: RIME2 LAMP sensitivity annealing peaks generated in the real time LAMP assay for the amplified target resulting from different concentrations.

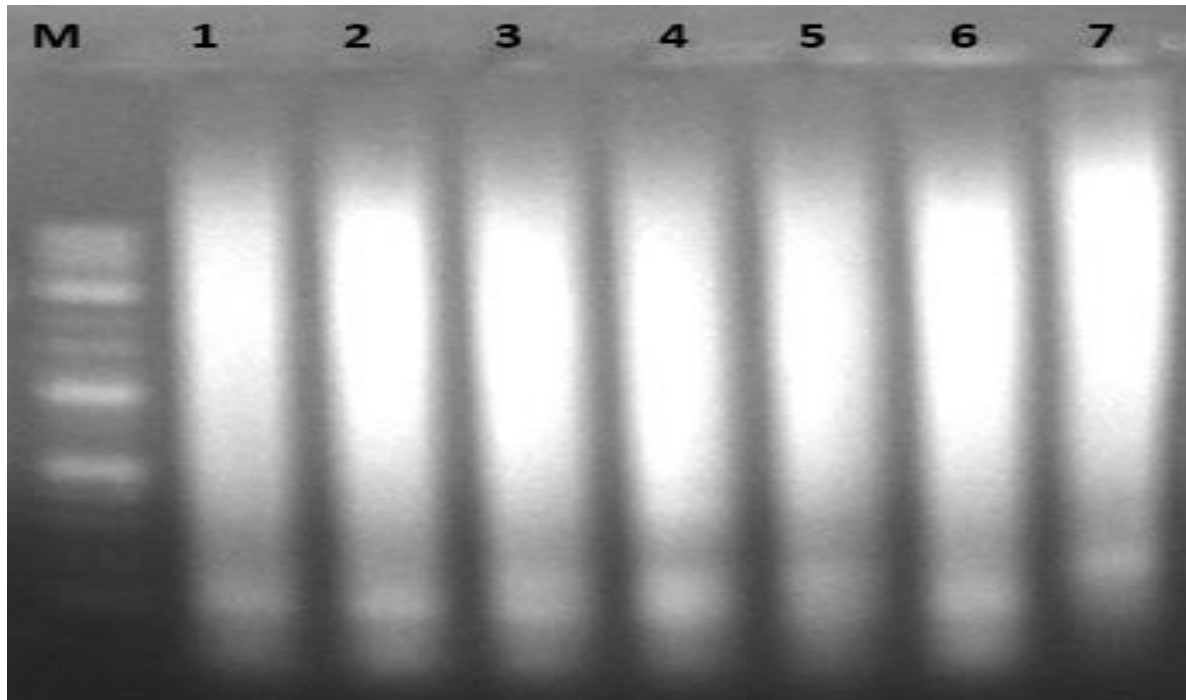


Figure 5.13: Gel agarose electrophoresis showing results of sensitivity of RIME2 LAMP primers **M:** DNA ladder (1kb), L1:10⁻¹, L2: 10⁻², L3: 10⁻³, L4: 10⁻⁴, L5: 10⁻⁵, L6: 10⁻⁶ & L7: 10⁻⁷

5.5.1.3 Field samples screened using RIME2 LAMP primers

Exponential amplification (figure 5.14) and melting curves showing positive LAMP reactions were observed as visualised in the LAMP real-time machine (figure 5.15) and also confirmed with gel electrophoresis (figure 5.16). A total of 48 serology positive DNA samples from the previous studies were screened for the presence of *T. equiperdum*, Free State (n = 12), Mpumalanga (n = 12), Northern Cape (n = 12), and North West (n = 12). The RIME 2 LAMP assay detected 34/48 (70.8%) samples for *T. equiperdum* infections across the four sampled provinces. Out of 48 samples, 10 samples were from donkeys and 38 from horses. Eight donkey samples were positive, among these positives one of the negative samples from the previous studies was positive. Twenty-six (26) samples were positive from horses (figure 5.17). Horses obtained infection rate of 68.4% while donkeys had 80.0%. Mpumalanga province showed higher prevalence (91.7%) amongst all screened provinces, followed by FS (83.3%), NC (66.6%) and NW (41.7%) with the lowest prevalence (table 5.5).

Table 5.5. Results of overall prevalence for Donkey and Horse DNA samples screened using RIME2 LAMP assay

Province	LAMP (+ve)		Total LAMP+ve(%+ve)	Horses		Donkeys	
	Horses	Donkeys		No. of samples	LAMP+ve(%+ve)	No. of samples	LAMP +ve(%+ve)
Free State (N=12)	8	2	10 (83.3)	10	8(80.0)	2	2(100.0)
Mpumalanga (N=12)	11	NDSC	11 (91.7)	12	11(91.7)	NDSC	-
Northern Cape (N=12)	4	4	8 (66.7)	8	4(50.0)	4	4(100.0)
North West (N=12)	3	2	5 (41.7)	8	3(37.5)	4	2(50.0)
Total = 48	26	8	34	38	26(68.4)	10	8(80.0)

NDSC: No donkey samples collected

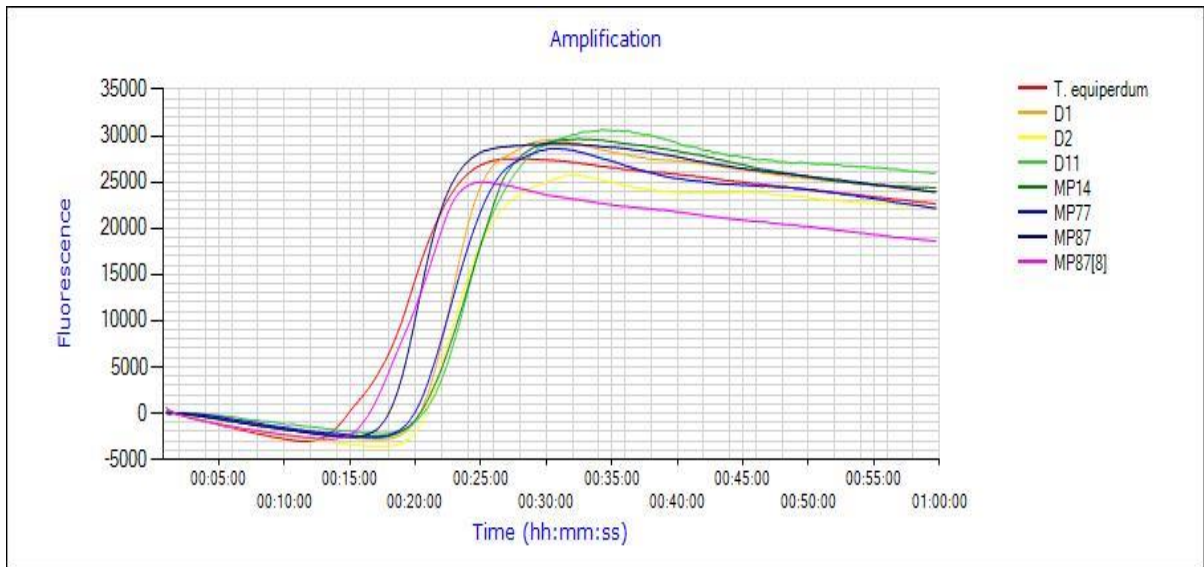


Figure 5.14: RIME 2 LAMP Amplification plot at 65°C for 1 hr when field samples were screened. *Trypanosoma equiperdum* (positive control), D1 & D2 (donkey samples from North-West), MP (14, 77 & 87 horse samples from Mpumalanga).

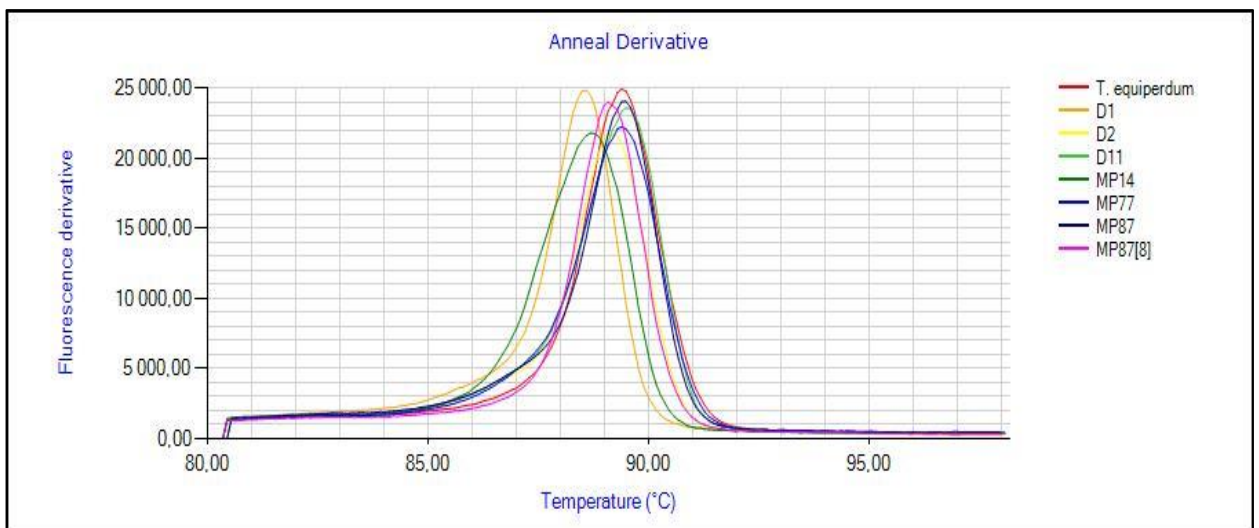


Figure 5.15: Annealing (melt) curves of the field samples, some samples produced a single peak with *Trypanosoma equiperdum* at 89.5°C, indicating similar amplicons.

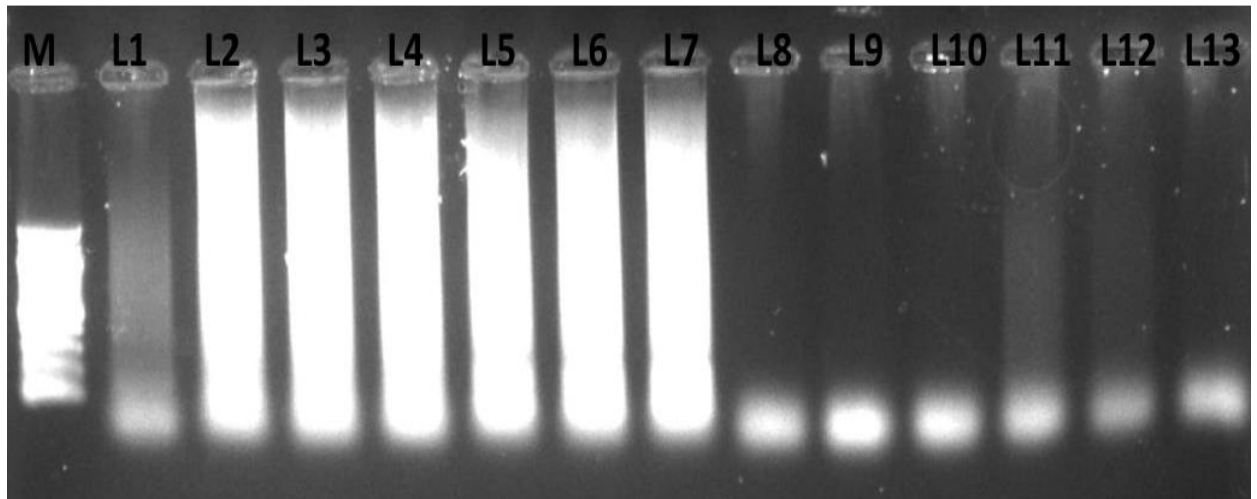


Figure 5.16: Gel agarose electrophoresis showing results of RIME2 LAMP primers **M:** DNA ladder (1kb), L1: DDW (negative control), L2: *T. equiperdum* (positive control), L3: D1, L4: D10, L5: D11, L6: MP 77 & L7: MP87 donkey and horse samples from the sampled provinces.

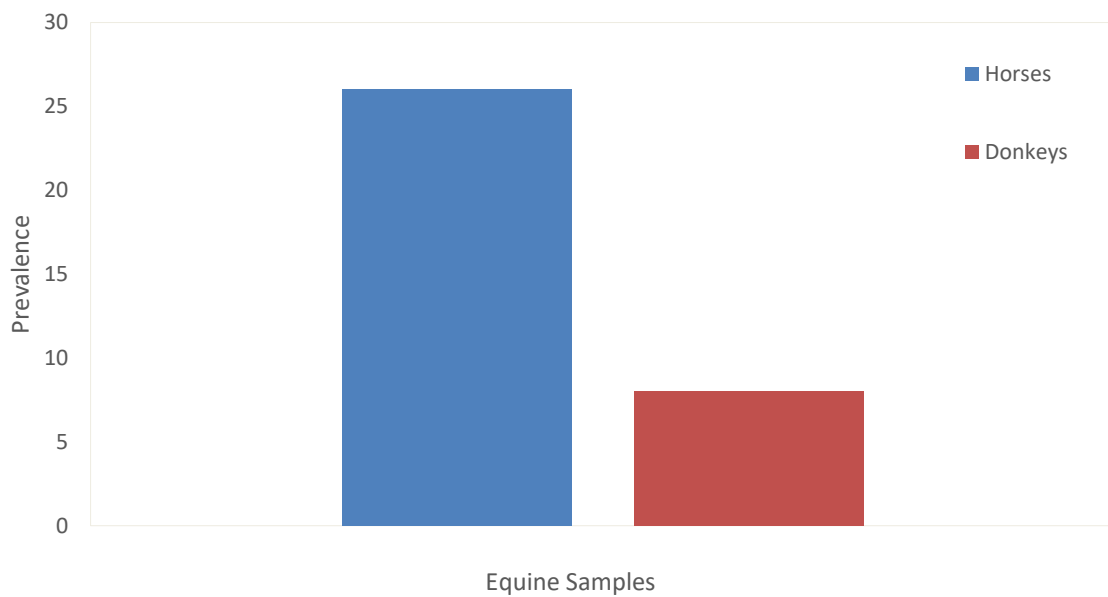


Figure 5.17: The overall *Trypanosoma equiperdum* positives obtained using RIME 2 LAMP primers for all tested horse and donkey samples was 26 and 8 respectively across all the sampled provinces.

5.5.1.4 Comparison between RIME 2 LAMP and conPCR assays

Out of 48 screened equine samples from the 4 sampled provinces, 14(29.2%) were negative while 34 (70.8%) samples were positive by RIME 2 LAMP assay. In contrast, 18 (37.5%) samples were negative while 30 (62.5%) were positive by conPCR (table 5.6). Mpumalanga province obtained the highest prevalence of (91.7%), followed by Free State (83.3%), then Northern Cape (66.7%) and North-West with the least prevalence (41.7%) for LAMP, whilst for PCR, Mpumalanga and Free State provinces obtained highest prevalence of (83.3%), followed by Northern Cape (58.3) and North West (25.0%).

Table 5.6. RIME 2 LAMP and conPCR amplification results for *T. equiperdum* infections across the sampled Provinces (p<0.04)

Province	LAMP		PCR	
	Positive(%+ve)	Negative	Positive(+ve)	Neg
Free State (N= 12)	10(83.3)	2	10(83.3)	2
Mpumalanga (N = 12)	11(91.7)	1	10(83.3)	2
Northern Cape (N =12)	8(66.7)	4	7(58.3)	5
North West (N = 12)	5(41.7)	7	3(25.0)	9
Total = 48	34(70.8)	14	30(62.5)	18

5.6 Statistical analysis

The RIME2 LAMP detected 34/48 (70.8%) from the field samples, while conventional PCR detected 30 out of 48 (62.5%) samples (table 5.7). The test performed on RIME 2 LAMP and PCR showed a consistent sensitivity of 89.5% and 88.2% respectively, and a specificity of 100% and 81.5% respectively. Kappa test was used to measure the degree of agreement between RIME 2 LAMP and conventional PCR which was 0.814 between the assays (table 5.8). The significance of the differences between the frequencies of positive results obtained in the tests was measured by Fischer's exact test. There was a significance difference observed with a p-value less than 0.05 at 95% confidence interval [$\chi^2 = 32.94$, (df = 3) p <0.0001]

Table 5.7. Results of the comparison of RIME 2 LAMP and conPCR for the detection of *T. equiperdum* ($p < 0.0001$)

		LAMP		Total
		Negative	Positive	
PCR	Neg	14	4	18
	Pos	0	30	30
Total		14	34	48

Table 5.8. Symmetric Measures between RIME 2 LAMP and PCR assays

		Value	Asymp. Error ^a	Std.Approx. T ^b	Approx. Sig.
Measure of Agreement	Kappa	.814	.088	5.739	.000
Number of Valid Cases		48			

5.7 Discussion

In this study we report on the successful development of a rapid and sensitive LAMP assay for the detection of trypanosome species of the sub-genus *Trypanozoon* including *Trypanosoma equiperdum* based on the amplification of RIME gene sequence (Hasan *et al.*, 1984). The LAMP reaction is rapidly and efficiently conducted in a single step and could be an ideal diagnostic tool for point of care (Abdullahi *et al.*, 2015). In this study, we report on the development of a RIME 2 LAMP method which is simple to perform and highly specific for detecting the trypanosomes of the sub-genus *Trypanozoon*. For the development of the RIME 2 LAMP assay an appropriate RIME gene with this accession no. K10801.1 (Hasan *et al.*, 1984) was selected and used to design LAMP primers. A set of six primers that recognizes eight distinct sequences on a target gene was selected and used in the study. Different reaction temperatures were selected that varied from 60 to 65°C, all of which gave good results (data not shown).

Experiments were at least repeated three times and also done in triplicates. There was 100% agreement in LAMP test replicates. Amounts of LAMP amplicons produced were detectable within 15 minutes of the reaction. Efficiency and rapidity of the assay may be assigned to the addition of loop primers to the regular LAMP primers (Liu *et al.*, 2008). In detail, only gDNA samples of the subgenus *Trypanozoon*: *T. equiperdum*, *T. b. brucei* and *T. evansi* were amplified under optimized amplification conditions. Amplification on the melting curves attained with a consistent T_m of ~ 89.5°C (figure 5.5.5) indicated similar amplicons with similar sequences. In most reactions melting curves of *T. evansi* DNA's were observed later with a T_m of ~ 89.7 consistently. This could possibly be due to their genetic difference. Melt-curve analyses are used to indicate different reaction products based on their shape and peaks (Njiru *et al.*, 2017). The use of gel electrophoresis also supported our results by revealing or confirming the false amplification that was seen in DDW. The specificity of the assay was confirmed using DNA samples from other protozoan parasites as well as non-target DNAs and tested negative to prove high specificity of the assay. All the specificities of the assays were in agreement with the specificity of the current study RIME 2 LAMP, none detected DNA's of other parasites that do not belong to the sub-genus *Trypanozoon*.

The LAMP is considered to be highly sensitive as compared to PCR methods, due to the ability of detecting as few as six DNA copies, i.e. 0.001 trypanosomes/ml from 1 ml blood, and to detect DNA extracted from other protozoan infected red blood cells (Liu *et al.*, 2008). In our study, the analytical sensitivity of RIME 2 LAMP was tested by subjecting tenfold serially diluted DNA of *T. equiperdum* to LAMP. The results showed that 1fg (1×10^{-7} ng/ μ l) DNA can be correctly diagnosed for parasite which is equivalent to 0.001 trypanosomes/ml, this indicate that RIME2 LAMP assay can detect very low parasitemia in animals. Njiru *et al.*, (2008a) and Grab *et al.*, (2011) conducted similar studies using a RIME gene for African human trypanosome species. The detection limit of the current study was similar to that of Njiru *et al.*, (2008a) and Grab *et al.*, (2011) with approximately 0.001 trypanosomes/ml but less than 10 trypanosomes/ml in the findings of Njiru *et al.*, (2011). The results of the study were in agreement with the previous studies conducted when the same gene (RIME) was used. It has also showed that the diagnostic sensitivity of the TgsGP LAMP (Njiru *et al.*, 2011) is higher than the sensitivity of the RIME 2 LAMP. The analytical sensitivity of SRA LAMP assay was 1×10^{-6} when a template (DNA or supernatant) was preheated before being added to a reaction, with the best detection limit of dilution 1×10^{-7} recorded with supernatant prepared from the buffy coat (Njiru *et al.*, 2008b). These findings were similar to those of the current study when *T. equiperdum* DNA was used even though the test was less sensitive when DNA was preheated. In the studies conducted by Tong *et al.*, (2018) and Njiru *et al.*, (2010), RoTat 1.2 VSG gene was used to detect *T. evansi*, with the analytical sensitivity of the findings of Tong *et al.*, (2018) being compared with the current study while that of Njiru *et al.*, (2010) was less sensitive than to the current study. These results support the reliability and robustness of the LAMP assay developed.

To evaluate the LAMP method, 48 field samples were subjected to LAMP assay. In this study, 34 out of the 48 samples were positive while 14 samples were negative for RIME 2 LAMP. Due to lack of reports on LAMP as a diagnosis tool for detecting *T. equiperdum*, no comparison of the current findings to previous studies was made. There are successful LAMP reports on other *Trypanozoon* species such as *T. brucei gambiense*, *T. b. rhodesiense* and *T. evansi* where different gene was used. In the study by Njiru *et al.*, (2008a), RIME LAMP assay was conducted on the same gene to diagnose *Trypanozoon* species which resulted in a 100% (75 isolates) detection in comparison to the 88.2% (34/48) observed in the current study. This could be due to the fact that the samples

screened by Njiru *et al.*, (2008a) were collected from HAT patients and *T. equiperdum* was not screened. Moreover, *T. equiperdum* is a tissue parasite unlike other *Trypanozoon* species and it is rarely found in the blood (Hagos *et al.*, 2010; OIE, 2015; Pascucci *et al.*, 2013). Furthermore, the samples screened in the current study were from the positive ELISA TeGM6-4r serology results, which normally indicate the exposure to the infection not necessarily the current infection. Njiru *et al.*, (2011), stated that the RIME LAMP cannot distinguish the species of the sub-genus *Trypanozoon*, but in the case of this current study, the RIME 2 LAMP can be used to differentiate the species of other trypanosomes in South Africa, since there are no reports or evidence of *T. evansi* detection. Nevertheless, the study was conducted in the areas where there is no evidence or information of other trypanosomes existence. In the melt-curves most of the *T. evansi* DNA's amplified later than *T. equiperdum* and *T. brucei brucei* DNA's, that attribute could also be used to differentiate *Trypanozoon* species. Secondly, the SRA LAMP detected all the 49 (100%) *T. b. rhodesiense* HAT patients, none of the animal *Trypanozoon* species were detected. This could be due to that the SRA gene is conserved and specific to *T. b. rhodesiense* (Njiru *et al.*, 2008b). Lastly, in the study conducted by Tong *et al.*, (2018) using RoTat 1.2 VSG gene, obtained 3% positive rate which was lower than 88.2% of the current study. This could be due to number of samples and the samples used in the current were from seropositive animals. The TgsGP LAMP assay detected 50% (Njiru *et al.*, 2011) less than the results of the current study. The RIME is known as a multi-copy gene while TgsGP is a low-copy number target, so the sensitivity of RIME LAMP was expected to be higher (Njiru *et al.*, 2011). Donkeys were more susceptible than horses with a higher infection rate of 8/10 (80%) and 26/38 (68.4%) respectively. This could be associated to the fewer number of donkey samples used in this study. Usually, donkeys are known to be resistant because of the anti-complementary activity present and they often remain carriers without obvious clinical signs (Brun *et al.*, 1998; Vulpiani *et al.*, 2013). There was a significance difference observed between the groups.

In comparison, the LAMP assay was more sensitive than the PCR method for the detection of *T. equiperdum*. This indicates that LAMP assay is sensitive enough to detect DNA at low level of infections. To that end, RIME 2 LAMP revealed four positive samples that were negative with conventional PCR. According to Parida *et al.*, (2008), the principle of LAMP positivity is based on the time of positivity, which varies based on the designed primer set and nature of the selected template. In most LAMP amplifications, it was

observed after 30–40 min in the amplification cycle. The high sensitivity of LAMP is due to four primers used in the reaction, which target six distinct sequences on the target DNA and can detect DNA as few as six copies (Alhassan *et al.*, 2007). These new developed techniques (RIME 2 LAMP and convPCR) can be employed in the areas where *T. evansi* and *T. brucei* subspecies does not exists and other trypanosome species. Polymerase chain reaction has limitations in resource-poor settings, including the need for a thermocycler and a reliable electricity supply. Isothermal DNA amplification offers some solutions: the first isothermal, the one-step transcription-based nucleic acid amplification system (Wastling & Welburn, 2011). The LAMP assay, when compared to other molecular tests is less expensive or comparable. Moreover, the relative temperature stability of the LAMP reagents enables this technique to be deployed in different fields (Chaouch *et al.*, 2013).

REFERENCES

- Abdullahi, U.F., Naim, R., Taib, W.R.W., Saleh, A., Muazu, A., Aliyu, S. & Baig, A.A. (2015). Loop-mediated isothermal amplification (LAMP), an innovation in gene amplification: Bridging the gap in molecular diagnostics; A review. *Indian Journal of Science and Technology*, 8:1–14.
- Adams, E.R., Gomez, M.A., Scheske, L., Rios, R., Marquez, R., Cossio, A., Albertini, A., Schallig, H. & Saravia, N.G. (2014). Sensitive diagnosis of cutaneous leishmaniasis by lesion swab sampling coupled to qPCR. *Parasitology*, 141:1891–1897.
- Ahmed, H.A., Picozzi, K., Welburn, S.C. & MacLeod, E.T. (2013). A comparative evaluation of PCR- based methods for species- specific determination of African animal trypanosomes in Ugandan cattle. *Parasites & Vectors*, 6:1–7.
- Alhassan, A., Govind, Y., Tam, N.T., Thekiso, O.M.M., Yokoyama, N., Inoue, N. & Igarashi, I. (2007). Comparative evaluation of the sensitivity of LAMP, PCR and in vitro culture methods for the diagnosis of equine piroplasmiasis. *Parasitology Research*, 100:1165–1168.
- Brun, R., Hecker, H. & Lun, Z-R. (1998). *Trypanosoma evansi* and *T. equiperdum*, distribution, biology, treatment and phylogenetic relationship. *Veterinary Parasitology*, 95–107.
- Cai, X., Lei, Z., Fu, W., Zhu, Z., Zou, M., Gao, J., Wang, Y., Hong, M., Wang, J., Li, W. & Xu, D. (2013). Optimisation of reverse transcription loop-mediated isothermal amplification assay for the rapid detection of pandemic (H1N1) 2009 virus. *African Journal of Microbiology Research*, 7:3919–3925.
- Chaouch, M., Mhadhbi, M., Adams, E.R., Schoone, G.J., Limam, S., Gharbi Z, Darghouth, M.A., Guizania, I. & BenAbderrazaka, S. (2013). Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Leishmania infantum* in canine leishmaniasis based on cysteine protease B genes. *Veterinary Parasitology*, 198:74–78.
- Desquesnes, M., Dargantes, A., Lai, D-H., Lun, Z-R., Holzmüller, P. & Jittapalpong, S. (2013). *Trypanosoma evansi* and Surra: a review and perspectives on transmission,

epidemiology and control, impact, and zoonotic aspects. *Biomedical Research International*, 2013:1–20.

Dhama, K., Karthik, K., Chakraborty, S., Tiwari, R., Kapoor, S., Kumar, A. & Thomas, P. (2014). Loop-mediated isothermal amplification of DNA (LAMP): a new diagnostic tool lights the world of diagnosis of animal and human pathogens: a review. *Pakistan Journal of Biological Sciences*, 17:151–166.

Grab, D.J., Nikolskaia, O.V., Inoue, N., Thekiso, O.M., Morrison, L. J., Gibson, W. & Dumler, J. S. (2011). Using detergent to enhance detection sensitivity of African trypanosomes in human CSF and blood by loop-mediated isothermal amplification (LAMP). *PLoS Neglected Tropical Diseases*, 5: e1249.

Hagos, A., Abebe, G., Philip, B., Bruno, M.G & Claes, F. (2010). Serological and parasitological survey of dourine in the Arsi–Bale highlands of Ethiopia. *Tropical Animal Health and Production* 42:769–776.

Hagos, A., Degefa, G., Yacob, H.B., Fikru, R., Alemu, T., Feseha, G., Claes, F. & Goddeeris, B.M. (2010b). Seroepidemiological survey of *Trypanozoon* infection in horses in the suspected dourine–infected Bale highlands of the Oromia region, Ethiopia. *Revue Scientifique Et Technique De L`Office International Des Epizooties*, 29:649–654.

Hagos, A., Goddeeris, B.M., Yilikal, K., Alemu, T., Fikru, R., Yacoba, H.T., Feseha, G. & Claes, F. (2010). Efficacy of Cymelarsan® and Diminisan® against *Trypanosoma equiperdum* infections in mice and horses. *Veterinary Parasitology*, 171:200–206.

Hasan, G., Turner, M.J. & Cordingley, J.S. (1984). Complete nucleotide sequence of an unusual mobile element from *Trypanosoma brucei*. *Cell*, 37:333–341.

Iseki, H., Alhassan, A., Ohta, N., Thekiso, O.M.M., Yokoyama, N., Inoue, N., Nambota, A., Yasuda, J. & Igarashi, I. (2007). Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites. *Journal of Microbiological Methods*, 71:281–287.

Iwamoto, T., Sonobe, T., & Hayashi, K. (2003). Loop-Mediated Isothermal Amplification for Direct Detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in Sputum Samples. *Journal of Clinical Microbiology*, 41:2616–2622.

Kuboki, N., Inoue, N., Sakurai, T., Di Cello, F., Grab, D.J., Suzuki, H., Sugimoto, C. & Igarashi, I. (2003). Loop-mediated isothermal amplification for detection of African trypanosomes. *Journal of Clinical Microbiology*, 41:5517–5524.

Liu, Z., Hou, J., Bakheit, M.A., Salih, D.A., Luo, J., Yin, H., Ahmed, J.S. & Seitzer, U. (2008). Development of loop-mediated isothermal amplification (LAMP) assay for rapid diagnosis of ovine theileriosis in China. *Parasitology Research*, 103:1407–1412.

Mandal, M., Banerjee, P.S., Kumar, S., Ram, H., Garg, R. & Pawde, A.M. (2015). Development of loop-mediated isothermal amplification (LAMP) for detection of *Babesia gibsoni* infection in dogs. *Veterinary Parasitology*, 209:50–55.

Mori, Y. & Notomi, T. (2009). Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of Infection and Chemotherapy*, 15:62–69.

Nagamine, K., Hase, T. & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, 16(3):223–22.

Nagamine, K., Kuzuhara, Y. & Notomi, T. (2002). Isolation of single-stranded DNA from loop-mediated isothermal amplification products. *Biochemical and Biophysical Research Communications*, 290:1195–1198.

Ngotho, M., Kagira, J.M., Gachie, M.B., Karanja, S.M., Waema, M.W., Maranga, D.N. & Maina, N.W. (2015). Loop mediated isothermal amplification for detection of *Trypanosoma brucei gambiense* in urine and saliva samples in Nonhuman Primate Model. *BioMedical Research International*, 2015

Njiru, Z.K., Mbae, C.K. & Mburugu, G.N. (2017). Loop-mediated Isothermal amplification test for *Trypanosoma gambiense* Group 1 with stem primers: A molecular xenomonitoring test for sleeping sickness. *Journal of Tropical Medicine*, 1–7.

Njiru, Z.K., Yeboah-Manu, D., Stinear, T.P. & Fyfe, J.A. (2012). Rapid and sensitive detection of *Mycobacterium ulcerans* using a loop-mediated isothermal amplification test. *Journal of Clinical Microbiology*, JCM–06460.

Njiru, Z.K., Ouma, J.O., Enyaru, J.C. & Dargantes, A.P. (2010). Loop-mediated isothermal amplification (LAMP) test for detection of *Trypanosoma evansi* strain B. *Experimental Parasitology*, 125:196–201.

Njiru, Z.K. (2011). Rapid and sensitive detection of human African trypanosomiasis by loop-mediated isothermal amplification combined with a lateral-flow dipstick. *Diagnostic Microbiology and Infectious Disease*, 69:205–209.

Njiru, Z.K. (2012). Loop mediated isothermal amplification technology: Towards point of care diagnostic. *PLoS Neglected Tropical Diseases*, 6: e1572.

Njiru, Z.K., Constantine, C.C., Guya, S., Crowther, J., Kiragu, J.M., Thompson, R.C.A. & Dávila A.M.R. (2005). The use of ITS rDNA PCR in detecting pathogenic African trypanosomes. *Parasitology Research*, 95:186–192.

Njiru, Z.K., Mikosza, A.S.J., Armstrong, T., Enyaru, J.C., Ndung'u, J.M. & Thompson, A.R.C. (2008b). Loop-Mediated Isothermal Amplification (LAMP) Method for Rapid Detection of *Trypanosoma brucei rhodesiense*. *PloS Neglected Tropical Diseases*, 2:1–8.

Njiru, Z.K., Mikosza, A.S.J., Matovu, E., Enyaru, J.C.K., Ouma, J.O., Kibona, S.N., Thompson, R.C.A. & Ndung'u, J.M. (2008a). African trypanosomiasis: Sensitive and rapid detection of the sub-genus *Trypanozoon* by loop-mediated isothermal amplification (LAMP) of parasite DNA. *International Journal for Parasitology*, 38:589–599.

Njiru, Z.K., Traub, R., Ouma, J.O., Enyaru, J.C. & Matovu, E. (2011). Detection of Group 1 *Trypanosoma brucei gambiense* by loop-mediated isothermal amplification. *Journal of Clinical Microbiology*, 49:1530–1536.

Notomi, T., Mori, Y., Tomita, N. & Kanda, H. (2015). Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *Journal of Microbiology*, 53:1–5.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28: e63–e63.

OIE, (2015). *Dourine. The Center for Food Security and Public Health, Iowa State University, College of Veterinary Medicine*, 1-4.

Parida, M., Posadas, G., Inoue, S., Hasebe, F. & Morita, K. (2004). Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile Virus. *Journal of Clinical Microbiology*, 42:257–263.

- Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L. & Morita, K. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology*, 18:407–421.
- Pascucci, I., Di provvido, A., Cammà, C., Di Francesco, G., Calistri, P., Tittarelli, M., Ferri, N., Scacchia, M. & Caporale, N. (2013). Diagnosis of dourine in outbreaks in Italy. *Veterinary Parasitology*, 193:30–38.
- Saiki, R.K., Gelfand, D.H., Stofel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988). Amplification of a Short DNA Stretch by Repeated Cycles of *in vitro* DNA Polymerization. *Science*, 239:487–491.
- Salih, D.A., El Hussein, A.M. & Singla, L.D. (2015). Diagnostic approaches for tick-borne haemoparasitic diseases in livestock. *Journal of Veterinary Medicine and Animal Health*, 7:45–56.
- Thekiso, O.M., Bazie, R.S., Coronel-Servian, A.M., Sugimoto, C., Kawazu, S.I., & Inoue, N. (2009). Stability of loop-mediated isothermal amplification (LAMP) reagents and its amplification efficiency on crude trypanosome DNA templates. *Journal of Veterinary Medical Science*, 71:471–475.
- Thekiso, O. M., Kuboki, N., Nambota, A., Fujisaki, K., Sugimoto, C., Igarashi, I. & Inoue, N. (2007). Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomiasis. *Acta Tropica*, 102:182–189.
- Thekiso, O.M., Inoue, N., Kuboki, N., Tuntasuvan, D., Bunnoy, W., Borisutsuwan, S., Igarashi, I. & Sugimoto, C. (2005). Evaluation of loop-mediated isothermal amplification (LAMP), PCR and parasitological tests for detection of *Trypanosoma evansi* in experimentally infected pigs. *Veterinary Parasitology*, 130:327–330.
- Thekiso, O.M., Rodriguez, C.V., Rivas, F., Coronel-Servian, A.M., Fukumoto, S., Sugimoto, C., Kawazu, S.I. & Inoue, N. (2010). Detection of *Trypanosoma cruzi* and *T. rangeli* infections from *Rhodnius pallescens* bugs by loop-mediated isothermal amplification (LAMP). *The American Journal of Tropical Medicine and Hygiene*, 82:855–860.

- Thekiso, O.M.M., Rambritch, N.E., Nakano, R., Bazie, R.S., Mbatia, P., Namangala, B., Malele, I., Skilton, R.A., Jongejan, F., Sugimoto, C., Kawazu, S. & Inoue N. (2010). Loop-mediated isothermal amplification (LAMP) assays for detection of *Theileria parva* infections targeting the PIM and p150 genes. *International Journal for Parasitology*, 40:55–61.
- Tomita, N., Mori, Y., Kanda, H. & Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature Protocols*, 3: 877.
- Tong, Q., Chen, R., Kong, Q., Goossens, J., Radwanska, M., Di Lou, Ding, J., Zheng, B., Fu, Y., Wang, T., Stefan, M. & Lu, S. (2018). DNA detection of *Trypanosoma evansi*: Diagnostic validity of a new assay based on loop-mediated isothermal amplification (LAMP). *Veterinary Parasitology*, 250:1–6.
- Vulpiani, M.P., Carvelli, A., Biologist, D.G., Iannino, F., Paganico, D. & Ferri, N. (2013). Reemergence of Dourine in Italy: Clinical Cases in Some Positive Horses. *Journal of Equine Veterinary Science*, 33:468–474.
- Wang, D., Brewster, J.D., Paul, M. & Tomasula, P.M. (2015). Two Methods for Increased specificity and sensitivity in loop-mediated isothermal amplification. *Molecules*, 20: 6048–6059.
- Wang, Y., Wang, Y., Xu, H., Dai, H., Meng, H. & Ye, C. (2014). Rapid and sensitive detection of *Listeria ivanovii* by loop-mediated isothermal amplification of the smcL gene. *PLOS one*, 1–12.
- Wastling, S.L. & Welbrun, S.C (2011). Diagnosis of human sleeping sickness: sense and sensitivity. *Trends in Parasitology*, 27:394–402.
- Zende, R., Kshirsagar, D., Vaidya, V., Waghamare, R., Todankar, R. & Shirke, A. (2017). Loop-mediated isothermal amplification assay (LAMP): A rapid tool for diagnosis of foodborne and zoonotic pathogens: A review. *International Journal of Livestock Research*, 7:23–35.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The difficulties in diagnosing *T. equiperdum* has led to complications in finding reliable data on the prevalence and distribution of the disease, as well as for implementation of monitoring, treatment and control programmes (Hagos *et al.*, 2010). For instance, in resource-poor endemic areas, lack of effective point of care diagnostic tests applicable is a critical barrier for the effective treatment and control of infectious diseases (Njiru, 2012). Furthermore, in order for veterinarians to select effective treatment and prevention programs, molecular diagnostic techniques are known to be essential for such practices (Laohasinnarong, 2011). Primarily, *in vitro* culture and microscopy are the preferred traditional methods due to the fact that they are simple to perform and cheap techniques. Albeit, such techniques occasionally give inconclusive results and are time consuming. The use of nucleic amplification techniques with high sensitivity and specificity are useful tools to solve such limitations (Laohasinnarong, 2011). Molecular-based assays, such as polymerase chain reaction (PCR), real time PCR and LAMP provide a more sensitive alternative to traditional parasitological and serological techniques.

To date, the diagnosis of *T. equiperdum* infection is still strongly based on serological evidence. Furthermore, there are no defined serological assays and molecular markers for clinical use for the true *T. equiperdum* (Hagos *et al.*, 2010; Ahmed *et al.*, 2018). Differentiation of *T. equiperdum*, *T. brucei* subspecies and *T. evansi* in the areas where the two organisms coexist remains a challenge due to the absence of a specific antigen and molecular marker for clinical use (Gizaw *et al.*, 2017). Ten years ago, the discovery of the loop-mediated isothermal amplification (LAMP) technique has given new stimulus towards development of point of care diagnostic tests based on amplification of pathogen DNA, a technology that has been the precinct of well-developed research centres (Njiru, 2012).

In this study, LAMP, a robust method was successfully developed for detection of *T. equiperdum* infections, and due to absence of other species of the genus *Trypanozoon*, it can be employed as great potential test that can be applied easily in trypanosomiasis-endemic region in South Africa. Its high specificity suggests that this is a very helpful method to diagnose individual cases of trypanosomiasis especially in the cases where

both *T. evansi* and *T. equiperdum* exist. According to Parida *et al.*, (2008), the specific significance of the LAMP assay is the substantial reduction in time required for the confirmation of results in 30 min as compared to 3–4 h in the case of conventional PCR. Polymerase chain reaction methods, are sensitive and specific but require training and expensive thermo-cycling equipment, making them difficult to be used in the field. There is no *T. equiperdum*-specific PCR method available for detection of *T. equiperdum*, however, the subgenus *Trypanozoon*-specific PCR can be used to detect *T. equiperdum* DNA (Gizaw *et al.*, 2017). Therefore, the newly developed PCR method can be deployed to detect *T. equiperdum* infections in South African equids and be used as supplementary or confirmatory to serological techniques.

Serological techniques and microscopic methods have been used to detect infected equids and these are the current mainstay. However, they are time consuming and can result in misdiagnosis in areas of low prevalence and tend to give false results. Therefore, the SYBR Green I real-time PCR assay described in this study for the detection and quantitation of *T. equiperdum* has been shown to be rapid, easy to handle, sensitive, and specific. Thus, these features make it an excellent tool for laboratory detection of *T. equiperdum* since is a tissue parasite that is rarely found in the blood. The high degree of sensitivity and specificity observed with the DNA extracted from blood suggests that the assay would be a useful tool for field investigation of *T. equiperdum* infections. To add on that, all samples that tested positive for *T. equiperdum* by the PCR assay were also positive when analysed with the SYBR Green I qPCR assays. Therefore, SYBR Green I qPCR assay can be adopted as another technique for *T. equiperdum* infections in South African equids. It can quantify levels of parasitaemia in blood or tissue samples.

To our knowledge this species has never been detected from the equines using either one of these techniques. Therefore, the results of the study, suggest that a newly RIME conventional, RIME 2 LAMP, PCR and real-time PCR methods developed can be useful tools to screen equines samples in the areas where *T. equiperdum* is suspected, in addition to serological and parasitological methods established.

6.2 Recommendations

Previous report on dourine in South Africa based on serology test studies had already suggested that *T. equiperdum* the causative agent of dourine, is endemic, so these methods can be utilized as supplementary tools and possibly they can be used in the diagnosis of *Trypanozoon* species.

South African OVI *T. equiperdum* strain available, was established in 1979, since that era, no *T. equiperdum* strain was established. Therefore, a new South African *T. equiperdum* strain is needed.

The establishment of in vitro culture, will increase the availability of new methodologies for direct detection of *T. equiperdum* in equines and

It will also be possible to screen trypanocidal drugs for efficacy against this parasite in experimental infections if a reliable diagnostic test becomes available.

REFERENCES

- Ahmed, Y., Hagos, A., Merga, B., Van Soom, A., Duchateau, L., Goddeeris, B.M. & Govaere, J. (2018). *Trypanosoma equiperdum* in the horse – a neglected threat? *Vlaams Diergeneeskundig Tijdschrift*, 87:66–75.
- Hagos, A., Abebe, G., Philip, B., Bruno, M.G & Claes, F. (2010). Serological and parasitological survey of dourine in the Arsi–Bale highlands of Ethiopia. *Tropical Animal Health and Production*, 42:769–776.
- Hagos, A., Degefa, G., Yacob, H.B., Fikru, R., Alemu, T., Feseha, G., Claes, F. & Goddeeris, B.M. (2010b). Seroepidemiological survey of *Trypanozoon* infection in horses in the suspected dourine–infected Bale highlands of the Oromia region, Ethiopia. *Revue Scientifique Et Technique De L`Office International Des Epizooties*, 29:649–654.
- Laohasinnarong, D. (2011). Loop-mediated Isothermal Amplification (LAMP): an alternative molecular diagnosis. *Journal of Applied Animal Science* 4:9–19.
- Njiru, Z.K. (2012). Loop mediated isothermal amplification technology: Towards point of care diagnostic. *PLoS Neglected Tropical Diseases*, 6: e1572.
- Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L. & Morita, K. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology*, 18:407–421.