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

**Malitaba A. Mlangeni[1],** M.O. Taioe[2], M. Syakalima[3], K. Suganuma[4], N. Inoue4, O. Thekisoe[1]

**[1]Unit for Environmental Sciences and Management, North-West University, South Africa**

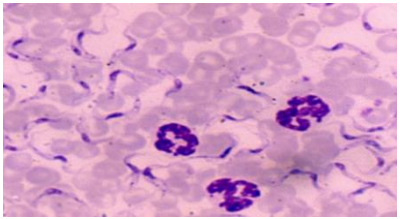
**[2]National Zoological Gardens, Pretoria, South Africa**

**[3]Animal Health Program, North-West University, South Africa**

**[4]National Research Center for Protozoan Diseases, Obihiro University, Japan**

# Introduction

- *Trypanosoma equiperdum* is a protozoan parasite causing a disease called dourine in equines.



- Transmission: - sexually from infected to healthy animals
  - to foals during birth and through maternal milk
- Dourine has a worldwide distribution including South Africa

Mlangeni, 2014

Mlangeni, 2014

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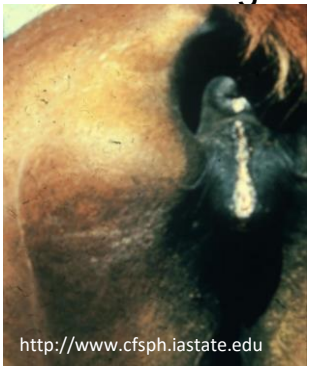
# Clinical Signs

- Swellings and local oedema of the genital organs

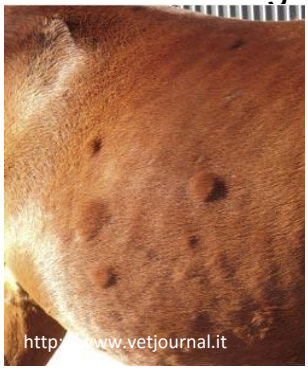
- There are three stages of the disease:

First stage




In mares there is a discharge from the vagina, slight fever and loss of appetite

Second stage

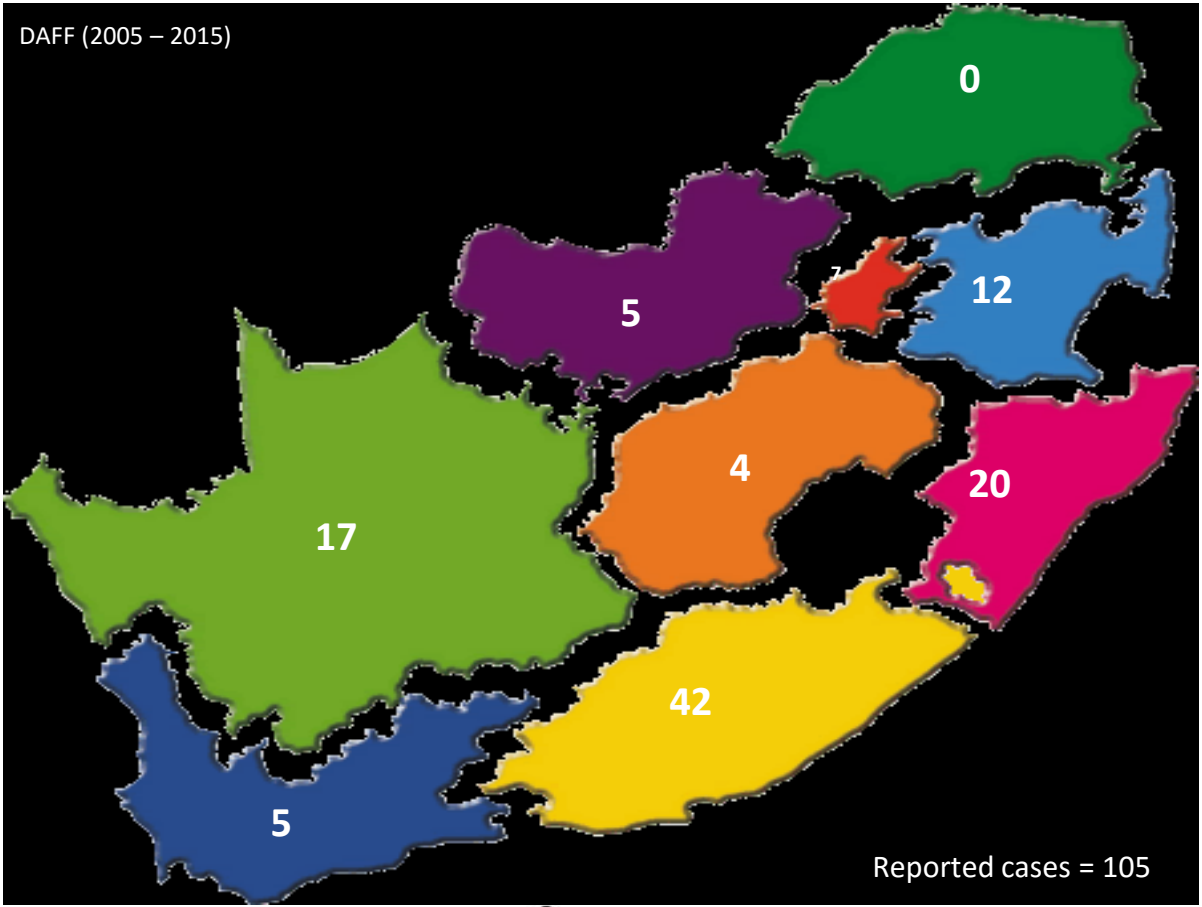



Cutaneous plaques and round rash eruptions

Third stage

Stiffness and weakness of the limbs with lack of coordination

# Dourine cases per province: 2005 - 2015



# Diagnosis

In South Africa

- Confirmation of clinical signs by veterinarians
- The complement fixation test (CFT) is currently used as a diagnostic method for dourine

# Treatment

- No effective treatment have been established
- Diminazene aceturate, quinapyramine and melarsomine hydrochloride are reported to be effective in other countries
- Slaughter of infected equines in South Africa (No treatment)

# Problem statement

- The CFT is the only official diagnostic method approved by OIE.

- CFT is an antibody detection test which indicates exposure of animals to infection not necessarily current infection.

  -It is time-consuming and also gives false positive results (Clausen et al. 2003; Cencek et al. 2008; Potts et al. 2013; Gizaw et al. 2017).

- The lack of accurate diagnostic techniques of *Trypanosoma equiperdum* remains a global challenge.

# Objective of the study

**Aim:**

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

**Objectives:**

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

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

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

# Materials and methods

## Development of LAMP assay

**LAMP primers designed targeting the RIME gene using Primer explorer V4 online software**

**FIP:** F2 + F1c
**BIP:** B2 + B1c

>K01801.1 ribosomal mobile element (RIME) in rRNA gene

CTCAAATGAAATACCACCACTCGGAACGTTGCTTCACTTATCGAATGAAGAGACCAATGGTTGTTTTGCG
CAGTCTTCGGGCTGTGCGCCGTCTAGGCTGGGCATAATTGTGGTGTTTCTTTCACCGCCACGGGAGGGGT
GTTGGTTTTTTATCCTTCTCTCCGTTTCTCCTTTTCTCTCTTTCTCCCTTTTTTGGGCGTTTGAGGGCTG
GTGACCCGTGGGCGCCCAGCCGTGCGGAACTATTTTTTTTTTTTTTGAAATTTTCTTCGTGCCTTCGTGA
GAGTTTCCCCCCACTGGCGCGGCCATCAGCCATCACCGTAGAGCCCTGAGATGCTATCGGTCTGGCGACT
GTGGCAGAGTCTCCCTTTTTCATTCATCCTAACACACCCTCTATAATTTTTGTTGTATTTCCGCATATGA
ACGGAATACAGCAGATGGG
TTTTTTTGGAGTTCCACATC
CCCAGGGACCGACAAATGAAA
CGGGGTACTGGACCCTGGCACACC
TTTAGAATTTTCTTCGTGCCTTCGTC
CCCTGAGATGCTATCGGTCTGGCGACTGTGGCAGAGTCTCCCTTTTTCATTCATCCTAACACACCCTCTA
TAATTTTTGTTGTATTTCCGCATATGAACGGAATACAGCAGATGGGGCGAGAAGCCTCCCACCCTGGACT
CTCCCAAAGATGCCAATTGGCAATCCTTTTTTTTGGAGTTCCACATCACCGGACAGCAGCATCTTAGTGC
GGATGATACTGCTGATGGCACGCTGGCACGTGCCCAGGGACCGACAAATGAAAAACGGTGTCCCAGCAAG
ACGCTGCTGATCCCTGTGCTGTATGATGCCGTAGCCGGAGCAAAGCAGTGTTCCCCATGAAAGGAACGCT
GCTGGCTCTTGGCTTCC
CAGGGCGTGTGGAACTA
ACATCTGCCAGATGGGGAGTTTGGCTGGGGCGGCATATCTGTTACACGACAACGCAGGTGTCCTAAGGCG
AG

F2
F1c
F3
B1c
B2
B3

CCTGGACTCCCAAAGA
CGGACAGCAGCA
CAGCAAGACGCTGCTGAT
CCATGAAAGGAACGCTGC
GGATGATACGGGGTACTGGA

**160 bp**

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# Materials and methods

**Molecular procedure**

*Trypanosoma equiperdum* DNA

LAMP and conPCR

OptiGene II (LAMP) and PCR (Mlangeni, 2016)

**Optimization**

Only positive and negative controls were used

**Sensitivity**

Eight 10-fold serial dilutions were prepared

**Specificity**

DNA of different *Trypanosoma* species and other protozoan parasites were used

**Field samples**

# Validation of LAMP and conPCR

- Forty eight samples collected from horses and donkeys were screened

- These samples were tested positive using ELISA TeGM6-4r for serology in the previous studies

**LAMP**

**PCR**

# Development of qPCR and conPCR

Optimization
- Only negative and positive controls were used

Standardization
- Five 10-fold dilutions were prepared and reactions were done in triplicates

Sensitivity
- Seven 10-fold dilutions were prepared and used

Specificity
- DNA's of different Trypanosoma species and other protozoan parasites were used

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# Validation of qPCR and conPCR

- Forty eight samples collected from horses and donkeys were screened

- These samples were tested positive using ELISA TeGM6-4r for serology in the previous studies

# Results

## Lamp specificity amplification plot

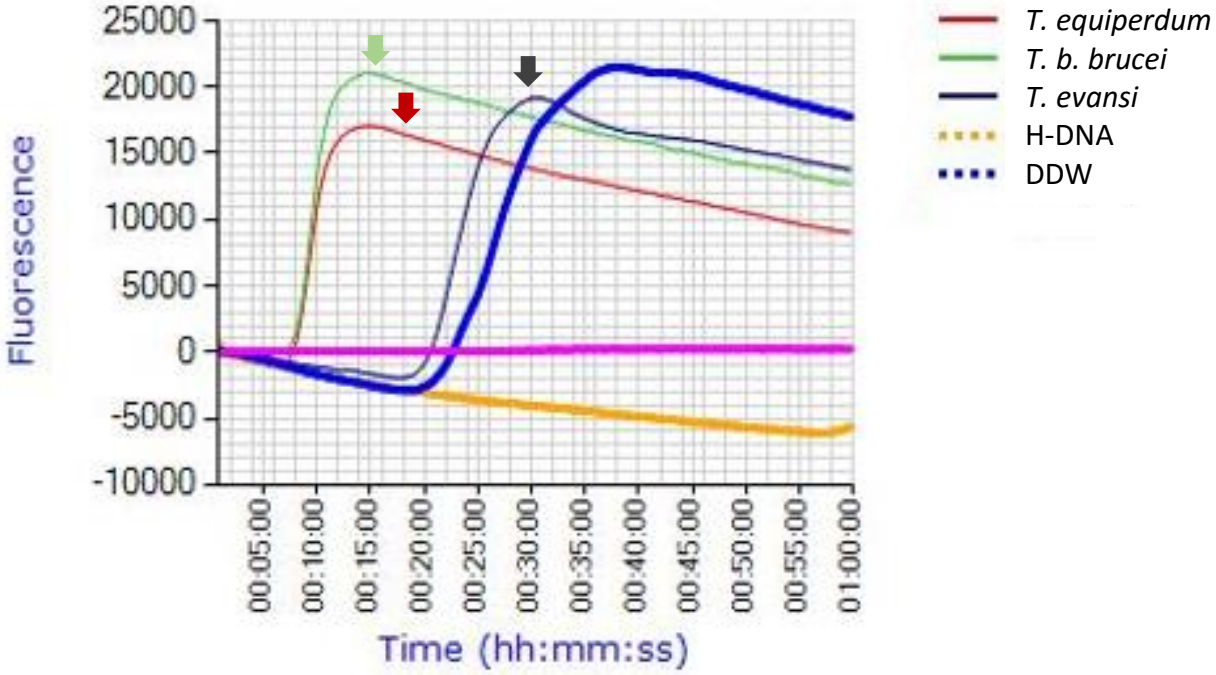



**Figure 1:** Specificity amplification at 65°C for 1 hr. The RIME2 LAMP assay was specific, no reactivity was recorded with non-target DNA (H-DNA) & DDW.

# Melt curve

**Lamp specificity melt curve**

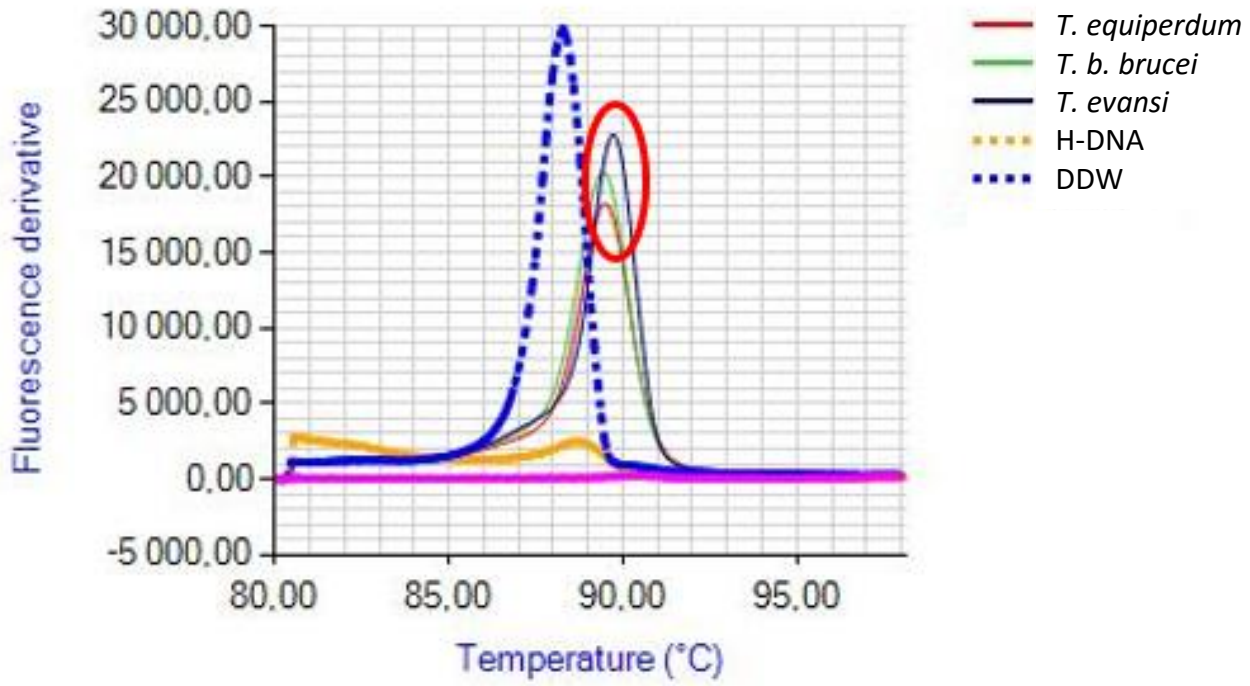



**Figure 2:** Specificity annealing curves of the ***Trypanosoma equiperdum*** and ***T. brucei brucei*** and ***T. evansi*** produced a single peak, at 89.5°, indicating similar amplicons.

# LAMP specificity gel electrophoresis

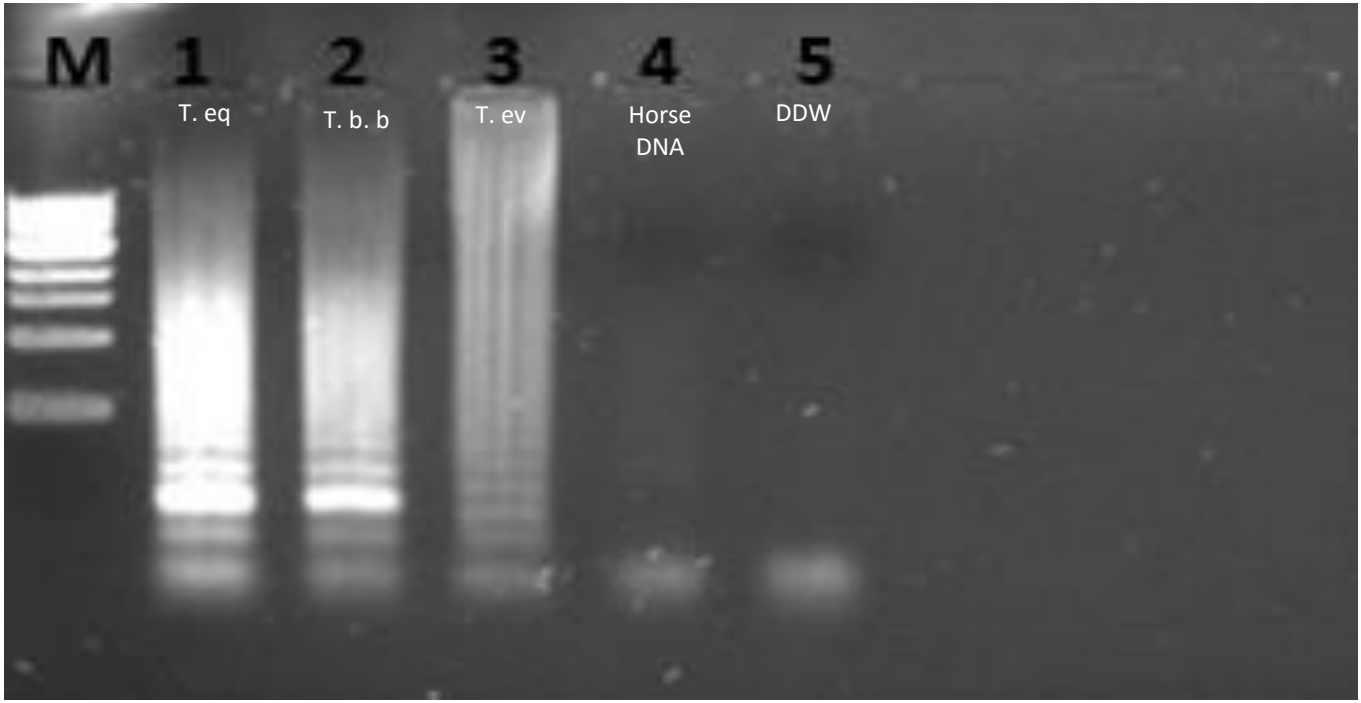
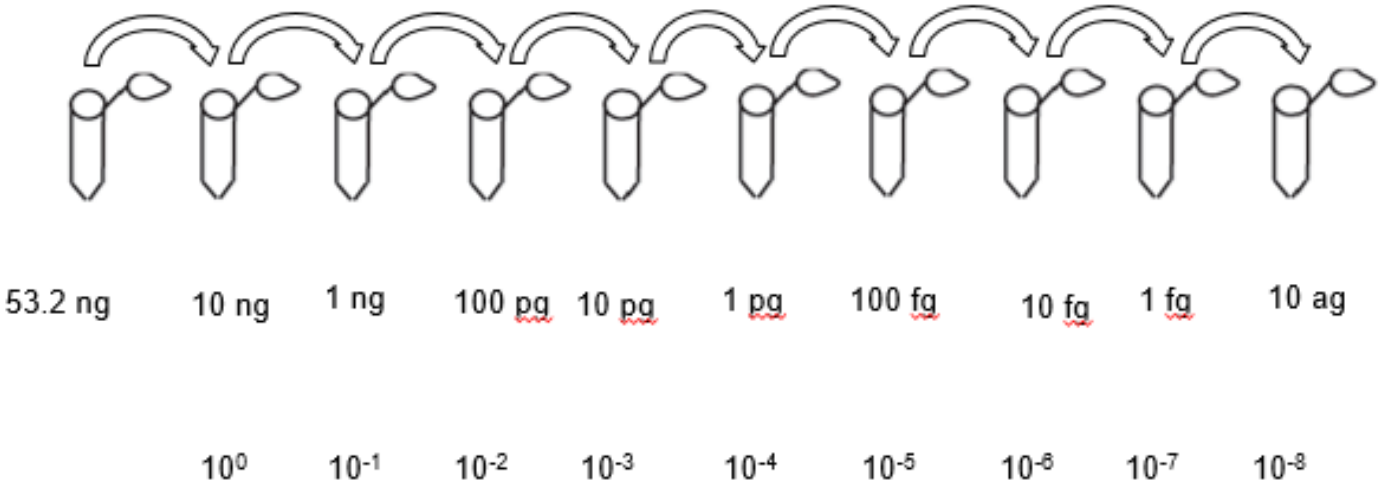



**Figure 3:** Gel agarose electrophoresis showing results of specificity of RIME2 LAMP primers **M**: DNA ladder (1kb), **L1:** *T. equiperdum* (T. eq) **L2:** *T. brucei brucei* (T. b. b) **L3:** *T. evansi* (T. ev), **L4:** H-DNA & **L5:** DDW

# Sensitivity of LAMP and PCR assays

## *Trypanosoma equiperdum* DNA serial dilutions



# Amplification plot

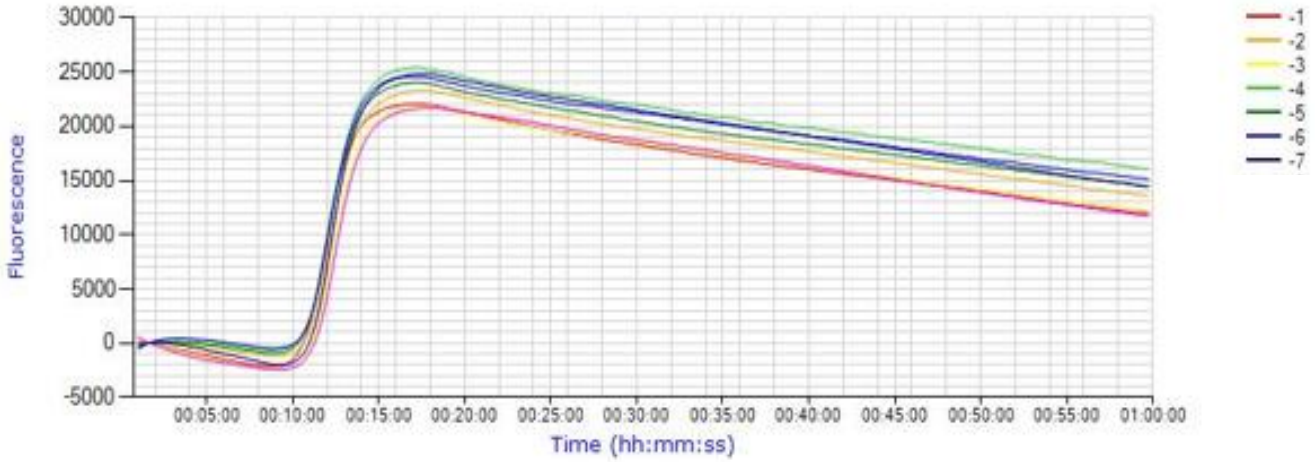



**Figure 4:** Sensitivity $10^{0}$, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$ at 65°C for 1 hr. Amplification had a detection limit of $10^{-7}$ which is equivalent to 0.001 number of trypanosome cells/ml.

# Melt curve

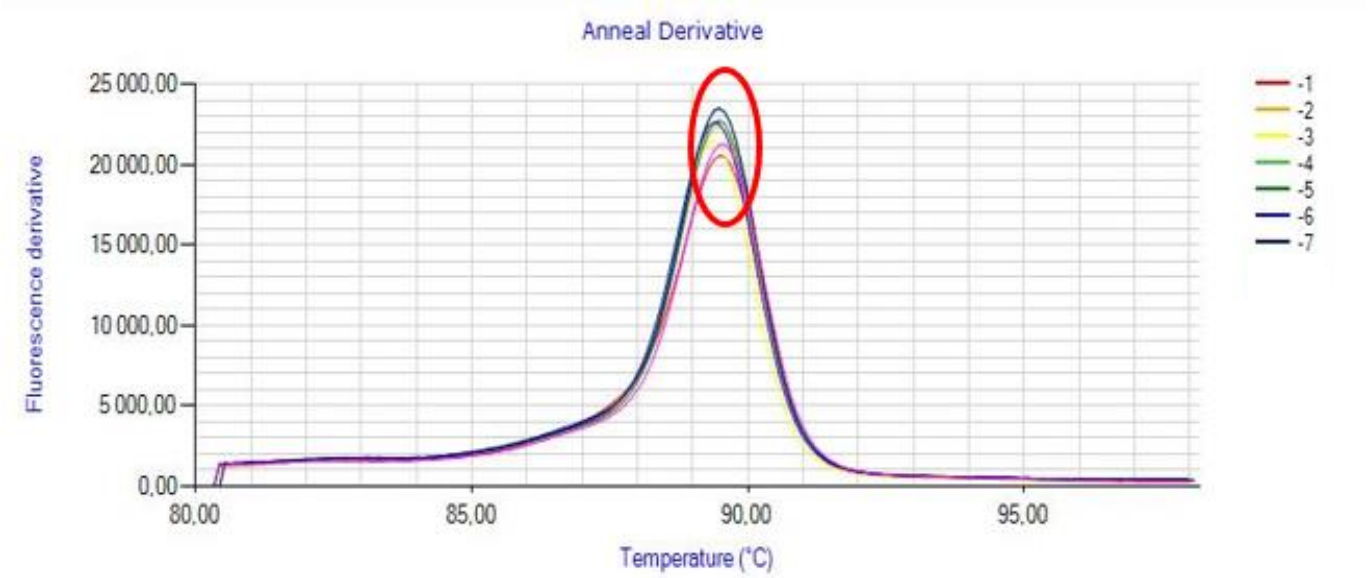



**Figure 5:** Sensitivity annealing peaks generated in the real time LAMP assay for the amplified target resulting from different concentrations.

# Polymerase chain reaction

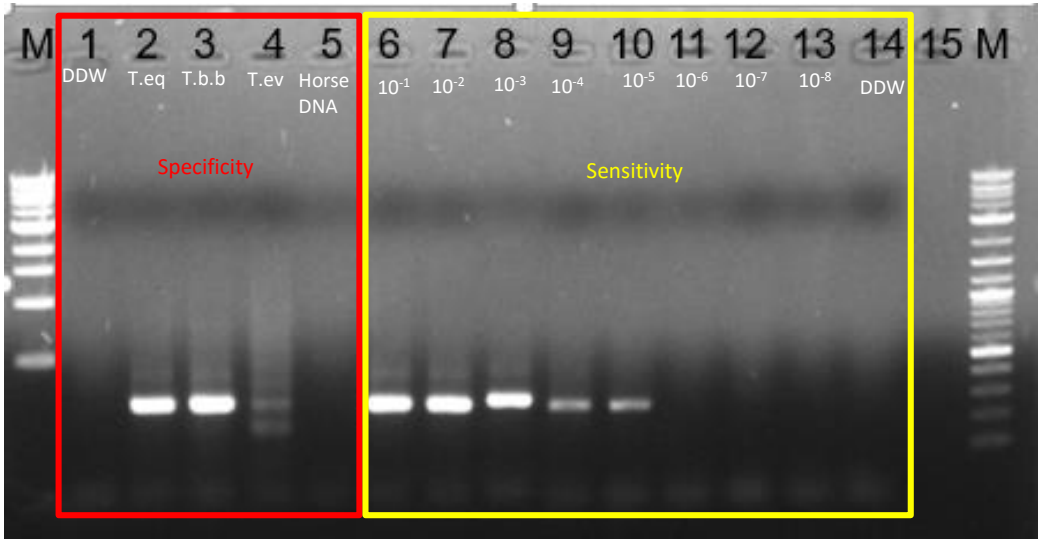



**Figure 6:** Gel image of 1% agarose gel electrophoresis. **M:** Ladder (1kb), 1. DDW, 2. *Trypanosoma equiperdum,* 3. *T. brucei brucei,* 4. *T. evansi*, 5. H-DNA for specificity, 6. $10^{-1}$ , 7. $10^{-2}$ , 8.$10^{-3}$ , 9.$10^{-4}$ , 10. $10^{-5}$ , 11. $10^{-6}$ , 12. $10^{-7}$ , 13. $10^{-8}$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

# LAMP and conPCR

LAMP and PCR amplification results for *T. equiperdum* infections across the sampled Provinces

| Province | LAMP | | PCR | |
|---|---|---|---|---|
| | Pos | Neg | Pos | Neg |
| Free State (n = 12) | 10 | 2 | 10 | 2 |
| Mpumalanga (n = 12) | 11 | 1 | 10 | 2 |
| Northern Cape (n=12) | 8 | 4 | 7 | 5 |
| North-West (n = 12) | 5 | 7 | 3 | 9 |
| Total = 48 | 34 | 14 | 30 | 18 |

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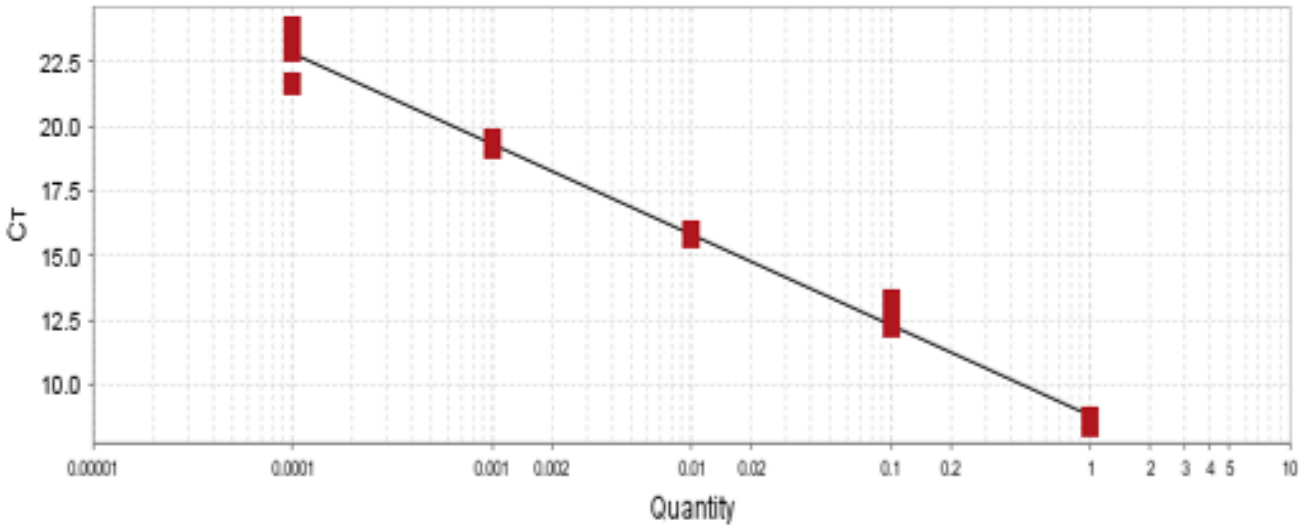
# Statistical analysis

| | | LAMP | | Total |
|---|---|---|---|---|
| | | Neg | Pos | |
| **PCR** | Neg | 14 | 4 | 18 |
| | Pos | 0 | 30 | 30 |
| **Total** | | 14 | 34 | 48 |

**Symmetric Measures**

| | | Value | Asymp. Std. Error[a] | Approx. T[b] | Approx. Sig. |
|---|---|---|---|---|---|
| Measure of Agreement | Kappa | .814 | .088 | 5.739 | .000 |
| N of Valid Cases | | 48 | | | |

# Standard curve SYBR Green




Target: Target 1 Slope: -3.508 Y-Inter: 8.847 $R^2$: 0.99 Eff%: 92.773 Error: 0.096

**Figure 7:** Standard curve of RIME qPCR assay

# Melt-curve

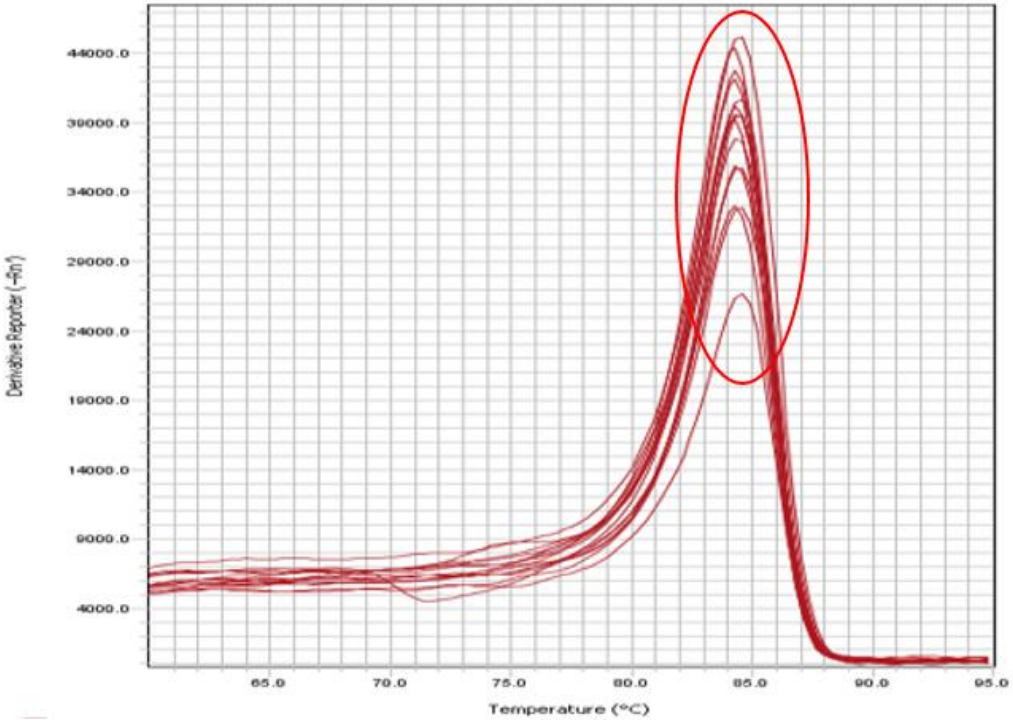
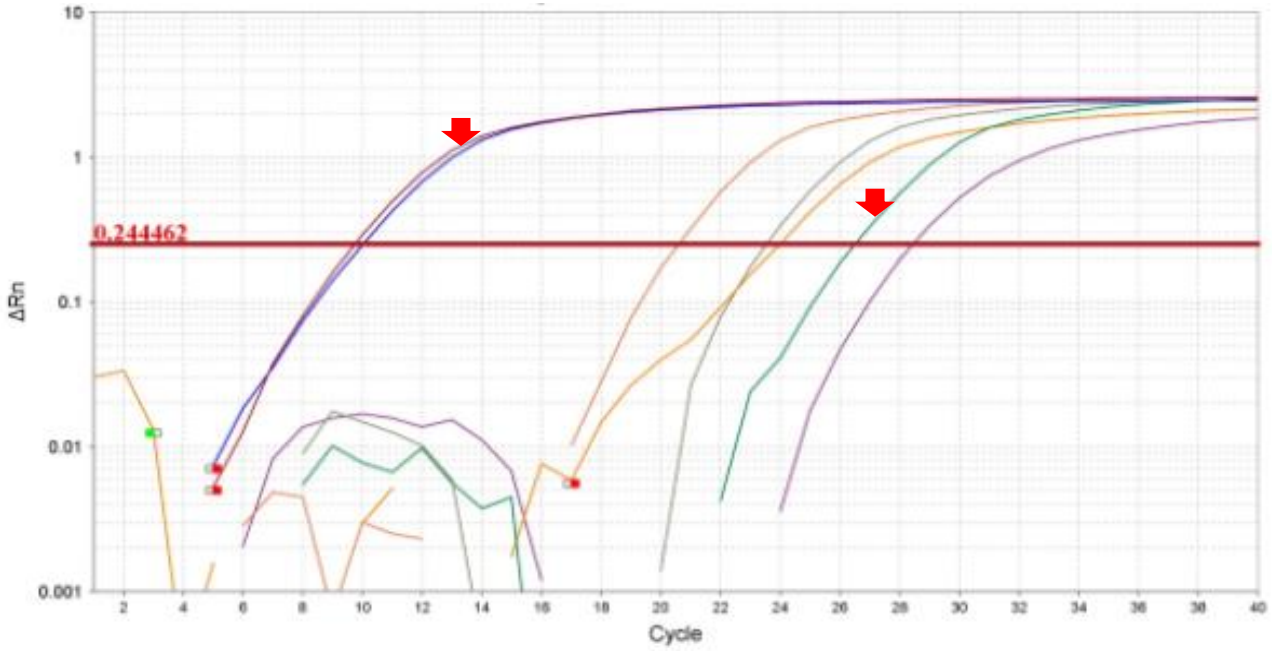



**Figure 8:** Melt curve plot RIME qPCR assay showing singular peaks with temperature ranging from 84.3°C to 85.0°C

# Specificity of RIME qPCR using SYBR green




*Babesia caballi*, DDW, *Theileria equi*, *Trypanosoma congolense*, *T. brucei brucei*, *T. evansi* and *T. equiperdum*

**Figure 9:** Specificity amplification plot for RIME qPCR assay

# Melt-curve

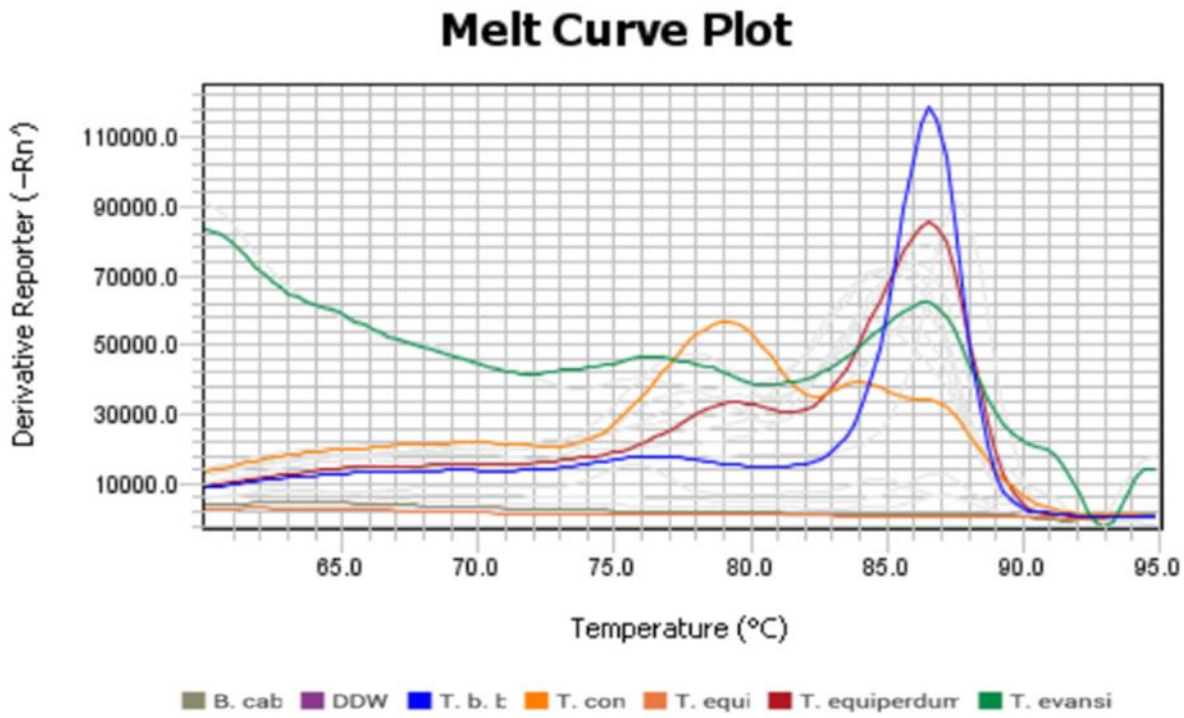



**Figure 10:** Melt curve plot showing multi peaks with temperature at ~ 87.0°C. No amplification was observed in non-target DNA and negative control (DDW).

# ConPCR-sensitivity

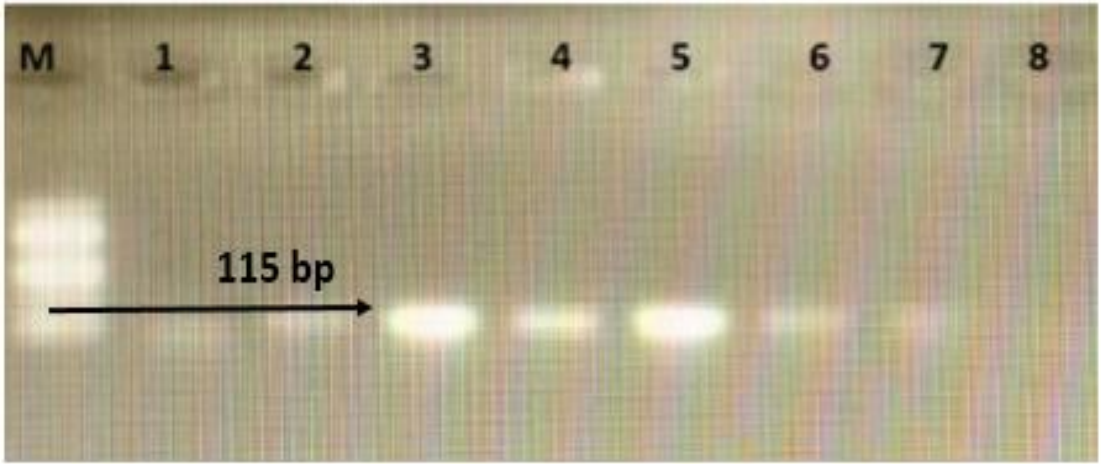



**Figure 11:** RIME1 primers sensitivity gel image of 1% agarose gel electrophoresis. **M:** Ladder (1kb), 1. DDW, 2. *Trypanosoma equiperdum,* 3. $10^{-1}$, 4. $10^{-2}$, $5.10^{-3}$, $6.10^{-4}$, 7. $10^{-5}$, 8. $10^{-6}$, PCR had a detection limit of $10^{-5}$ which is equivalent to 1 trypanosome/ml.

# ConPCR-specificity

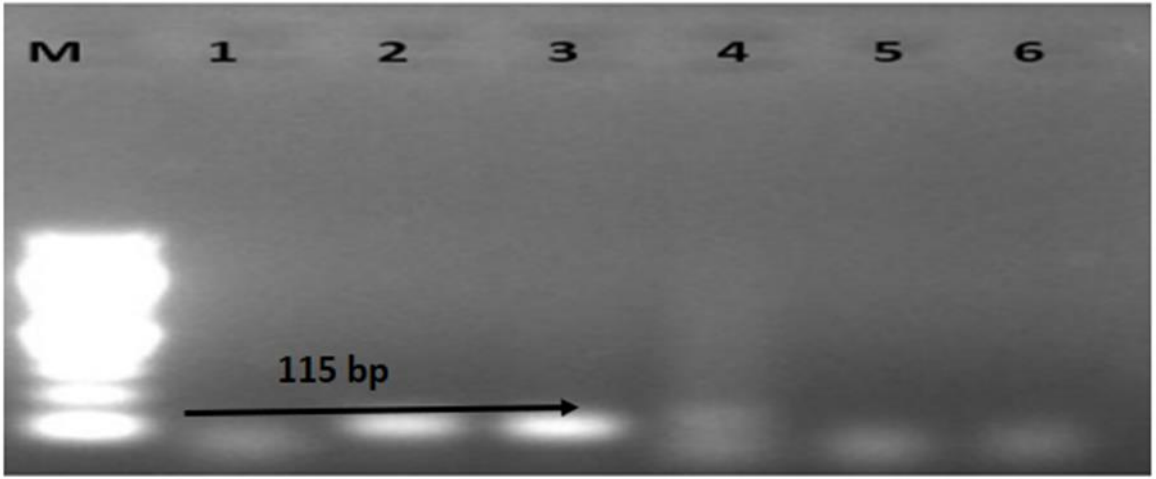



**Figure 12:** RIME 1 primers specificity gel image of 1% agarose gel electrophoresis. **M:** Ladder (1kb), 1. DDW, 2. *Trypanosoma equiperdum,* 3. *T. brucei brucei,* 4. *T. evansi,* 5. *T. congolense* & 6. H-DNA.

# ConPCR v's qPCR

| Province | Horses | | | Donkeys | | |
|---|---|---|---|---|---|---|
| | No. of samples | qPCR | ConPCR | No. of samples | qPCR | ConPCR |
| Free-State (n=12) | 10 | 5 | 5 | 2 | 2 | 2 |
| Mpumalanga (n=12) | 12 | 9 | 8 | NDSC | - | - |
| Northern Cape (n=12) | 8 | 3 | 3 | 4 | 2 | 2 |
| North West (n=12) | 8 | 2 | 2 | 4 | 2 | 1 |
| Total = 48 | 38 | 19 | 18 | 10 | 6 | 5 |
| Percentage (%) | - | 50.0 | 47.4 | - | 60.0 | 50.0 |

NDSC: no donkey samples collected

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# Discussion

- In this study, we report on the successful development of a LAMP and conPCR assays targeting the RIME gene.

- LAMP is simple to perform and highly specific.

- Different reaction temperatures were selected that vary from 60 to 65°C, still giving a good result (Data not shown).

- Amounts of LAMP products produced were detectable within 15 minutes of the reaction meaning that our LAMP assay can be conducted in 30 min maximum time.

# Discussion Cont.

- The detection limit of RIME-LAMP assay for serially diluted *T. equiperdum* DNA was 5.3 x $10^{-7}$ ng/µl which is equivalent to 0.001 trypanosomes/ml.

- conPCR using F3 and B3 primers detected 30/48 samples
  -Samples were sent to Inqaba biotech for sequencing to confirm that they are true *T. equiperdum.*

- conPCR had the detection limit of 5.3 x $10^{-5}$ ng/µl which is equivalent to 1 trypanosome/ml.

# Discussion Cont.

- LAMP showed slightly higher detection than conPCR with 70.8% and 62.5% respectively.

- SYBR green and probe based qPCR standardization with serially diluted *T. equiperdum* DNA had efficiency 92.7% & 94.9%, slope of -3.5 and R-value of 0.99.

  - The detection limit was 5.3 x $10^{-5}$ ng/µl for both assays but the specificity for qPCR assays was not stable.

# Discussion Cont.

- Melt-curve analysis was used to differentiate between between false positives and curves of other *Trypanozoon* species

- Real-time PCR showed higher detection than conPCR 52.1% and 47.9% respectively

  -The level of agreement was 0.917 between the assays

- These data sets suggest that both PCR techniques were equally effective for detection of *T. equiperdum*

# Conclusion

- Due to the absence of tsetse and other non-tsetse transmitted animal trypanosomes (*T. b. brucei, T. congolense, T. evansi and T. vivax*) in 8 out of 9 provinces of South Africa, the RIME-LAMP, conPCR and qPCR assays can be used as supplementary tools to clinical signs and microscopy for diagnosis of *T. equiperdum* infections in equines in South Africa.

# Acknowledgements


- Unit for Environmental Sciences and Management
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Obihiro University of Agriculture and Veterinary Medicine

National Research Foundation

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