Molecular characterization of South African *Ehrlichia ruminantium* field isolates and development of a qPCR assay for the evaluation of a multivalent inactivated vaccine

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Declaration

I hereby declare that this thesis which I submit for the degree Philosophiae Doctor at the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa is my original research work and has not been submitted by me to any institution or University for the conferment of an award.

…… …April 2023….

Zinathi Dlamkile Date

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Table of Contents

List of abbreviations

16S rRNA	16S ribosomal ribonucleic acid
β	Beta
°C	Degrees Celcius
δ	Delta
γ	Gamma
µg	Microgram
µl	Microliter
µM	Micromolar
ARC-OVR	Agricultural Research Council- Onderstepoort Veterinary Institute
BA	Bovine Aorta
bp	Basepairs
BHK	Baby hamster kidney
CO_2	Carbon dioxide
CyX	Cycloheximide
CPE	Cytopathic effect
D	Day
DALRRD	Department of Agriculture, Land Reform and Rural Development
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EC	Eastern Cape
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FA	Freund's adjuvant

GP	Gauteng province
G	Group
IFAT	Indirect fluorescent antibody test
IFN	Interferon
IL-2R	Interleukin 2 receptor
KZN	KwaZulu-Natal
LP	Limpopo
MAFFT	Multiple Alignment, Fast Fourier Transform
MAP1	Major antigenic protein 1
MDBK	Madin Darby bovine kidney
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum likelihood
MHC	Major histocompatabilty complex
ML	Multi locus
MLST	Multi-Locus Sequence Typing
MLVA	Multi locus variable number tandem repeat analysis
MP	Mpumalanga
NCBI	National Center for Biotechnology Information
NK	Natural killer
NW	North West
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Post challenge
PV	Post vaccination
REC	Research Ethics Committee

RFLP	Restriction fragment length polymorphism
RT qPCR	Reverse transcription quantitative real-time polymerase chain reaction
ST	Sequence Type
SPG	Sucrose Phosphate Glutamate
SNP	Single nucleotide polymorphism
US	United States
TNF	Tumor necrosis factor
UNG	Uracil-N-glycosylase
UP	University of Pretoria
UV	Ultra violet
V1	Variable1
VNTR	Variable number tandem repeat

List of figures

List of tables

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Thesis Summary

Heartwater is one of the major tick-borne diseases of both domestic and some wild ruminants in Africa and some Caribbean islands. The disease is caused by *Ehrlichia ruminantium*, a member of the phylum Proteobacteria and order Rickettsiales. *Amblyomma* genus ticks transmit *E*. *ruminantium*. Heartwater is a cause for concern, it challenges livestock farmers from introducing high producing exotic breeds to upgrade local stock. The exotic breeds are highly susceptible to *E*. *ruminantium,* and the mortality rate of susceptible animals ranges from 20 to 90% depending on the animal's species, breed, and previous exposures. *Ehrlichia ruminantium* has diverse genotypes and immunogenicity caused by recombination that occurs between different isolates in the field. The diversity of *E*. *ruminantium* isolates render the commercially available vaccine ineffective against some virulent isolates. A development of an inactivated vaccine that can cross protect against virulent isolates is crucial. To develop an effective vaccine researchers must, isolate field *E*. *ruminantium* by *in vitro* culture system to provide the antigen required for the vaccine, characterize the isolates by genotyping to determine the

possibility of their use in an inactivated cocktail vaccine. Then, the vaccine can be developed and tested. This study was therefore carried out to characterize South African *E*. *ruminantium* field isolates by Multi-Locus Sequence Typing (MLST) and develop a reverse transcription quantitative real-time polymerase chain reaction assay for the evaluation of a multivalent inactivated vaccine.

A total of 1004 DNA samples were extracted from *Amblyomma hebraeum* ticks collected from cattle in Mpumalanga, Limpopo, and KwaZulu-Natal provinces of South Africa, and were tested for *E*. *ruminantium* by pCS20 Sol1 qPCR. The *E*. *ruminantium* positive samples were characterized by MLST using five housekeeping genes. Out of 1004 samples tested, overall *E*. *ruminantium* infection rate was 22% and provincially 19%, 22% and 27% in Mpumalanga, KwaZulu-Natal and Limpopo respectively. Sequencing of the housekeeping genes showed genetic variation in the sequenced regions and phylogenetic analysis revealed three main lineages, clade 1 made up of worldwide isolates (eastern, southern Africa, and Caribbean isolates); clade 2 comprised only West African isolates; and clade 3 consisted of the unique South African isolates, Omatjenne, Kümm2, and Riverside. MLST can clearly distinguish the South African genotypes from the distinct West African genotype. The genetic variation in the sequenced regions does not change the makeup and function of the protein encoded by the genes.

In vitro culture using gut and salivary glands from *E*. *ruminantium* positive field ticks as an inoculum was initiated. First attempt of *E*. *ruminantium in vitro* culture, 19 days post inoculation, the cultures were *E*. *ruminantium* negative and at 25 days the cultures were contaminated. The second attempt had to be discontinued due to national lockdown caused by the worldwide corona virus pandemic. There is *E*. *ruminantium* positive tick material stored at -80 °C and that can be used in future to initiate *in vitro* culture of *E*. *ruminantium*. *Ehrlichia ruminantium in vitro* culture from tick material must be optimized and is a possible alternative for isolating field *E*. *ruminantium* without the need of infecting an animal. Also, the optimization of the method would make it easier to sequence

genomes of *E*. *ruminantium* field isolates and fast track the development of an efficient vaccine.

Methods to determine success of a developed vaccine need to be developed. One of the assays the current study developed was a reverse transcription quantitative real-time PCR (RT qPCR) assay to evaluate a multivalent inactivated vaccine against heartwater. This method requires selection and validation of housekeeping genes to obtain reliable results in immune markers for protection. Most housekeeping genes commonly used for qPCR analyses were selected from previous studies and the sheep homologues were selected from GenBank. The housekeeping genes were validated using Ct mean, CV of RPKM values and CT values and the ΔCt method to estimate the expression stability of each housekeeping gene. The genes were ranked for RT qPCR data normalization using an online tool, RefFinder. Immune markers for protection differentially expressed between surviving animals and animals that were treated (considered dead) were identified using RNA-seq data from a previous study.

The most suitable choice combination of housekeeping genes that showed the least expression variation throughout the experimental conditions is SDHA, RPL22, YWHAZ and GAPDH. The immune markers for protection were identified and were upregulated in animals that survived heartwater challenge: CD41–ITGA2B, CD156A-ADAM8, CXCR5, CD8B, CD5, GZMB-LIKE, IFN-γ and TGFB1. Based on the developed RT qPCR the immune markers for protection were differentially expressed throughout the experimental conditions. The upregulation of the identified immune markers for protection does not inform protective immunity against the disease.

In conclusion, the study revealed genetic diversity among *E*. *ruminantium* field isolates from *A*. *hebraeum* ticks in three provinces of South Africa. The RT qPCR assay developed to evaluate a multivalent inactivated vaccine against heartwater can be validated and

applied in further studies. Also, there is a need for alternative genotypic genes that reveal immunogenicity of isolates for identifying best vaccine isolates for the development of an effective vaccine against heartwater. Optimization of *E*. *ruminantium in vitro* culture using tick salivary gland and gut is important.

Chapter 1

Introduction and Literature Review

1.1 Introduction

Ehrlichia ruminantium, previously called *Cowdria ruminantium* causes heartwater disease in some wild and domestic ruminants (Cowdry, 1925). Ticks of the *Amblyomma* genus are responsible for transmitting the pathogen (Bezuidenhout, 1985). Heartwater occurs in different forms: peracute, acute, subacute, or chronic depending on the type and age of animal infected, how the animal was infected and pathogenicity of strain that infected the animal (Van de Pypekamp and Prozesky, 1987). Heartwater is typified by high fever, nervous signs, hydropericardium, hydrothorax, oedema of the lungs and brain (Allsopp, 2010).

Heartwater is fatal to susceptible animals with mortality rate of up to 90%. The susceptible rate varies depending on the type of animal and prior exposures (Mahan et al., 1992). Heartwater presents a major obstacle to livestock farmers from introducing high producing susceptible exotic breeds to upgrade local stock (Meltzer et al., 1996). According to Mukhebi et al. (1999) the disease is estimated to cost the Southern African Development Community an estimate of US$ 47.6 million per year. Crucial losses caused by heartwater include the cost of antibiotics and acaracides used to treat animals and decreased produce by farmers (CABI, 2020). A safe and effective method to control heartwater could reduce the negative economic repercussion exerted by the disease on farmers.

1.2 Historical background

On the 17th of February 1838, Louis Trichardt noted in his diary that his sheep suffered a fatal death due to a disease locally called “nintas” three weeks after the tick infestation they acquired (Bezuidenhout, 1987a). In 1877 at the Cattle and Sheep Disease Commission in Grahamstown, John Webb described a disease "boschsickness" (heartwater) fatal in sheep in the Eastern Cape (Provost and Bezuidenhout, 1987). John Webb was certain the disease was first observed in the Eastern Cape immediately after William Bowker found a bont tick on a cow that came from Zululand in 1837 (Bezuidenhout, 2009b).

In 1898, Edington and Dixton separately demonstrated that heartwater can be transmitted by inoculating blood from an infected animal to healthy susceptible animals (Provost and Bezuidenhout, 1987). Even though no organisms could be observed in the blood or tissues of the infected animal, it was suggested that heartwater was caused by a living microorganism believed to be a filterable virus (Provost and Bezuidenhout, 1987). It was Loundry in 1900 who confirmed the vector of heartwater as the bont tick, *Amblyomma hebraeum* in South Africa (Cowdry, 1925). The breakthrough of Edington and Dixton allowed the possibility to replicate laboratory heartwater infection, making it easy to define the disease. Subsequently, Cowdry was able to prove that heartwater is caused by a pathogen and named it *Rickettsia ruminantium* (Cowdry, 1925). In 1947, the organism was classified to the genus *Cowdria*, and renamed after Cowdry and called *Cowdria ruminantium* (Bezuidenhout, 2009a).

1.3 Classification of *E. ruminantium*

The causal agent of heartwater is an obligate intracellular bacterium formerly called *Cowdria ruminantium*. The heartwater causing agent was previously classified as a member of the family Rickettsiaceae, order Rickettsiales, tribe Ehrlichieae and genus *Cowdria*. With the emergence of molecular phylogenetics the order Rickettsiales was

reorganised into two families, Rickettsiaceae and Anaplasmataceae causing different assignment of the genera in those families (Allsopp, 2010). The genotypic results of 16S rRNA genes, *groESL* and surface protein genes, assigned the heartwater agent to the family Anaplasmataceae, genus *Ehrlichia* (Dumler et al., 2001).

Phylum: Proteobacteria

Class: Alphaproteobacteria

Order: Rickettsiales

Family: Anaplasmataceae

Genus: *Ehrlichia*

Species: *Ehrlichia ruminantium*

1.4 Life cycle of *E. ruminantium*

Certain *Amblyomma* species can disseminate *E*. *ruminantium* transstadially. However, *E*. *ruminantium* can be passed on transovarially even though it is not frequent as proven by Bezuidenhout and Jacobsz (1986). *Ehrlichia ruminantium* divides by binary fission within intracytoplasmic vacuoles (Pienaar, 1970). In culture, the organism has different morphological forms that vary in particle size: large colonies of reticulate bodies, minute moderate bodies indicated by an electron-dense core and electron-dense elementary bodies (Jongejan et al., 1991). Subsequent to infected blood meal by a tick, the pathogen is first observed in the gut epithelial cells and salivary gland acini cells eventually become infected (Kocan and Bezuidenhout, 1987a). The predominant form of organism in both the gut and salivary glands is reticulate bodies. The heartwater agent can be found in tick haemocytes and malpighian tubules (Kocan et al., 1987b).

In vertebrate host the organism is transmitted via saliva when the tick is indulging on blood meal and by regurgitation of previous meal constituents. Initial development of organisms appears to take place in reticulo-endothelial cells (Cowdry, 1926). The infected reticulo-endothelial cells finally disrupt, and the organisms escape into the general circulation to occupy endothelial cells of various organs and tissues (Du Plessis, 1975). Apart from endothelial cells, *E*. *ruminantium* is detected in the brain, followed by the kidneys. However, the organisms were saturated in the lungs in mice infected with the Welgevonden strain (Prozesky and Du Plessis, 1985).

1.5 Transmission

Ehrlichia ruminantium is transmitted by members of the three-host life cycle tick of the *Amblyomma* genus. There are twelve species of *Amblyomma* species found to be able of disseminating *E. ruminantium* and are outlined in table 1.1. Among the *Amblyomma* species in Africa, *A*. *variegatum* is the most broadly widespread vector (Walker and Olwage, 1987). Bezuidenhout (1985) attributes Lounsbury as the first to prove that *A*. *hebraeum,* is the principal vector of heartwater in South Africa. Infections in *A*. *hebraeum* ticks are transmitted transstadially even though a report by Bezuidenhout and Jacobsz (1986) proved that transovarial transmission does occur. Infection acquired in the vector's juvenile stage can be spread by both young and adult stages, despite the host for the juvenile not being diseased (Bezuidenhout, 1987a). A reptile tick, *A*. *dissimile* has been shown to experimentally transmit *E*. *ruminantium*. However, its role is insignificant in transmitting heartwater among ruminants but potentially serve as a source in the reptile inhabitants (Jongejan, 1992).

Table 1.1: *Amblyomma* tick species proven to be experimental and natural vectors of heartwater.

***Amblyomma* spp**	**Mode of transmission**
African	
A. hebraeum	I[a], II[b], III[c]
A. variegatum	I, II, III
A. pomposum	I, II
A. gemma	?[d]
A. lepidum	I, II
A. tholloni	I, II, III
A. sparsum	I
A. astrion	I, III
A. cohaerens	I, III
A. marmoreum	I, III
American	
A. maculatum	I,II, III
A. cajennense	I

[a]I from larval to nymphal stage

[b]II from nymphal to adult stage

[c]III from larval through nymphal to adult stage

[d]? stage(s) not reported

Table is from Bezuidenhout (1987a) and Walker and Olwage (1987) with modification.

1.6 Distribution

Heartwater occurs in regions where ticks from the *Amblyomma* genus capable of transmitting the organism are present (Petney et al., 1987). In southern Africa, the main vector of heartwater is *A. hebraeum* (Bezuidenhout, 2009b). The endemic areas for the vector includes sub-Saharan Africa, Madagascar, Reunion, Mauritius, Zanzibar, the Comoros Islands, and Sao Tomé and three Caribbean islands: Guadeloupe, Marie-Galante, and Antigua (Spickler, 2015). Figure 1 shows the distribution of some *Amblyomma* spp. capable of transmitting heartwater in the continent of Africa (Pascucci

et al., 2007). The vector occurs in bush veld and low veld, therefore in South Africa it is endemic in six of the nine provinces: Limpopo, Mpumalanga, Kwa-Zulu Natal, Gauteng, Eastern Cape and North West Province (Purnell, 1984).

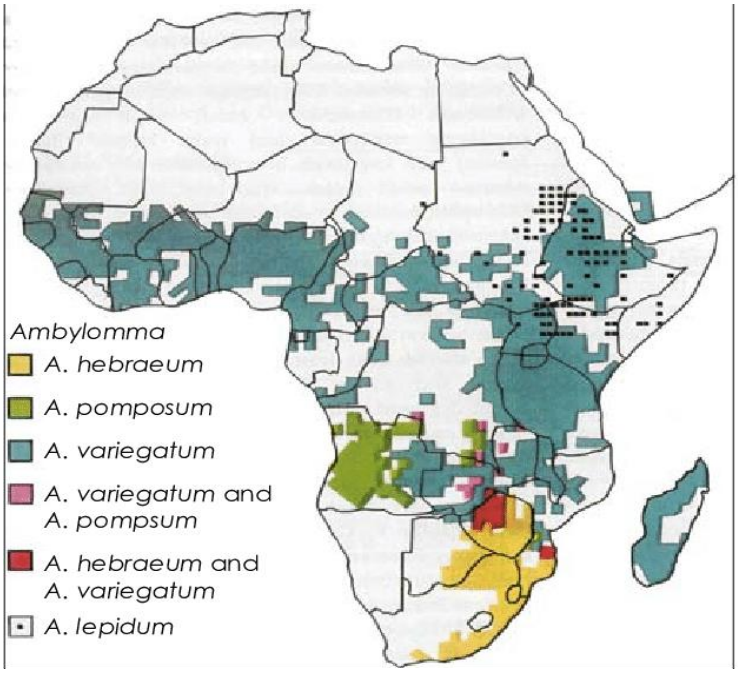

Figure 1: Distribution of some African vectors of heartwater (Pascucci et al., 2007).

1.7 Laboratory Diagnosis

The laboratory diagnosis of heartwater in live animals was filled with hurdles until the development of molecular techniques for detection of *E. ruminantium* infection. Laboratory diagnosis of heartwater was by a serological test called indirect fluorescent antibody test (IFAT). The IFAT target antigen was the Kümm stock in peritoneal macrophages in infected mice (Du Plessis and Malan, 1987a). The test cross reacted with *Ehrlichia* and *Anaplasma* spp causing error in result (Du Plessis and Malan, 1987b). Following IFAT, a competitive enzyme linked immunosorbent assay (ELISA) was developed using a monoclonal anti-major antigenic protein 1 (MAP1) antibody (Jongejan et al., 1991b). This test also gave false positive reactions with related *Ehrlichia* spp (Katz et al., 1996). To remedy the problem, the competitive ELISA was modified by making use of a partial fragment of MAP1 called MAP1B in an indirect ELISA (van Vliet et al., 1995).

There was improvement in the specificity of the test compared to previously developed serological tests. However, MAP1B ELISA cross reacted with *E*. *canis* and *E*. *chaffeensis* (Katz et al., 1996).

With the advent of molecular techniques, the problem of cross reaction was solved with the development of probes targeting the pCS20 region, the small subunit ribosomal RNA (16S RNA) gene, or the *map*1 gene. The 16S rRNA has been extensively used as a taxonomic and phylogenetic tool for classifying microorganisms (Allsopp, 2010). The pCS20 region is best used for the detection of *E*. *ruminantium* while the *map*1 is best used to provide immunotypic information about *E*. *ruminantium* isolates (Allsopp et al., 1998).

1.8 Characterization of *E*. *ruminantium* strains

Heartwater is a disease that affects the economy in sub-Saharan Africa, Madagascar, and some Caribbean islands (Mukhebi et al., 1999). The disease presents a great challenge to the improvement of livestock and productivity in the farming sector (Provost and Bezuidenhout, 1987). Heartwater is controlled by use of acaricide to control tick infestation, antibiotic therapy of cases showing symptoms of the disease, prophylactic use of antibiotics and immunization (Yunker, 1996). The commercially available vaccine is made by cryopreserving blood from an infected sheep with *E*. *ruminantium* strain Ball3 (Van der Merwe, 1987). The vaccine lacks ability to elicit protective immunity against all genotypes of *E*. *ruminantium*. The lack of cross protection is caused by the wide genetic and antigenic distinction of *E*. *ruminantium* strains in the field (Allsopp, 2010). Therefore, it is necessary to characterize strains of *E*. *ruminantium* to pave a path for development of a more efficient vaccine.

Different tools to characterize *E*. *ruminantium* strains have been developed and are mostly based on conserved regions. Markers that have been used to characterize *E*.

ruminantium strains include the small subunit (16S) ribosomal RNA, pCS20, *map*1 and core function genes.

1.8.1 Small subunit (16S) ribosomal ribonucleic acid

The16S ribosomal ribonucleic acid is a good marker for strain characterization because it is present in all pathogens, support precise comparative sequence alignments to be created and has species-specific variations (Collins et al., 2002). The 16S rRNA has been successfully used to characterize strains of *E*. *ruminantium* and closely related *Ehrlichia* species (Mahan, 1995a, Allsopp et al., 1999, Allsopp et al., 2007). There are eight known 16S genotypes of *E*. *ruminantium* that are intimately related to each other when compared to other *Ehrlichia* species. These are Mara 87/7, Pretoria North, Ball3, Welgevonden, Gardel, Senegal, Crystal Springs and Omatjenne (Collins et al., 2002).

Phenotypic behavior of heartwater isolates, in terms of their ability to elicit protective immunity against other isolates and establishment in culture vary. *Ehrlichia ruminantium* stocks having different phenotypes also reveal differences in their 16S rRNA genes (Allsopp, 2010). However, it has been demonstrated that the 16S rRNA gene is unable to discriminate between non-pathogenic from pathogenic isolates (Allsopp et al., 2003). The difference of the 16S rDNA sequences between the Senegal and Crystal Springs (Zimbabwe) isolates served as earliest proof of genetic diversity within the *Cowdria* genus (Dame et al., 1992, Van Vliet et al., 1992). Allsopp et al. (1997) targeted the highly variable V1 loop region of the 16S rRNA gene to characterize organisms associated with heartwater and two new *E*. *ruminantium* genotypes (Ehrlichia spp Ball3 (U03777), Omatjenne (U03776)) and one *Ehrlichia* sp. (Germishuys (U03776)) were determined. There is no *E*. *ruminantium* homologous sequence for Germishuys, instead a close relation to *Ehrlichia* 16S sequence similar to that of *E. canis* is observed. The Germishuys was isolated from a naturally tick challenged infected sheep and results of brain smear examination and pathology, revealed that the sheep seemed to be suffering from heartwater. The initial isolation of the Omatjenne genotype was by challenging a mouse

with a lysate of a *Hyalomma truncatum* tick collected from apparently healthy cattle that tested positive for heartwater in a farm free of heartwater in Namibia (Du Plessis, 1990). The Crystal Springs (Allsopp et al., 1997) and Mara87/7 (Allsopp et al., 1999) genotypes are predominantly found in heartwater rampant areas in South Africa, and the Omatjenne genotype is prevalent, both in and out of the heartwater rife areas (Allsopp et al., 1997).

Small-subunit rRNA molecules are structurally and functionally preserved amongst all living microorganisms and availed a practical initial way for phylogenetic categorization of organisms. However, 16S gene differences cannot be relied upon as an exclusive method for genotyping, therefore Wayne et al. (1987) suggested that before defining different genotypes, whole genomic DNA/DNA hybridization variables should be ascertained.

1.8.2 pCS20

Another DNA probe that has been utilized for *E*. *ruminantium* strain characterization is the pCS20 probe, a cloned piece of DNA. The first pCS20 probe was developed by Waghela et al. (1991) to identify *E*. *ruminantium* from *Amblyomma variegatum* ticks. The approximately 1300 bp fragment is specific for *E*. *ruminantium* and results in no cross reaction with other *Ehrlichia* species (Allsopp, 2010). When compared to other available probes for testing for positivity of *E*. *ruminantium*, the pCS20 is the most highly sensitive (Allsopp et al., 1999, Allsopp et al., 1998). The ability of the method to detect even the minute amount of pathogen was boosted by making new primers and probes (Van Heerden et al., 2004). With the new primers and probes it was possible to obtain sequences of the pCS20 region of fourteen different *E*. *ruminantium* isolates and revealed high conservation of the West African isolates compared to more variation amongst southern African isolates (Van Heerden et al., 2004). The probe has been extensively used for detection of *E*. *ruminantium* in domestic animals, ticks and wildlife species (Guo et al., 2019). The pCS20 probe is suggested as the probe of choice for initial screening of *E*. *ruminantium*. However, the probe fails to differentiate between the various *E*.

ruminantium genotypes (Allsopp et al., 2007) and cannot differentiate between virulent and avirulent isolates (Allsopp et al., 2003). It is advised that the 16S probes must be used if inter species phylogenetic information is required (Allsopp et al., 1999).

1.8.3 Major Antigenic Protein gene (*map*1)

Ehrlichia ruminantium expresses an immunodominant protein, major antigenic protein 1 (MAP1), located on the surface of the organism. This protein was used as a serodiagnostic tool of heartwater (van Vliet et al., 1995). The gene encoding this protein is designated *map*1. A subunit serological assay using recombinantly expressed MAP1 protein proved its ability to recognize *E. ruminantium* antibodies in sera from domestic ruminants that were exposed to several different *E*. *ruminantium* isolates. However, the test cross reacted with other *Ehrlichia* spp, limiting its use in serodiagnosis of heartwater (Reddy et al., 1996). Although the *map1* gene exhibit high degree of sequence polymorphisms between isolates, it does not provide any genotypic information (Allsopp et al., 1999). The *map*1 gene is an unsuitable candidate for use as a typing marker to provide a phylogeographic correlation between *E*. *ruminantium* strains (Allsopp et al., 2001, Cangi et al., 2016).

1.8.4 Multi Locus Sequence Typing (MLST)

Multi Locus Sequence Typing (MLST) is a technique that investigate nucleotide sequence features from various conserved structural or functional genes known as housekeeping genes to obtain consolidated alleles defined as a sequence type (ST). These STs allows for differentiation of isolates and give precise distinction with enough resolution for pathogen characterization without the need of full-genome sequencing (Sullivan et al., 2005). MLST is an extensively used system to characterize pathogens molecularly (Maiden, 2006). Mayer et al. (2002) supports the use of MLST and says “MLST enables the genotypic characterization of isolates and the study of the global dispersion of some new variants of pathogens”. Additionally, the information deduced from MLST method is relevant to the application of evolutionary and population studies (Jolley et al., 2000). It is

believed that MLST lacks ambiguity, is portable, reproducible, has high differentiation abilities to distinguish isolates and can be automated (Sullivan et al., 2005).

The first MLST scheme for *E*. *ruminantium* based on eight different housekeeping genes (*gltA*, *groEL*, *lepA*, *lipA*, *lipB*, secY, *sodB* and *sucA*) was developed by Adakal et al. (2009). The same tool was used by Nakao et al. (2011) on a group of reference strains and field sample originating from different geographical areas. Based on studies of Nakao et al. (2011) and Adakal et al. (2010), *sodB* was the less variable gene between the investigated strains, conversely, the gene with many variable sites was *secY*. The MLST scheme was able to differentiate between closely related strains and a great level of genetic dissimilarity among the examined strains was observed. However, no notable relation was observed amongst genotypes and geographic origins. Though the phylogeography of *E*. *ruminantium* isolates is unclear, *E. ruminantium* isolates cluster into two major groups: Group1 (West Africa) and Group2 (worldwide) which encompasses West, East, and southern Africa, Indian Ocean, and Caribbean strains when using MLST (Nakao et al., 2011, Cangi et al., 2016).

1.8.5 Other tools

Additional tools that have been used to characterize *E*. *ruminantium* isolates include restriction fragment length polymorphism (RFLP) and multi locus (ML) variable number tandem repeat (VNTR) analysis (MLVA). RFLP entails DNA amplification with primers, PCR products are then digested with restriction enzymes and visualized by gel electrophoresis (Cheriyedath, 2019). Genotypic diversity within West African and southern Africa *E*. *ruminantium* isolates was revealed by the clustering of *map*1 restriction profiles. de Villiers et al. (2000) used *SmaI* and *KspI* restriction enzymes on DNA and could not differentiate among the four West African isolates examined, Kerr Seringe, Senegal, Sankat 430 and Pokoase 417. Faburay et al. (2008) suggested that alternatively, the exact selected amplified gene, *map*1 must be used rather than whole genomic DNA. Although the *map*1 restriction profiles showed genotypic diversity, the

profile did not reveal any geographical clustering and clusters resulting from the RFLP method did not correspond with cross protection with other strains of *E*. *ruminantium* (Faburay et al., 2008).

A complete genome sequence of the Welgevonden isolate proved that *E*. *ruminantium* has many tandem repeated sequences (Collins et al., 2005). Multi locus variable number tandem repeat analysis assay is based on direct polymerase chain reaction (PCR) amplification of specific loci that are repeated elements (Yazdankhah and Lindstedt, 2007).The first development of MLVA based on VNTR structures, for the purpose of characterizing the heterogeneity of *E*. *ruminantium* was by Pilet et al. (2012). The tool revealed a great level of variables among the tested strains, with 10 different profiles out of 13 *E*. *ruminantium* strains. The VNTR profiles could not show any differences between virulent and attenuated strains.

1.9 *In vitro* propagation of *E. ruminantium*

E. *ruminantium* is an intracellular parasite dependent on a living host for survival and growth (Bell-Sakyi, 2004). Cultivation of *E*. *ruminantium* outside its host animal has resulted in the production of large quantities of relatively pure organisms, attenuation of the organism for use in immunization, preparation of antigen for serological tests and screening of chemotherapeutics (Jongejan et al., 1980a). Initial reports on *in vitro* propagation of *E*. *ruminantium* met with failure or limited success (Bezuidenhout et al., 1985). However successful isolation and propagation of *E*. *ruminantium* in endothelial cells (Bezuidenhout et al., 1985) and tick cells (Bell-Sakyi et al., 2000) have been reported.

1.9.1 *In vitro* propagation in tick cell lines

Isolation and cultivation of tick-borne pathogens in tick cell lines is an important tool to investigate the interactions between tick cells and disease-causing pathogens (Bell-Sakyi

et al., 2007). Currently there are 63 cell lines made from 18 ixodid and argasid tick species and one each from the sand fly *Lutzomyia longipalpis* and the biting midge *Culicoides sonorensis* in a biobank established by Bell-Sakyi et al. (2018).

The first successful attempt at short term propagation of *E*. *ruminantium* in primary cultures of tick cells from moulting nymphs of *A*. *hebraeum* and *A*. *variegatum* dates to 1974 (Bell-Sakyi, 2004). Continuous growth of the Gardel stock of *E*. *ruminantium* was established in *Ixodes scapularis* cell line IDE8 for over 500 days by Bell-Sakyi et al. (2000). Additionally, a *Rhipicephalus appendiculatus* cell line RAN/CTVM3 was infected with the Gardel stock of *E*. *ruminantium* that was initially cultured in *I*. *scapularis* cell line IDE8 (Bekker et al., 2002a). Bell-Sakyi et al. (2002) successfully tested the capacity of different tick cell lines from non-vector (*I*. *scapularis*, *I. ricinus*, *Boophilus decoloratus*, *B. microplus* and *R*. *appendiculatus*) tick species to support growth of *E. ruminantium*, proving that *E*. *ruminantium* can be propagated in cells from other tick genera. Also, the absence of the elementary bodies in tick cell culture, the infective stage of *E*. *ruminantium* in ruminants and endothelial cell cultures is an indication that *E*. *ruminantium* develops differently in vectors from ruminant host cells (Bell-Sakyi et al., 2000). Furthermore, *E*. *ruminantium* cultivated in ruminant host cell and vector cell cultures exhibit different pathogenicity and immunogenicity for animals they were tested on (Bell-Sakyi et al., 2002).

1.9.2 *In vitro* propagation in mammalian cell lines

Jongejan et al. (1980b) initiated primary kidney cell cultures derived from heartwater diseased goats. The goats were infected by intravenously injecting varying period old primary kidney cultures and the goats suffered from heartwater symptoms. Despite this success, Jongejan and authors could not detect *E*. *ruminantium* when the kidney cell cultures were Giemsa stained and the cultures were not pathogenic. Bezuidenhout et al. (1985) established the first successful cultivation of *E*. *ruminantium* by making use of bovine umbilical cord endothelial cells. Following the success of Bezuidenhout et al.

(1985), other authors used endothelial cells of different kinds of ruminants, and from varying anatomical areas to cultivate *E*. *ruminantium* and they are ovine pulmonary artery (Byrom et al., 1991), bovine endothelial cells isolated from brain microvasculature (Martinez et al., 1993, Totté et al., 1993). In addition, Smith et al. (1998) cultivated *E*. *ruminantium* in endothelial cells of three species of African wild mammals, sable antelope (*Hippotragus niger*), buffalo (*Syncerus caffer*) and eland (*Taurotragus oryx*) suggesting that *in vitro* cultivation of *E*. *ruminantium* is possible in these species and may be natural hosts of the organism. Furthermore, Logan et al. (1987) successfully established primary neutrophil culture of *E*. *ruminantium* that can be an alternate method to infect secondary cell lines.

1.9.3 Inoculum

The type of inoculum used to isolate and cultivate *E*. *ruminantium* plays a significant role in an *in vitro* culture system (Bezuidenhout et al., 1985). Different inoculums have been used in both the tick cell and mammalian cell culture systems. The inoculum used in the first successful continuous growth of *E*. *ruminantium* in tick cell line IDE8 was elementary bodies obtained from a supernatant of an infected bovine pulmonary endothelial cell culture (Bell-Sakyi et al., 2000). Other researchers used infected endothelial cells from various anatomical sites to infect tick cells (Bekker et al., 2002a, Bell-Sakyi et al., 2002). The possibility of using infected tick cells as an inoculum to infect other tick cell lines has been reported (Bell-Sakyi et al., 2002, Bell-Sakyi, 2004). It was only later, that *in vitro* isolation of *E*. *ruminantium* from sheep blood (leukocyte fraction of the blood) into tick cell (IDE8) cultures was reported (Zweygarth et al., 2008).

A stabilate prepared from *A*. *hebraeum* nymphae was used as an inoculum to inoculate a calf endothelial cell line (Bezuidenhout et al., 1985). Two years later, Bezuidenhout (1987b) used infected choroid plexus, freshly drawn heparinized blood, liver and spleen suspensions made from moribund mice to initiate cultures in endothelial cells. A heparin-derived plasma was successfully inoculated in bovine and ovine vascular endothelial

cells, proving that *E*. *ruminantium* is present in plasma of infected animals (Byrom et al., 1991).

Apart from the tick-derived stabilate prepared by Bezuidenhout et al. (1985), there is no literature on the use of tick material (gut and salivary glands) as an inoculum in isolation and growth of *E*. *ruminantium* in endothelial and tick cell *in vitro* culture systems.

1.9.4 Culture media

The base of culture medium used in cultivating *E*. *ruminantium* in tick cells is L-15 Leibovitz medium. Bell-Sakyi et al. (2000) supplemented the medium with 10% tryptose phosphate broth (TPB), 5% heat inactivated fetal calf serum, 0.1% bovine lipoprotein and 100 Units/ml penicillin and 100 µg/ml streptomycin as choice of antibiotics for the continuous growth of *E*. *ruminantium* in IDE8 cell line. Dulbecco's Modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12) containing 15 mM HEPES and 1.2 g/l sodium bicarbonate used for infected and uninfected endothelial cell cultures can also be used for maintaining tick cells provided it is supplemented with the above mentioned nutrients (Zweygarth et al., 2008).

The first *in vitro* culture of *E*. *ruminantium* in an endothelial cell line was achieved using Eagle's medium containing 10% bovine serum, sodium benzylpenicillin, streptomycinsulphate and fungizone (Bezuidenhout et al., 1985). Other media that have been used include Glasgow minimal essential medium (GMEM) (Bezuidenhout, 1987b), Leibovitz L-15 supplemented with 0.45% glucose (Byrom and Yunker, 1990), Dulbecco's minimal essential medium (DMEM) and RPMI 1640 (Martinez et al., 1993). The media is supplemented with varying amounts of foetal bovine serum and tryptose phosphate broth (TPB). However, *E*. *ruminantium* has been successfully isolated *in vitro* using serum and TPB free medium supplemented with bovine lipoproteins and transferrin (Zweygarth et al., 1997).

1.9.5 Propagation of *E. ruminantium* in cell cultures

Endothelial cells inoculated with *E*. *ruminantium* are incubated at 37°C with 5% CO_2 and tick cells are incubated at 30 ± 2°C without CO_2 with weekly medium change (Bekker et al., 2002a). Post inoculation, the cultures are monitored for cytopathic effect (CPE) that occurs in a form of a line across the monolayer of endothelial cells (Byrom et al., 1991). When infected cells are observed under an inverted microscope, the line consists of spherical, refractile cells that have space in the middle where a portion of cells had detached. In contrast to endothelial cells, tick cells exhibit very little obvious CPE (Bell-Sakyi et al., 2000). The cytopathic effect is evident 3-14 days post inoculation depending on the type of inoculum used to infect the cells. Using tick stabilate as an inoculum, *E*. *ruminantium* colonies were first observed 11 days after inoculation and passaged after 4-7 days of visible CPE (Bezuidenhout, 1987b). When plasma of infected ruminants was used to infect bovine aorta and bovine pulmonary artery endothelial cells, *E*. *ruminantium* colonies were observed after 11-14 days (Byrom et al., 1991). However, when bovine brain capillary endothelial cells were inoculated with supernatant of infected bovine umbilical endothelial cells the CPE was detected in 7 days and was passaged 8 days after initial inoculation (Martinez et al., 1993).

Bell-Sakyi et al. (2000) observed *E*. *ruminantium* on day 21 post inoculation of an IDE8 cell line from a non-vector species infected with supernatant from infected bovine pulmonary artery endothelial cells. The first and second subcultures were carried on days 51 and 66 respectively and rickettsiae was detected within 12 and 9 days respectively after subculture. An *A*. *variegatum* cell line inoculated with supernatant from infected bovine pulmonary artery endothelial cells exhibited *E*. *ruminantium* infection after 9-10 weeks (Bell-Sakyi, 2004). *Ehrlichia ruminantium* infection was detected in 18 and 9 days when IDE8 cells were infected using leukocytes isolated from sheep inoculated with Ball3 and Welgevonden stocks of *E*. *ruminantium* respectively (Zweygarth et al., 2008).

Heavily infected cultures are harvested for sub-cultivation by means of trypsin (Bezuidenhout, 1987b) or cell scraper that can be either a Pasteur pipette curved at the narrow end (Zweygarth et al., 1997) or a sterile 21 gauge needle with a bent tip (Zweygarth and Josemans, 2001) to form a small hook. A 21-gauge needle is utilized to separate clumps and disintegrate the cells. The cell suspension is centrifuged (800 x g for 10 minutes) to separate the elementary bodies from the cells and the supernatant is distributed into culture flasks containing confluent monolayer of uninfected cells (Byrom and Yunker, 1990).

1.9.6 Detection of *E. ruminantium* in cell cultures

Cultures are observed for common well-being and development of *E. ruminantium* weekly by microscopic examination. Cultures showing CPE are sampled by removing a small portion of the cell monolayer with a sterile bent 21-gauge needle to make a smear on slide. The slide is air-dried, fixed using methanol, stained with Giemsa (Bezuidenhout, 1987b) or RapiDiff (Zweygarth and Josemans, 2003) or Leukostat (Byrom and Yunker, 1990) and examined for cytoplasmic inclusions (colonies) typical of *E. rurninantium*. The organisms appear as small and dark colonies and as they multiply the colonies become larger and less dense. Ultimately the colonies disrupt and give rise to individual elementary bodies (Byrom et al., 1991).

1.10 Immune response to *E. ruminantium* and vaccine development

Plotkin (2008) states that “cell mediated immune functions play a critical role in protection against intracellular infections, and in almost all diseases, $CD4^+$ T cells are necessary to help B cell development”. Since *E. ruminantium* is a pathogen dependent on a cell for its survival, protective immunity to heartwater in vertebrate host is believed to be essentially cell mediated (Du Plessis et al., 1991) although *E. ruminantium* antibodies are detected (Mahan et al., 1995b) in serum and colostrum and are likely to play a role in immunity (Deem et al., 1996). Therefore, production of a heartwater vaccine that elicits a cellular immune response is important. In South Africa, the available method of vaccination

against heartwater is the “infection and treatment” method that involves intravenously inoculating sheep blood infected with Ball3 strain of *E*. *ruminantium* and subsequently treating with tetracycline (Oberem and Bezuidenhout, 1987). Although the vaccine has been useful in controlling the disease, it has its disadvantages. Alternative vaccines have been explored with inactivated, attenuated, and DNA vaccines receiving more attention.

1.10.1 Inactivated vaccines

Inactivated vaccines are vaccines that contain organisms that have been grown in cell culture and rendered non-viable before vaccination. Inactivated vaccines are mixed with an adjuvant, a medium that supplements an accurate immune response by an antigen when utilized in conjunction with specific vaccine antigens (Schmidt and Lenz, 2014). A number of adjuvants have been tested with an *E*. *ruminantium* antigen: Freund's adjuvant (FA) (Mahan et al., 1994), Montanide ISA50 (Martinez et al., 1996), and Quil A (Mahan et al., 1998). It was found that FA has inflammatory side-effects and is commercially not acceptable making it unsuitable for use in the field (Martinez et al., 1996). Montanide ISA50 is an adjuvant with acceptable safety, has comparable efficacy as FA and is the choice of adjuvant to formulate an inactivated vaccine in (Mahan et al., 1998).

Martinez et al. (1994) reported the first successful use of heartwater inactivated vaccine made from elementary bodies of *E*. *ruminantium* (Gardel strain) mixed with FA. The vaccine protected more than 50% of the immunized goats against a homologous intravenous challenge as opposed to 100% mortality of the negative control group. Mechanisms of immune resistance on the survivors were not determined but the authors hypothesized that the protection is T cell mediated. The hypothesis that immune response to *E*. *ruminantium* is T cell mediated was proved by Totté et al. (1997). Totté et al. (1997) observed *E*. *ruminantium* specific T cell cultures initiated from animals vaccinated with inactivated elementary bodies in FA and restimulated with *E*. *ruminantium* lysate to be 95-100% $CD4^+$ T lymphocytes, major histocompatabilty complex (MHC) class II restricted and produce interferon gamma (IFN-γ). Also, activation of $CD8^+$ T cells to a reduced

degree compared to $CD4^+$ T cells was observed from *E*. *ruminantium* stimulated peripheral blood mononuclear cells (PBMC) collected from goats immunized by an inactivated vaccine (Gunter et al., 2002, Esteves et al., 2004a). However, $CD8^+$ T cells need the assistance of $CD4^+$ T cells to express IFN-γ (Esteves et al., 2004b).

IFN-γ has been reported to suppress multiplication of *in vitro E*. *ruminantium* (Totté et al., 1993). Even though IFN-γ reduces growth of *E*. *ruminantium in vitro*, infected endothelial cells treated with IFN-γ fails to generate cell surface expression of MHC class II molecules (Totté et al., 1996). The core function of MHC class II molecule is antigen peptide presentation on the cell surface for recollection by antigen presenting cells to stimulate $CD4^+$ T cells, causing the coordination and regulation of effector cells (Wieczorek et al., 2017). Therefore, if the immune cells fail to present the antigen, there will be no immune protection mounted to fight the infection. Totté et al. (1996) suggests that *E*. *ruminantium* produces inhibitory factors that minimizes the ability of endothelial cells to produce MHC class II molecules.

Additionally, PBMC from vaccinated goats with inactivated *E*. *ruminantium* in ISA adjuvant expresses increased interleukin 2 receptor (IL-2R) after antigenic recall *in vitro* (Gunter et al., 2002). Upon activation, IL-2R is momentarily indicated on T cells surface (Manning and Nakanishi, 1996). Binding of IL-2 to IL-2R activates the functions of IL-2 which include activation of the cytotoxic function of natural killer (NK) cells, T lymphocytes, monocytes, and enhance the secretion of IFN-γ as well as tumor necrosis factor-beta (TNF-β) (Skupin-Mrugalska, 2019).

More than one field isolate that has been tested and found to offer broad protection against virulent isolates can be included in an inactivated vaccine to improve the protective capability of the vaccine. Also, through the breakthrough of inactivated vaccines, antigens that elicit protective immune response have been determined; and

have made it possible for developing a subunit vaccine against the disease (Mahan et al., 1999).

1.10.2 Attenuated vaccines

Another type of vaccine that has received interest is the attenuated vaccine. An attenuated vaccine consists of live, whole pathogen particles with reduced virulence, causing no clinical disease but stimulate an immune response (Mak and Saunders, 2006). The possibility of using an attenuated heartwater vaccine was proven by Jongejan (1991a) using the Senegal isolate. This isolate was the first to be attenuated *in vitro* through passages on bovine umbilical endothelial cells (Jongejan, 1991a). The vaccine protected 100% of goats and sheep against a virulent homologous needle challenge. However, the protection levels were less satisfactory with a decrease to 75% when the same vaccine was tested against a natural field tick challenge (Faburay et al., 2007, Adakal et al., 2010). The Senegal strain has been found to attenuate faster than other *E. ruminantium* strains due the observed attenuator gene *ntrX* (Gordon et al., 2023). The attenuation in the Senegal strain take place by gene conversion, causing a 4 base pair deletion which is exclusivel to the Senegal strain. Collins et al. (2003) attenuated the virulent Welgevonden isolate through passages in a canine macrophage monocyte cell line DH82 and recorded a 100% protection against virulent homologous needle challenge. Since the cell line DH82 is cancerous, the Welgevonden isolate was re-adapted to grow in bovine endothelial cells and has been reported to provide cross protection against a needle challenge of Ball3, Gardel, Mara87/7, Kwanyanga and Blaauwkrans isolates (Zweygarth et al., 2005). However, the attenuated Welgevonden vaccine is yet to be tested against a natural field tick challenge. With all the effort directed in heartwater attenuated vaccines, there is no study that reports the cellular immune response conferred by attenuated vaccines.

1.10.3 Recombinant vaccines

The reports of inactivated *E*. *ruminantium* organisms to stimulate an immune response suggested that live organism is not a requirement for a vaccine to confer protective immunity. Therefore, presenting a possibility for the development of defined recombinant antigens as vaccines. Recombinant vaccines are proteins or DNA recombinants capable of stimulating an immune response, that are inserted into bacterial or mammalian cells, and express the antigen in these cells (Mukkeed, 2022). Recombinant vaccines are affordable to manufacture; eliminate the need of cold chain storage and can be utilized to halt an outbreak in areas where a disease is not endemic (Allsopp, 2015).

Recombinant vaccines made from DNA recombinants has been investigated and shown to offer protective immunity (Nyika et al., 1998, Pretorius et al., 2007, Tshilwane et al., 2019). Splenocytes from mice immunized with the *map*1 gene vaccine proliferated in response to *E*. *ruminantium* antigen and secreted IFN-γ and IL-2. The protective capacity of the DNA vaccine was improved by protein boost against a homologous challenge and significantly protected the mice (Nyika et al., 2002). Pretorius et al. (2007) tested four *E*. *ruminantium* open reading frames as a DNA vaccine and the vaccine protected the sheep against a homologous needle challenge.

Proteins of *E*. *ruminantium* as possible vaccine components have been investigated. Sebatjane et al. (2010) investigated proteins of the five open reading frames different from those investigated by Pretorius et al. (2007) for potential use as vaccine. Liebenberg et al. (2012) identified *E*. *ruminantium* proteins that stimulate responses mediated by $CD4^+$ T cells and have been found to be protective. Thema et al. (2016) found that secreted proteins induce a Th1 immune response which characterizes the *E*. *ruminantium* protective immune response.

1.11 Justification

E. *ruminantium* causes heartwater in cattle, sheep, goats and some wild ruminants Raliniaina et al. (2010). According to World Organization for Animal Health, heartwater is

a notifiable disease and is one of the major causes of livestock loss in sub-Saharan Africa (Steyn, 2022). Heartwater is an obstacle to livestock farmers who want to introduce high yielding breeds to their farms to upgrade their breed due to their susceptibility to the infection with 20-90% mortality (Allsopp, 2015). *Ehrlichia ruminantium* has diverse immunogenicity with recombination occurring between different genotypes giving rise to new strains in the field (Allsopp, 2010). The diverse immunogenicity of *E*. *ruminantium* has proved to be a challenge for the development of new vaccines against heartwater. The "infection and treatment" vaccination technique currently in use in South Africa is laborious as daily monitoring of rectal temperature of the animal after vaccination is mandatory, the necessary use of antibiotics after immunization, the mandatory intravenous injection of the vaccine by a trained personnel, the necessity to keep a cold chain for the distribution of the vaccine posing a challenge for rural area farmers who do not have such facilities; and it does not cross protect against all virulent strains. Development of a more practical vaccine against heartwater is important for the future control of the disease. An inactivated multivalent cocktail vaccine may offer cross protection against virulent *E*. *ruminantium* field isolates (EU-funded MuVHA [Multivalent inactivated Vaccine against Heartwater in Africa]). Also, a clear understanding of the immune markers of protection against the agent is an important requirement for the achievement of this goal. Therefore, there is a need for characterization of new isolates and be tested as a multivalent inactivated cocktail vaccine for protection against virulent genotypes of *E*. *ruminantium* in the field.

1.12 Aim and Objectives

The aim of the study is to characterise new *E*. *ruminantium* field isolates and develop a reverse transcription quantitative real-time PCR assay (RT qPCR) for the evaluation of a multivalent inactivated vaccine. The aim will be achieved through the following objectives:

- Collection of *A*. *hebraeum* ticks from cattle
- Isolate and characterize *E*. *ruminantium* from *A*. *hebraeum* ticks using MLST

- Initiate *E*. *ruminantium in vitro* culture using tick salivary glands and gut
- Develop a RT qPCR for the evaluation of a multivalent inactivated vaccine

1.13 References

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Chapter 2

Characterization of *Ehrlichia ruminantium* from field *Amblyomma hebraeum* ticks collected from cattle in three South African provinces using Multi-Locus Sequence Typing

2.1 Abstract

Heartwater is one of the major tick-borne diseases of both domestic and some wild ruminants in Africa and some Caribbean islands. The disease is caused by *Ehrlichia ruminantium*, a member of the class Alphaproteobacteria and order Rickettsiales. The diversity of *E*. *ruminantium* isolates render the commercially available vaccine ineffective against some virulent isolates. To better understand the *E*. *ruminantium* genotypes circulating in South Africa, a total of 1004 *A*. *hebraeum* tick DNA samples from cattle in three South African provinces were tested by pCS20 Sol1 qPCR and characterized by Multi-Locus Sequence Typing (MLST) using five housekeeping genes. Out of 1004 samples tested, 222 (22%) were positive for *E*. *ruminantium*. The occurrence of *E*. *ruminantium* in the Mpumalanga, KwaZulu-Natal and Limpopo provinces was 19%, 22% and 27%, respectively. The *E*. *ruminantium* positive samples were screened for five housekeeping genes and sequenced. From the five housekeeping genes investigated, *lipB* and *sodB* were the most conserved. Phylogenetic analysis revealed three main lineages: clade 1 made up of worldwide isolates (eastern, southern Africa, and Caribbean isolates); clade 2 comprised only West Africa isolates; and clade 3 consisted of the unique South African isolates, Omatjenne, Kümm2, and Riverside. Genetic variation in the sequenced regions was observed in the form of single nucleotide polymorphisms (SNPs). These variations did not alter the function of the proteins encoded by the housekeeping genes. Using MLST to characterize *E*. *ruminantium* field isolates reveals genetic diversity and allows the South African genotypes to be clearly distinguished from the distinct West African isolates.

Key words

Erhlichia ruminantium, heartwater, characterisation, pCS20, MLST, phylogenetic, PCR

2.2 Introduction

Ehrlichia ruminantium, previously called *Cowdria ruminantium* is the causative agent of heartwater in some wild and domestic ruminants (Bezuidenhout, 2009). Heartwater occurs in regions where ticks from the *Amblyomma* genus capable of transmitting the organism are present (Petney et al., 1987). In South Africa, *E*. *ruminantium* is transmitted by *A*. *hebraeum* and in sub Saharan Africa, Indian ocean islands and the Caribbean predominantly by *A*. *variegatum* (Walker and Olwage, 1987). Heartwater is endemic in six of the nine provinces of South Africa: Limpopo (LP), Mpumalanga (MP), KwaZulu-Natal (KZN), Gauteng (G), Eastern Cape (EC) and North West Province (NW) (Purnell, 1984). Livestock farmers interested in introducing high producing susceptible exotic breeds to upgrade local stock are hindered by heartwater as mortality rate of susceptible animals ranges from 20 to 90% (Mahan et al., 1992).

Currently there is no efficient vaccine against heartwater, the commercially available vaccine in South Africa consists of sheep blood infected with the Ball3 strain and employs the “infection and treatment” vaccination method (Van der Merwe, 1987). However, the Ball3 vaccine does not cross protect against all virulent and field isolates. The limited cross protection is a result of high genetic diversity caused by recombination of *E*. *ruminantium* giving rise to new isolates (Cangi et al., 2016). A prerequisite to develop effective control strategies of heartwater is the comprehension of genetic diversity of *E*. *ruminantium* strains. Different methods of genotyping *E*. *ruminantium* have been investigated and are mostly based on conserved molecular markers. Allsopp et al. (2003) used the genes *groESL*, citrate synthase (*gltA*), 16S rRNA and pCS20 (a cloned DNA fragment containing two partial reading frames) to phylogenetically characterize *E*. *ruminantium* isolates. The sequences of *groESL*, *gltA* and pCS20 revealed SNPs spread throughout the sequenced regions (Allsopp et al., 2003). In addition to the above

mentioned markers, Allsopp and Allsopp (2007) used the functional genes (*rnc*, *ctaG*, *ftsZ*, *nuoB* and *sodB*) to characterize *E*. *ruminantium* isolates from different geographic origins. Isolates from southern and eastern Africa grouped together while isolates from western Africa and one from southern Africa clustered together revealing a great genetic variability between southern and eastern African *E*. *ruminantium* isolates.

Multi Locus Sequence Typing (MLST) is a technique broadly used for molecular characterization of pathogens (Maiden, 2006). Adakal et al. (2009) developed a MLST scheme for *E*. *ruminantium* using eight housekeeping genes (*gltA*, *groEL*, *lepA*, *lipA*, *lipB*, *secY*, *sodB* and *sucA*). The same technique was used by Nakao et al. (2011) on various reference strains and field samples from different geographic origins. Based on both studies, *sodB* was the most conserved gene between the strains investigated, conversely, the gene with many variable sites was *secY*. The MLST scheme was able to differentiate among closely related strains and significant level of genetic variations among the examined strains was observed. *Ehrlichia ruminantium* isolates cluster into two main groups: Group1 (West Africa) and Group2 (worldwide) which is represented by West, East, and southern Africa, Indian Ocean, and Caribbean strains when using this method (Nakao et al., 2011, Cangi et al., 2016). The aim of the study is to characterize *E*. *ruminantium* field isolates currently circulating in three South African provinces using MLST.

2.3 Ethical considerations

The study was approved by the research ethics committee and part of the study that involves animals was performed in accordance with the stipulation of the Animal Ethics Committee at the University of Pretoria, Faculty of Veterinary Science (research ethical clearance and animal ethical clearance number: REC205-19). Permission to conduct the study in terms of Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) was granted by Department of Agriculture, Land Reform and Rural Development (DALRRD) (reference number 12/11/1/1/6).

2.4 Materials and Methods

2.4.1 Study areas

Sample collection areas are shown in Figure 2.1 DNA extracted from ticks collected in MP in Utha and Welverdiend dip tanks was obtained from Mazhetese et al. (2022). Ticks were collected from cattle in Moddergat and Geodere-S dip tanks in MP. In LP, ticks were collected from cattle in Koedoeskop dip tank, Motlhabane Colchester 3 animal camp and a commercial farm in Groblersdal. In KZN, ticks were collected from cattle at the following dip tanks: Cecelia, Uthukela and Mpungamhlophe.

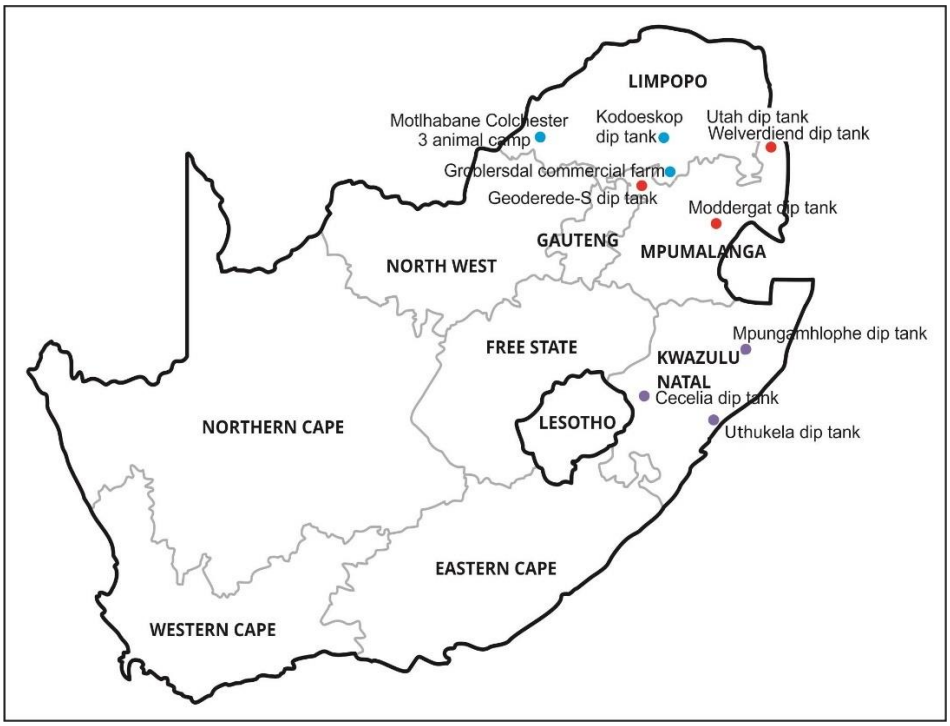

Figure 2.1: Map of South Africa showing where sample collection areas (coloured dots) are situated in the provinces of Limpopo, Mpumalanga and KwaZulu-Natal.

2.4.2 Tick collection

Tick sampling was carried out between October 2020 and January 2022 where rainfall was expected to increase and tick population to have been abundant. *Amblyomma*

hebraeum ticks were handpicked from cattle with permission from the cattle owners at dip tanks. About 3-5 adult ticks were handpicked from each animal and the minimum number of cattle sampled per collection site was 20. Collected ticks were kept alive in a humid environment and transported to the Research and Training laboratories, Department of Veterinary Tropical Diseases, University of Pretoria adhering to the biosafety rules and regulations stipulated by the Section 20 permit (DALRRD). Using a stereo microscope the ticks were identified based on entomological keys by Walker et al. (2003). Microscopy was done to correctly identify *A*. *hebraeum* ticks to be tested.

2.4.3 DNA extraction

Individual ticks were washed with 70% ethanol, rinsed with distilled water then air dried in a laminar flow. DNA was extracted from two legs of a tick using Qiagen DNeasy Blood and Tissue kit according to the manufacturer's instructions. Two legs were cut off close enough to the body of the tick to get the hemolymph from the tick and cut into small pieces in 180 µl lysis buffer (ATL). The rest of the tick was kept alive for gut and salivary glands extraction and preserved in sucrose phosphate glutamate buffer for possible culturing (see chapter 3 methods section 3.3.2) for vaccine development and genome sequencing if the tick is positive for *E*. *ruminantium*. The proteinase K digestion was carried out at 56 °C overnight. The genomic DNA was eluted from the column with 100 µl elution buffer. The DNA was kept at -20°C for further analysis.

2.4.4 Detection of *E. ruminantium* using pCS20 qPCR

Detection of *E*. *ruminantium* was performed by targeting a conserved genomic DNA fragment known as the pCS20 region using the TaqMan real time polymerase chain reaction (qPCR) previously described by Cangi et al. (2017). Each reaction was performed in a final volume of 25 µl containing 2 µl DNA as template, a final concentration 0.25 µM each primer (pCS20 Sol1 Forward and pCS20 Sol1 Reverse in Table 2.1), 0.2 µM probe (Sol1 TM probe in table 2.1) and TaqMan Universal PCR MasterMix 1X. Thermal cycling consisted of 1 cycle of uracil-N-glycosylase (UNG) incubation at 50 °C for 2 minutes and 1 cycle of AmpliTaq Gold pre-activation at 95 °C for 10 minutes. This

was then followed by 40 cycles of denaturing at 95 °C for 15 seconds and annealing at 55 °C for 1 minute and kept at 4 °C.

2.4.5 Characterization of *E. ruminantium* isolates using MLST

MLST was conducted on pCS20 qPCR positive samples using the housekeeping genes *lipA*, *lipB*, *secY*, *sodB*, *sucA* to characterize *E. ruminantium* field samples (Cangi et al., 2016). Each housekeeping gene was amplified using conventional PCR with the primers listed in table 2.1. PCR amplification was performed in a 20 µl reaction with 1X Phusion Flash High Fidelity PCR Master Mix (Thermo Fisher Scientific, Lithuania), 2.5 µl template DNA and 0.5 µM final concentration of each primer (each primer set with its annealing temperature is indicated in table 2.1). The PCR program consisted of 94 °C for 3 minutes; followed by 40 cycles of denaturing at 94 °C for 50 seconds, primer annealing (see temperature in table 2.1) for 50 seconds and extension at 72 °C for 50 seconds, followed by a final extension of 72 °C for 10 minutes. The PCR products were run on a 1.5% agarose gel stained with ethidium bromide and visualised with UV light illumination and photography. The PCR products were sequenced at Inqaba Biotec using Sanger sequencing.

Table 2.1: Oligonucleotides used for pCS20 qPCR and PCR amplification of housekeeping genes.

Primers/Probe	Sequence 5'- 3'	Annealing temperature °C	Product size (bp)
pCS20 Sol1F	ACAAATCTGGYCCAGATCAC	55	280
pCS20 Sol1 R	CAGCTTTCTGTTCAGCTAGT		
Sol1 TM probe	6-FAM-ATCAATTCACATGAAACATTACATGCAACTGG-BHQ1		
lipA F	GGATCCTCATGAGCCTCAAA	62	430
lipA R	CTGCCGACCTTAAATCATCCATA		
lipB F	TGGAAGATTGAATCCTTACCTG	59	452
lipB R	CCATGATATGTTATCCATTTTC		
secY F	CCAGGCATTAATCCAGATGT	61	690
secY R	GCGGAACATATGTAGATGCAGT		
sodB F	TGCCAGAACTGCCTTATCAA	61	507
sodB R	AAGCGTGTTCCCATACATCC		
sucA F	TGAAAGGCTTTGGCTACAGG	59	566
sucA R	CCTGACCAATTACAGCAGCA		

2.4.6 Sequences and phylogenetic analysis

Sequences obtained from the housekeeping genes were processed using CLC Genomics Workbench version 7.5.1 (CLC Bio, Boston, MA, USA). The homologous sequences of the five genes were identified in the genome sequences available from NCBI (https://www.ncbi.nlm.nih.gov/genome/microbes/): Welgevonden (NC_005295.2/ CR767821.1), Gardel (NC_006831.1/CR925677.1), Blaauwkrans (CP063043), Grootvallei (CP040120), Kwanyanga (CP040119), Mara87/7 (CP040118), Nonile (CP040117), Springbokfontein1 (CP040116), Springbokfontein2 (CP040115), Springbokfontein4 (CP040114), Springbokfontein5 (CP040113), Springbokfontein6 (CP040112), Springbokfontein7 (CP040111), Um Banein (CP063044), Crystal Springs (BDDK01000001 to BDDK01000034), Senegal (NZ_MQUJ00000000.1), Sankat 430 (BDDN01000001 to BDDN01000183), Kümm2 (CP033456), Omatjenne (CP033455), and Riverside (CP033454), as well as the incomplete genome sequence of Ball3 (personal data, property of Dr Junita Liebenberg).

Alignments of sequences from the housekeeping genes were created using the Multiple Alignment, Fast Fourier Transform (MAFFT) [version 7] program (Katoh and Standley, 2013) and subsequently modified using BioEdit (version 7.2.5) (Hall, 1999). A phylogenetic tree was constructed using concatenated nucleotide sequences of the housekeeping genes, *sodB*, *secY*, *lipB*, and *lipA* partial sequences, using the maximum likelihood (ML) method in MEGA7. The credibility for the internal branches was evaluated using bootstrapping (100 bootstrap replicates) (Felsenstein, 1985). Phylogenetic trees were constructed using MEGA7 and edited on Paint Tool for Windows 10.0.

2.4.7 Statistical analysis

Distributional patterns for the occurrence of *E*. *ruminantium* were described for each province and for study areas within each province. We determined how provinces and study areas differed in their occurrence using the non-parametric Kruskal-Wallis test. The

provinces and study areas were the independent variables while the occurrence served as the dependent variable. To measure the difference between the various provinces and study areas within the provinces, a post hoc test (Tukey's Honest Significant Difference) was performed at a significance level of 5% ($P<0.05$). All statistical analyses were performed in R Console version 3.2.1.

2.5 Results

2.5.1 Tick collection

The collected ticks were verified to be *A*. *hebraeum*. A total of 1004 DNA samples were extracted from *A*. *hebraeum* ticks collected from cattle in MP, LP and KZN provinces of South Africa, and were tested for *E*. *ruminantium*.

2.5.2 Detection and prevalence of *E*. *ruminantium* by pCS20 qPCR

The number of ticks collected in each locality and that of pCS20 positive ticks are shown in table 2.2. *Ehrlichia ruminantium* DNA was successfully amplified from 222/1004 ticks of *A*. *hebraeum* using the pCS20 Sol1 qPCR assay that amplifies a 280 bp gene fragment of all *E*. *ruminantium* (Figure 2.2). Occurrence of *E*. *ruminantium* was 19%, 22% and 27% in ticks collected in MP, KZN and LP provinces respectively. The overall *E*. *ruminantium* infection rate in ticks collected in the three *E*. *ruminantium* endemic provinces of South Africa was 22%. The positivity rate was the highest in samples collected in Moddergat dip tank followed by Motlhabane Colchester animal camp (43% and 34% respectively).

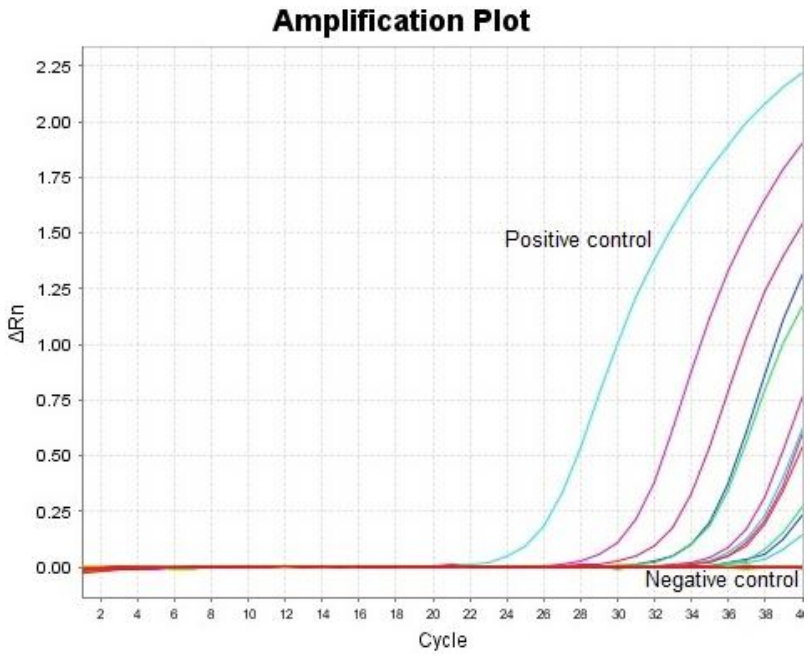

Figure 2.2: Amplification plot of *E*. *ruminantium* specific qPCR assay targeting the pCS20 gene fragment.

Comparing all the provinces together, the Kruskal-Wallis test showed that even though MP had the highest occurrence, this was not significant (p>0.0946). Also, the post hoc test across the provinces showed no significant difference (Figure 2.3). Pairwise comparison across the study areas showed significant differences between all the study areas in MP (P<0.005), study areas within LP province (p<0.005), but no significant differences between the study areas in the KZN Province (p>0.005; Figure 2.3).

Table 2.2: Occurrence of *E*. *ruminantium* in *A*. *hebraeum* ticks collected from cattle in the study areas.

Sample collection areas	Samples processed (n)	Number (%) pCS20 qPCR positive
Mpumalanga		
Utha	100	8 (8%)
Welverdiend	150	20 (13%)
Moddergat	58	25 (43%)
Geoderede-S	87	22 (25%)
Total	**395**	**75 (19%)**
Limpopo		
Groblersdal	28	4 (14%)
Koedoeskop	102	23 (23%)
Motlhabane Colchester	136	46 (34%)
Total	**266**	**73 (27%)**
KwaZulu-Natal		
Cecelia	140	31 (22%)
Uthukela	83	19 (23%)
Mpungamhlophe	120	24 (20%)
Total	**343**	**74 (22%)**

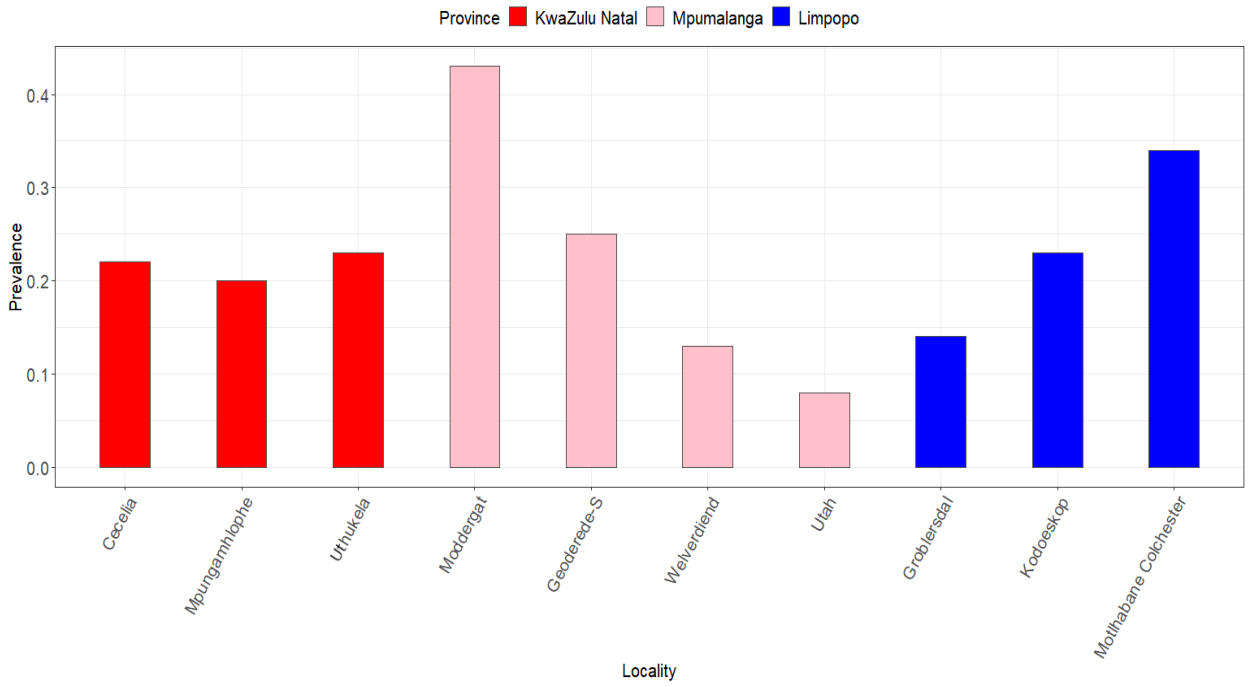

Figure 2.3: Bar graph showing the occurrence of *E*. *ruminantium* in *A*. *hebraeum* ticks collected from cattle in the study areas.

2.5.3 Characterization of *E. ruminantium* isolates using MLST

All the 222 pCS20 positive samples were tested for the five housekeeping genes (*lipA*, *lipB*, *secY*, *sodB*, *sucA*) to characterize the *E*. *ruminantium* field isolates. For some samples, amplification was not successful for all the MLST genes, which limited the possibility of obtaining sequences for all loci of all samples and some samples had multiple unspecific amplification. A total of 524 amplicons (all loci) were sent for sequencing: *lipA* = 133; *lipB* = 109; *sodB* = 125; *secY* = 98; *sucA* = 59. Out of the 28 samples from Utah and Welverdiend dip tanks, 14 allowed for the successful amplification all the housekeeping genes, with *sodB* amplicons available in 27 samples.

All the amplicons of samples for which the housekeeping genes were amplified were sequenced (Inqaba Biotechnical Industries (Pty) Ltd) and found to be 99.4-100% identical to selected reference genes: Ball3, Nonile, Kwanyanga, Mara87-7, Grootvallei,

Welgevonden, Crystal Springs, Kiswani, Omatjenne, Kümm2, Riverside Springbokfontein 1; 2; 4; 5; 6; 7 for each gene (see table 2.3) (Altschul et al., 1990). Out of the 133 *lipA* amplicons sent for sequencing 15 samples could not meet standard requirements for Sanger sequencing. Some samples had bad sequences and could not be analyzed further: *lipA* =19, *sodB* = 2, *secY* = 8 and *sucA* = 17. Since the identity of some samples were the same, a small number of sequences was included in the construction of the phylogenetic tree.

Table 2.3: Number of samples with percentage similarity identity with reference strains according to each housekeeping gene.

Gene	No of samples	% Similarity	Reference isolates
	23	100	SBF1, SBF4, Ball3
	17	100	Crystal Springs, Mara87-7, SBF2
lipA	15	99.7	Nonile, SBF1, SBF4, Ball3
	5	99.7	Grootvallei, SBF6, SBF7
	40	99.5	Kwanyanga
	52	100	Kwanyanga, Nonile, SBF1, Ball3
	24	100	Mara87-7, SBF2, SBF5
lipB	28	100	Grootvallei, SBF4, SBF6, SBF7
	6	100	Sao Tome
	1	99.8	Kwanyanga, Mara87-7, SBF2
	1	99.7	Mara87-7, SBF2, SBF5
	50	100	Grootvallei, Nonile, SBF1
sodB	36	100	Mara87-7, SBF2
	37	100	Kwanyanga, Blaauwkrans

Gene	No of samples	% Similarity	Reference isolates
	39	100	Grootvallei, SBF4, SBF6
	12	100	Nonile, SBF1, Ball3
	27	100	Kwanyanga, Mara87-7, SBF2
	2	99.8	Grootvallei, SBF4, SBF6
secY	1	99.8	Omatjenne, Kümm2, Riverside
	1	99.8	Nonile, SBF1, Ball3
	1	99.8	Kwanyanga, Mara87-7, SBF2
	4	99.6	Grootvallei, Nonile, SBF1
	3	99.6	Grootvallei, Kwanyanga, Mara87-7
	12	100	CHIP026MH1
	8	100	Kwanyanga
sucA	8	100	Grootvallei, SBF4, SBF6
	8	100	Nonile, SBF1, Kiswani
	3	99.8	Grootvallei, SBF1, SBF6
	3	99.8	Nonile, SBF1, Kiswani

A consistent pattern of SNPs extending throughout the sequenced genes was observed for all genes. Using concatenated nucleotide sequences of housekeeping genes, *sodB*, *secY*, *lipB*, and *lipA*, a MLST phylogenetic tree was constructed using only samples that successfully amplified all the four genes. The alignment of the concatenated sequences used to construct the tree is shown in Appendix 1. The *sucA* gene was only successfully amplified in a small portion of the samples and was excluded from the MLST analysis. Phylogenetic tree topology showed three clades: clade 1 (southern and East African and Caribbean isolates), clade 2 (West African isolates) and clade 3 (unique South African isolates: Omatjenne, Kümm2, and Riverside). It was observed that some samples were not identical to any of the reference isolates (samples blocked in red in Figure 2.4). However, they were all found to belong to the southern and East African/worldwide clade

(clade 1). No province-specific grouping was found among the analysed field strains, suggesting that similar isolates are found all over South Africa.

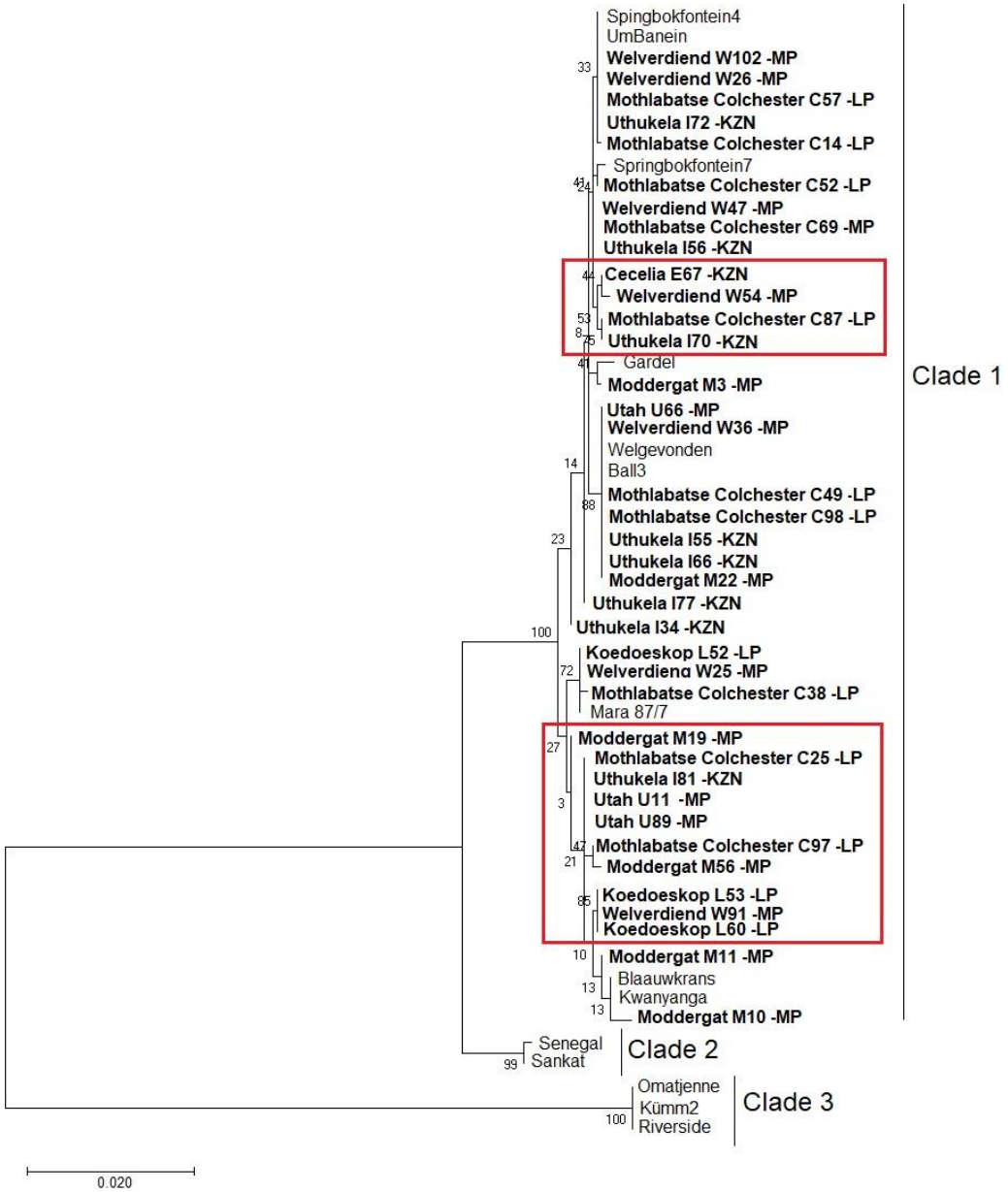

Figure 2.4: Phylogenetic tree showing relationship between *E*. *ruminantium* field isolates from three South African provinces and global reference isolates. The phylogenetic tree was constructed based on concatenated nucleotide sequences of housekeeping genes (*sodB*, *secY*, *lipB*, and *lipA* partial sequences), using MEGA7 and maximum likelihood method. Sequences from this study are shown in bold font. Bootstrap values are

represented as the numbers shown at the nodes and they were calculated from 100 replicates.

2.6 Discussion

Heartwater, caused by *E*. *ruminantium* is one of the major causes of livestock loss in sub-Saharan Africa with mortalities up to 90% (Allsopp, 2015). The high genetic diversity of *E*. *ruminantium* is a hindrance to the development of an efficient vaccine (Cangi et al., 2016). Understanding the genotypic characteristics of *E*. *ruminantium* isolates currently circulating is a prerequisite to developing an efficient vaccine.

In this study, we screened *A*. *hebraeum* ticks obtained from cattle in three provinces of South Africa to characterize circulating *E*. *ruminantium* isolates. The overall occurrence of *E*. *ruminantium* in the three South African provinces is 23% which is higher than Cameroon, Benin and Mozambique (6.6%, 6% and 15%) respectively; (Esemu et al., 2018, Guo et al., 2018, Matos et al., 2019). All the above mentioned authors used the nested pCS20 PCR to detect *E*. *ruminantium*. Mtshali et al. (2015) reported the infection rate of *A*. *hebraeum* ticks collected in KZN to be slightly over 25%, which is similar to the 22% reported in the current study. Mtshali et al. (2015) used the loop-mediated isothermal amplification (LAMP) assay to test *E*. *ruminantium* infection in *A*. *hebraeum* ticks. Guo et al. (2019) did not detect any *E*. *ruminantium* from *A*. *hebraeum* ticks collected in Msinga Mountainview dip tank, an area that is surrounded by the three sample collection areas of our study in KZN. However, in the current study, the overall occurrence of *E*. *ruminantium* in sample collection sites in KZN was 22%. A possible explanation for the discrepancy in these findings might be that Guo et al. (2019) may have missed low parasitaemia positive ticks as they used conventional nested pCS20 PCR, which is less specific and less sensitive than pCS20 Sol1 qPCR (Cangi et al., 2017), which was used in our study.

Altogether, there was genetic variation in the five analyzed housekeeping genes among the *E*. *ruminantium* field isolates. The topology of the phylogenetic tree had three *E*. *ruminantium* clades; clade 1 consisting of isolates from the current study; southern and East African and Caribbean (Gardel); clade 2 with exclusively West African isolates and clade 3 with the unique South African isolates. The most conserved genes were *lipB* and *sodB* as all the analysed isolates were 100% identical to previously known *E*. *ruminantium* isolates. The *sodB* gene was also highly conserved in *E*. *ruminantium* isolates obtained from *A*. *variegatum* samples in Burkina Faso (Adakal et al., 2010) and Uganda (Nakao et al., 2011), and it has been suggested that *sodB* might be a promising target for a molecular diagnostic test to identify *E*. *ruminantium* (Nakao et al., 2011). Adakal et al. (2010) found *lipB* to have the most polymorphic sites out of the genes used in our study, however, the current study found *lipB* to be one of the most conserved genes.

Study isolate E29 from Cecelia dip tank, KZN was observed to be 99.8% identical to *E*. *ruminantium* isolates, Omatjenne, Kümm2 and Riverside, and grouped with the same isolates for *secY* gene (circled red in Figure 2.5). The same phylogenetic tree topology of the three isolates (Omatjenne, Kümm2 and Riverside) grouping together was observed by Steyn and Pretorius (2020) and Liebenberg et al. (2020). Omatjenne genotype was isolated from a *Hyalomma truncatum* female tick collected from cattle in the heartwater free Otjiwarongo district of Namibia and contributed to the heartwater seropositivity of cattle in the area. Initially, the isolate was apathogenic until it was passaged through three generations of *A*. *hebraeum* and caused a disease similar to heartwater in sheep (Du Plessis, 1990). The same Omatjenne genotype was detected in blood from healthy sheep in LP, South Africa, which was seropositive for heartwater (Allsopp et al., 1997). In our study Kümm2 clustered with Omatjenne implying they are indeed closely related and concur with the findings of Zweygarth et al. (2002).

Kümm2 isolate is a component of the Kümm isolate and has an exact 16S rDNA genotype of Omatjenne isolate (Zweygarth et al., 2002). The isolate was obtained from naturally

infected goats in Rust de Winter, an area bordering LP and northern Gauteng provinces in South Africa (Du Plessis, 1981). The Riverside isolate was obtained from the blood of a sick Angora goat in one of the heartwater endemic areas in South Africa, in the EC province in a farm called Riverside situated in Makhanda (formerly known as Grahamstown) (Steyn and Pretorius, 2020).

Omatjenne, Kümm2 and Riverside genotypes can be initiated *in vitro* in the tick cell line IDE8, but not on bovine endothelial cells (Liebenberg et al., 2020) and they lack certain open reading frames which are present in other *E*. *ruminantium* isolates (Allsopp et al., 2003). Liebenberg et al. (2020) found variations in the membrane protein families of Omatjenne, Kümm2 and Riverside which may play a critical role in their ability to be propagated in other cells. Study isolate E29 may exhibit these unique genotypic characteristics since it is closely related to the three isolates. These genotypes were previously found in the EC (Steyn and Pretorius, 2020) and borders of LP and northern Gauteng provinces (Du Plessis, 1981) in South Africa. In our study, the unique genotype was observed in KZN. The movement of animals from one province to another, allowed by trade, may have played a role in disseminating the unique genotype provincially or some isolates have evolved.

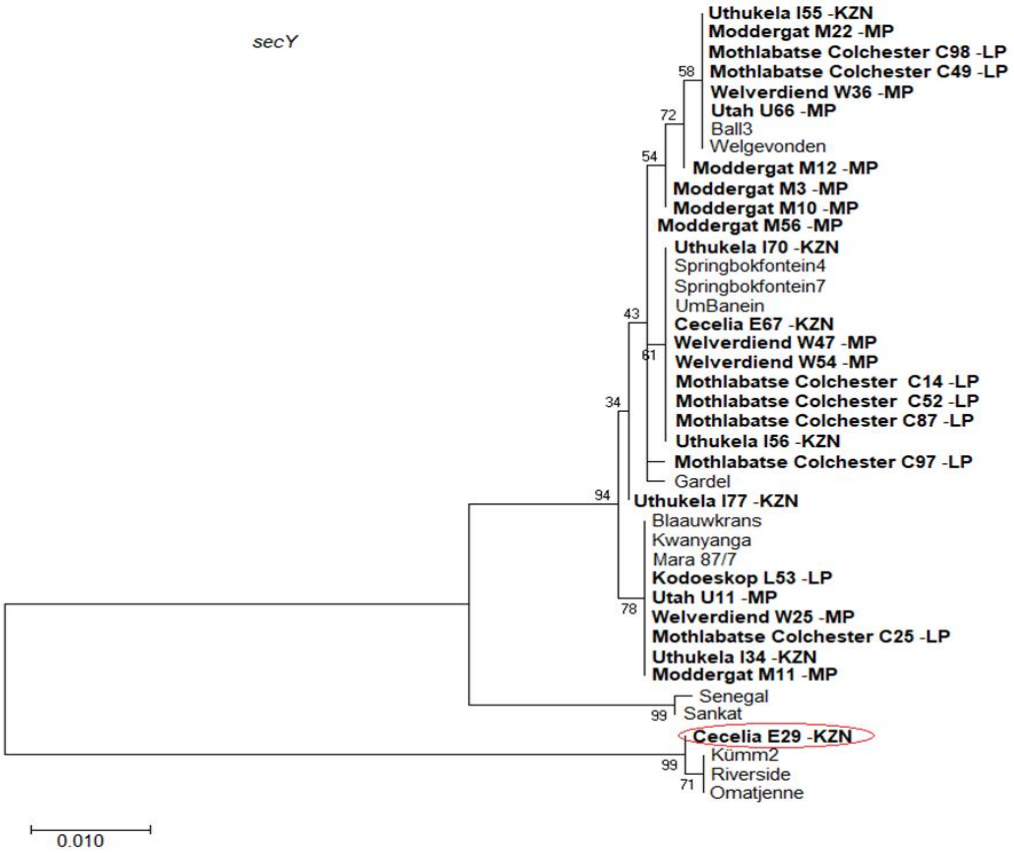

Figure 2.5: Phylogenetic tree showing relationship between *E*. *ruminantium* field isolates from three South African provinces and global reference isolates based on *secY* gene. Study isolate E29 from Cecelia dip tank KZN province circled in red clustered with the unique South African isolates. The numbers at the nodes represents bootstrap values that were calculated from 100 replicates.

2.7 Conclusion

Using the MLST scheme to characterise *E*. *ruminantium* isolates reveals genetic diversity, SNPs throughout the sequenced regions, and three main lineages. The SNPs do not change the makeup and function of the protein encoded by the genes. The three main lineages, one made up of the worldwide isolates, the other comprising of only West African isolates and the last one consisting of the unique South African isolates remains throughout the years despite the occurrence of recombination or evolution of isolates in the field. Since there are many genotypes at any location at any given time, especially in

southern Africa, there is a need for regular surveillance to understand the driving force of cross protection or lack thereof between *E*. *ruminantium* isolates. MLST can clearly distinguish the South African genotypes from the distinct West African genotype and to the best of our knowledge this is a first report of using a panel of housekeeping genes to characterize *E*. *ruminantium* field isolates from ticks in three South African provinces.

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Chapter 3

In vitro culture of *Ehrlichia ruminantium* using tick salivary glands and gut

3.1 Abstract

Ehrlichia ruminantium causes heartwater, an infectious, non-contagious disease in some domestic and wild ruminants. Heartwater disease is controlled by vigorous use of acaricides and live blood vaccine. The blood vaccine offers limited protection against some virulent *E*. *ruminantium* isolates and highlights the need for development of effective vaccines. The antigen used in an inactivated vaccine development is obtained by *in vitro* culture of *E*. *ruminantium*. The conventional method of propagating *E*. *ruminantium in vitro* employs *E*. *ruminantium* blood stabilate which is obtained by infecting a blood donor animal. There is a need for developing an alternative method of propagating *E*. *ruminantium in vitro* without the requirement of a blood donor animal. Therefore, this study is aimed at initiating *in vitro* culture of *E*. *ruminantium* using salivary glands and gut from *E*. *ruminantium* positive field ticks. Salivary glands and gut were extracted from *E*. *ruminantium* positive ticks and preserved in sucrose phosphate glutamate buffer. Confluent monolayer of bovine aorta endothelial cells in a 6-well cell culture plate was inoculated with the tick material and incubated at 37°C with 5% CO_2. The cell cultures were examined for cytopathic effect using inverted microscope and were examined for *E*. *ruminantium* by quick staining with Rapi-Diff II stain Rapid and tested by pCS20 Sol1 qPCR 19 days post inoculation. Microscopic examination from the cell culture plate showed no cytopathic effect. Dark purple colonies like *E*. *ruminantium* elementary were observed after staining. However, the cell cultures were *E*. *ruminantium* negative by pCS20 Sol1 qPCR. The cell cultures had microbial contamination 23 days post inoculation. A second attempt to initiate *E*. *ruminantium in vitro* culture was cut short due to the South African lockdown caused by the worldwide corona virus pandemic. *Ehrlichia ruminantium in vitro* culture from tick material could be a better alternative for isolating various *E*. *ruminantium* genotypes circulating in the field without the need of infecting an

animal. Therefore, there is a need to optimize this cell culture system and its optimization could provide an easier way for creating a biobank of *E*. *ruminantium* isolates that could be used for development of efficient heartwater vaccines.

Key words

Ehrlichia ruminantium, tick salivary glands, gut, *in vitro*, optimization, heartwater

3.2 Introduction

Ehrlichia ruminantium is a pathogen that causes heartwater, an infectious, non-contagious disease of cattle, sheep, goats, and some wild ruminants such as the springbok (Oberem and Bezuidenhout, 1987). *Amblyomma* genus ticks are responsible for disseminating heartwater and in southern Africa it is *A*. *hebraeum* and *A*. *variegatum* that transmit the disease (Bezuidenhout, 2009). Infected susceptible animals suffer from high fever and central nervous signs that leads to death within a few hours or days (Allsopp, 2010). Heartwater is prevalent in regions where the tick vector capable of transmitting the pathogen is present. The endemic areas includes sub-Saharan Africa, Madagascar, Reunion, Mauritius, Zanzibar, the Comoros Islands, and Sao Tomé and three Caribbean islands: Guadeloupe, Marie-Galante, and Antigua (Spickler, 2015).

Incubation of heartwater is about 10 to 30 days post infectious tick bite and in most cases an instant increase in body temperature presents as an initial symptom (Kocan and Bezuidenhout, 1987a). The multiplication of the parasite is at its peak during the febrile reaction when the temperature of the animal reaches 40°C. Infected animals suffer from fever, heavy breathing, loss of appetite, diarrhoea, repeated blinking of the eyes, hypersensitivity to touch and once recumbent they exhibit pedalling movements (Allsopp, 2010). Autopsy reveal hydrothorax and hydro-pericardium with oedema in the lungs.

Tetracycline antibiotics have been found to be efficient when administered immediately after the rise in body temperature (Van Amstel, 1987). Vaccination is one of the strategies

used to fight against heartwater. The commercially available vaccine in South Africa consists of sheep blood infected with the live Ball3 isolate and employs the “infection and treatment” vaccination method (Van der Merwe, 1987). However, the Ball3 vaccine does not cross-protect against all isolates due to high genetic diversity of *E*. *ruminantium* that give rise to new isolates (Cangi et al., 2016).

Etiological studies of heartwater suggest that *E*. *ruminantium* parasitizes endothelial cells and is confined in the endothelial cells of the smaller blood vessels throughout the body (Cowdry, 1925). The parasite accumulates as large densely packed masses which are spherical that grow within vacuole-like cytoplasmic spaces filled with a clear fluid in a single endothelial cell (Cowdry, 1926). Bezuidenhout et al. (1985) reported the first successful *E*. *ruminantium in vitro* culture system where a calf endothelial cell line known as E_5 was irradiated or chemically treated and inoculated with tick-derived stabilate, prepared from *Amblyomma hebraeum* nymphae. Subsequent successful continuous culture of various *E*. *ruminantium* isolates has been achieved using endothelial cells of several animal species and from varying anatomical sites (Jongejan et al., 1991b, Martinez et al., 1993, Totté et al., 1993, Smith et al., 1998).

In vitro culture of *E*. *ruminantium* in other cells could be achieved, including leukocytes (Logan et al., 1987). However, the culture system is not suitable for continuous *in vitro* culture as Logan et al. (1987) was able to maintain the cultures for only 5 days and the collection of host cells is laborious. Zweygarth and Josemans (2003), successfully propagated *E*. *ruminantium in vitro* in non-endothelial cells including: baby hamster kidney (BHK), Chinese hamster ovary (CHO-Kl) and Madin Darby bovine kidney (MDBK) cells. However, the cell lines required treatment of cycloheximide (CyX) for regular growth. Bezuidenhout (1987) observed that Vero, lamb foetal kidney and Mouse L-cells do not support *in vitro* growth of *E*. *ruminantium*.

E. *ruminantium* is transmitted by members of Ixodidae family with *A*. *hebraeum* being the predominant vector in the southern Africa region and *A*. *variegatum* in other parts of Africa (Bezuidenhout, 2009). In the tick vector, *E*. *ruminantium* is found in midgut and salivary glands (Cowdry, 1925b). Bell-Sakyi et al. (2000) successfully propagated *E*. *ruminantium* in *Ixodes scapularis* tick cell line IDE8 by infecting the cells with Gardel isolate from bovine pulmonary artery endothelial cells. Propagation of *E*. *ruminantium* is not only achieved in vector tick cells but is also possible in cell lines from non-vector (*Ixodes scapularis*, *I. ricinus*, *Boophilus decoloratus*, *B. microplus* and *Rhipicephalus appendiculatus*) tick species (Bell-Sakyi et al., 2002). There are 63 cell lines obtained from 18 ixodid and argasid tick species and one from sand fly and biting midge in the tick cell biobank with various geographic origins (Bell-Sakyi et al., 2018). Out of the 63 tick cell lines available, *E*. *ruminantium* can be propagated successfully in 10 cell lines from 4 ixodid tick genera (Bekker et al., 2002a, Bell-Sakyi, 2004, Bekker et al., 2005, Postigo et al., 2008, Zweygarth et al., 2008).

When using tick cells to propagate *E*. *ruminantium*, the choice of inoculum has been infected tick cell lines and infected endothelial cells (Bell-Sakyi, 2004) and leukocyte fraction of blood of infected sheep (Zweygarth et al., 2008). The use of leukocytes to inoculate tick cells was reported by Munderloh et al. (1999) for the purpose of isolating and propagating the human granulocytic ehrlichiosis agent. Use of tick organs to isolate and propagate various potential pathogenic tick-borne microorganisms on tick cell lines has been reported (Palomar et al., 2019).

Culture inoculums from ticks are currently prepared by first feeding an infected tick on a naive animal, once the animal develops fever blood is collected that is used for propagation. Ethical requirements for the use and infection of host animals have impacted on the feasibility of using this method, and it would be an advantage if the use of animals is eliminated. Therefore, there is a need for the optimization of *in vitro* isolation method of *E*. *ruminantium* directly from ticks to allow for field isolate collection and ultimately will assist in whole genome sequencing of *E*. *ruminantium* strains circulating in the field.

Successful optimization of *in vitro* cell cultures initiated directly from ticks could lead to extensive research that could provide genotypic and phenotypic information that informs isolates with best immunogenicity and broad protective capacity against various *E. ruminantium* isolates. Also, such an *in vitro* culture system will provide enough antigen for vaccine development. Hence the aim of the study is to initiate *E. ruminantium in vitro* culture using tick salivary glands and gut.

3.3 Materials and Methods

3.3.1 Culture medium

Infected and uninfected cells were grown in Dulbecco's modified eagle's medium/Ham's nutrient mixture: F12 containing L-glutamine, 15 mM HEPES and sodium bicarbonate (DME/F-12). The medium was supplemented with 10% heat inactivated fetal bovine serum and 100 IU penicillin and 100 µg/ml streptomycin.

3.3.2 Inoculum preparation

Ticks were disinfected with 70% ethanol, rinsed twice with sterile water, and allowed to dry on sterile filter paper. The ticks were tested by pCS20 Sol1 qPCR for *E. ruminantium* positivity (see methods in chapter 2 section 2.4.4 for detection of *E. ruminantium* using pCS20 Sol1 qPCR) by testing the tick DNA extracted from two legs of the tick cut close enough to the body to get the hemolymph (see methods in chapter 2 section 2.4.3 for DNA extraction).

3.3.3 Inoculum

In a laminar flow, under a microscope, the *E. ruminantium* positive tick was put in sterile petri dish containing sucrose phosphate glutamate buffer. Tick gut and salivary glands were extracted by removing the dorsal integument and cutting round the midline with a scalpel. The gut and salivary glands were removed and rinsed once in sucrose phosphate

glutamate buffer. The gut and salivary glands were homogenized in a 2 ml Precelly lysing tube containing culture medium using Precelly homogenizer program 1 once (centrifuge at 6800 x g for 90 seconds). The gut and salivary gland suspension was centrifuged at slow speed (2000 x g) for 10 minutes and the supernatant was used as the inoculum. Some *E*. *ruminantium* positive tick gut and salivary glands were preserved in sucrose phosphate glutamate buffer and stored at -80°C for later attempts at initiating *E*. *ruminantium in vitro* culture.

3.3.4 Initiating *in vitro* culture of *E*. *ruminantium* using tick material

Approximately 0.2 ml supernatant was used to inoculate a confluent monolayer of bovine aorta endothelial cells in a 6-well cell culture plate. Another confluent monolayer of bovine aorta endothelial cells was inoculated with culture medium and served as negative control. Both cultures were incubated at 37°C with 5% CO_2 in a slow rocking incubator for 1-2 h for cell absorption to occur. After 2 h the inoculum suspension was removed, and the cells were rinsed twice with calcium and magnesium free phosphate buffered saline (PBS-). Then 1 ml of culture medium was added, and the cultures were incubated at 37°C with 5% CO_2. After 24 h the media was changed by using a pipette to remove the media in the wells of the culture plate and added 1 ml of culture medium. Thereafter, media was changed once a week.

Briefly, the culture medium in the cell culture plate was discarded and the cells were washed twice with PBS- that is equal to half the volume of the growth medium. About 100 µl of trypsin was added to the cells and the plate was gently tilted to spread the trypsin over the entire surface of the cells. The cells detached and the cell layer separated after 5-10 minutes. The cells were incubated at 37°C for 1 minute to enhance the trypsinization process. Culture medium was added to the trypsin suspension to neutralize trypsin activity. The cell suspension was aspirated into a 2 ml sterile tube and centrifuged at 2000 x g for about 2 minutes. The supernatant was discarded carefully by a pipette making sure not to disturb the sedimented cells. The cells were gently dispersed with culture

medium using syringe with 18-gauge needle. The cells in suspension were transferred to a clean sterile 6-well cell culture plate containing culture medium and incubated at 37°C with 5% CO_2.

The cell cultures were examined using inverted microscope for cell growth and by visual inspection for any signs of microbial contamination (usually indicated by turbid media and dead/detached cells) daily. Also, using an inverted microscope the cells were examined for the cytopathic effect that demonstrate *E*. *ruminantium* in the endothelial cells. Small samples from the monolayer were removed by a sterile disposable cell scraper from which smears were made at days 7, 13, 19. The smears were air dried, methanol fixed and quick stained with Rapi-Diff II stain. Cell cultures which appeared to be infected with *E*. *ruminantium* colonies were tested for *E*. *ruminantium* by pCS20 qPCR.

3.4 Results

Ticks for salivary glands and gut extraction used as inoculum were *E*. *ruminantium* positive by the pCS20 Sol1 qPCR assay (Figure 3.1). Though the ticks were positive for *E*. *ruminantium*, the CT values of the positive ticks were above 31.

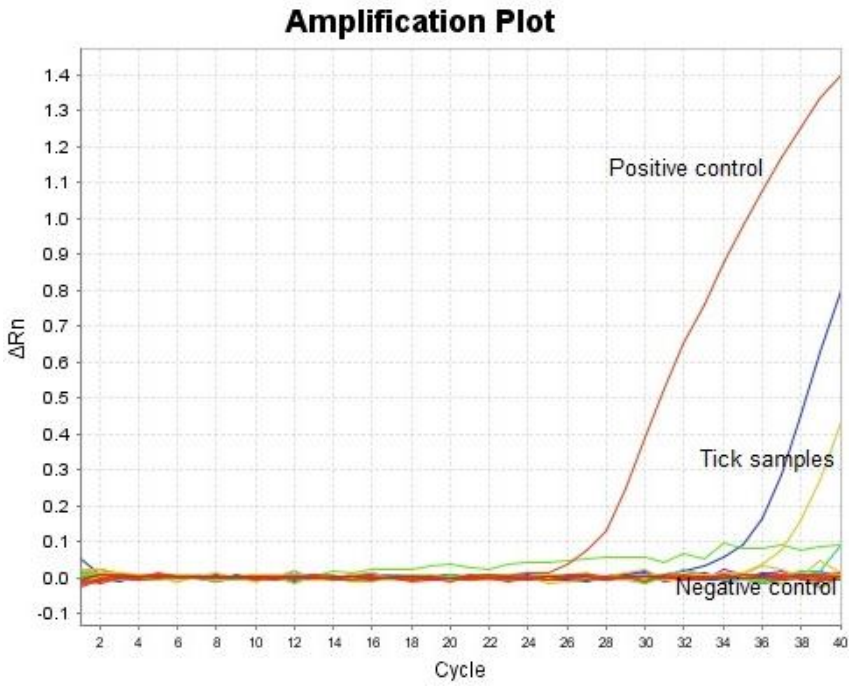


Figure 3.1: Representative amplification plot of *E*. *ruminantium* positive ticks used for extracting salivary glands and gut for *E*. *ruminantium in vitro* culture.

Throughout the 23 days, the cell cultures did not show any sign of infection, the medium was clear without any debris. Microscopic examination from the cell culture plate showed no cytopathic effect, cells looked normal and were multiplying. Dark purple colonies looking like *E*. *ruminantium* elementary bodies as shown in Figure 3.2 A and B were observed after staining with Rapi-Diff II stain 19 days post inoculation. However, the cell cultures were *E*. *ruminantium* negative by pCS20 Sol1 qPCR 19 days post inoculation. The cell cultures were negative even after 23 days post inoculation and the cell cultures showed signs of microbial contamination indicated by turbid media and detached cells. The contamination persisted and the cell cultures could not be salvaged. The contaminated cell cultures were discarded at day 25 post inoculation. The negative control cell culture remained negative throughout the 25 days.

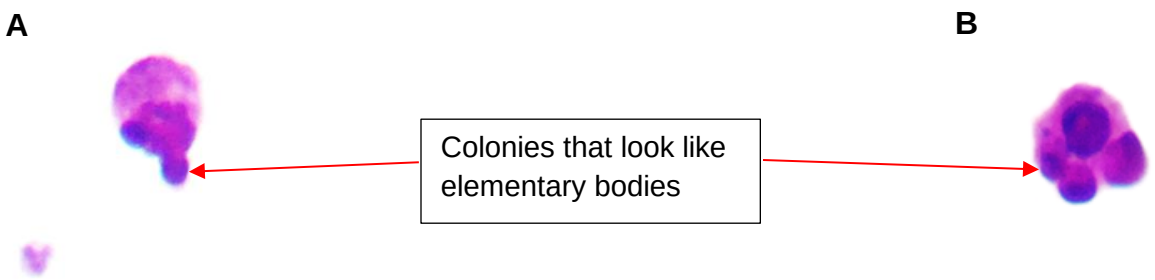
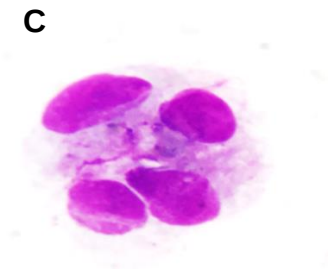


Figure 3.2 A & B: BPAE cells 19 days post inoculation with tick gut and salivary glands (A and B). Dark purple colonies that resemble elementary bodies of *E*. *ruminantium* were observed. C is the negative control cell culture inoculated with culture medium 19 days after inoculation. The dark purple colonies that resemble elementary bodies of *E*. *ruminantium* were not present.



3.5 Discussion

Ehrlichia ruminantium is an intracellular rickettsial pathogen transmitted by *Amblyomma* ticks and causes heartwater, a major cause of livestock loss in sub-Saharan Africa. The pathogen is found in different anatomical sites within the mammalian host, including neutrophils and reticulo-endothelial cells (Du Plessis, 1975, Logan et al., 1987) and in tick midgut and salivary glands within the tick vector (Cowdry, 1925b). In 1926, Cowdry described the multiplication of *Rickettsia ruminantium* within the endothelial cells of an infected animal (Cowdry, 1926). It was not startling when Bezuidenhout et al. (1985) successfully propagated *E*. *ruminantium* in calf endothelial cells. *Ehrlichia ruminantium* was thought to be an obligate parasite of endothelial cells since it is often found *in vivo* in endothelial cells. However, the pathogen can be successfully propagated *in vitro* in non-endothelial cells: bovine foetal testis, cat ovary, donkey fibroblastoid, E_2, horse testis,

lamb foetal testis, mouse connective tissue and African Green monkey kidney (Zweygarth, 2003).

The use of mammalian host endothelial cells to propagate *E*. *ruminantium* from blood and cell culture has been extensively investigated (Byrom and Yunker, 1990, Byrom et al., 1991, Jongejan, 1991a, Martinez et al., 1993). Initiating *E*. *ruminantium in vitro* culture from blood requires infecting a healthy live animal with an *E*. *ruminantium* isolate. It is of paramount importance to optimize a cell culture system that will not be dependent on infecting an animal to get the infected blood to culture *E*. *ruminantium*.

The *in vitro* culture system has been applied in the modification of virulence of *E*. *ruminantium* isolate. Jongejan (1991a) observed the attenuation of *E*. *ruminantium* on serial passages without the loss of immunogenicity. Also, the culture system has been used in the development of inactivated vaccine. Although the cell culture system has potential for breakthrough in *E*. *ruminantium* research Yunker (1995) says, “workers in different laboratories, and even different workers in a single laboratory, commonly experience difficulties in consistently growing the heartwater organism.” Therefore, highlighting the need of the optimization of the culture system.

The current study used *E*. *ruminantium* positive tick gut and salivary glands to initiate *E*. *ruminantium in vitro*. The attempts were unsuccessful due to contamination and possibly low rickettsial load in the ticks. It is known that ticks are hosts to several pathogens with high natural bacterial load on the surface of ticks (Munderloh et al., 1994). Lesley Bell-Sakyi (personal communication) suggested that the ticks should be immersed in 0.1% benzalkonium chloride for 5 min, then in 70% ethanol and then rinsed with sterile water before the extraction of tick material to reduce the tick surface bacterial load. Furthermore, the culture medium used in the current study did not contain an antifungal agent which would have assisted in the control of unwanted fungal growth in cell culture (Perlman et

al., 1961). Additionally, in the current study, the culture medium was not further supplemented with 2 mM L-glutamine meaning the medium lacked enough nutrients required for the growth of *E*. *ruminantium in vitro* (Zweygarth et al., 2013, Palomar et al., 2019).

Also, low rickettsial load in the ticks could have negatively influenced the method as CT values of the *E*. *ruminantium* positive ticks were above 31. Therefore, it will be of benefit if the infectious material can be concentrated. A way to concentrate the infectious material is to dissect the salivary glands and midgut from several positive ticks collected from a single animal. Bezuidenhout et al. (1985) highlights that the source of the inoculum, choice of cell line and techniques of infection are important factors that contribute to a successful *E*. *ruminantium in vitro* culture.

A successful optimization of isolating *E*. *ruminantium* directly from ticks will pave a path for rapid isolation of field isolates and generation of genome sequences. Probably the question of what causes the lack of protection among the various *E*. *ruminantium* isolates may be answered once the whole genomes of these field isolates is known, leading to development of efficient vaccines.

3.6 Conclusion

The success of mammalian culture system for *in vitro* culture of *E. ruminantium* contributed to the understanding of the development of the organism in the ruminant host. While the use of tick cell cultures has piloted an increased knowledge and understanding of *E. ruminantium* development in the vector. It is without a doubt that there is a need for optimization of the *in vitro* culture system using tick material. The *in vitro* system will provide an easier way of isolating various *E*. *ruminantium* genotypes circulating in the field. Also, the system will eliminate the need of infecting a live animal with an *E*. *ruminantium* isolate to get the infected blood for the propagation of the *E*. *ruminantium*

isolate. Even though the current attempts of the culture system were without success, future attempts at initiating *E*. *ruminantium in vitro* culture will be done using tick gut and salivary glands stored at -80°C.

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Chapter 4

Development of a reverse transcription quantitative real-time PCR assay for the evaluation of a multivalent inactivated vaccine

4.1 Abstract

Heartwater is a tick-borne disease caused by *E*. *ruminantium* and occurs in sub-Saharan Africa region and Caribbean islands. The disease poses a threat to livestock farmers that want to introduce exotic high yielding breeds as mortality rate is up to 90% in susceptible animals. Heartwater is also a threat to livestock in areas where the disease does not occur yet the tick vector capable of transmitting the diseases is present. In southern Africa, the disease is controlled by regular use of acaricides especially in the warm rainy season where the tick vector is abundant. Another control strategy is immunization by live virulent blood vaccine and subsequent treatment with tetracyclines to prevent death. The nature of the vaccine requires cold chain storage and trained personnel to administer the vaccine. The major drawback of the vaccine is its limited cross protection against some virulent *E*. *ruminantium* isolates. Efficient alternative vaccines are a requirement in order to conquer the fight against heartwater. The developed vaccines must be tested for their ability to elicit protective immunity against heartwater. Hence, the aim of the study is to develop a reverse transcription quantitative real-time PCR (RT qPCR) assay for the evaluation of a multivalent inactivated vaccine. Sheep were vaccinated with an inactivated multivalent heartwater vaccine developed by Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) and blood was collected at different time points. Housekeeping genes were selected and validated for relative quantification of mRNA-levels of immune markers for protection. The selection and validation method were by statistical algorithm that include Ct mean, CV of RPKM values and CT values and the ΔCt method and the genes were ranked using RefFinder. Immune markers for protection differentially expressed between surviving animals and treated animals were

identified using RNA-seq data from a previous study. Relative changes in gene expression of immune markers for protection were measured by RT qPCR. The best housekeeping genes were SDHA, RPL22, YWHAZ and GAPDH and the appropriate immune markers were CD41–ITGA2B, CD156A-ADAM8, CXCR5, CD8B, CD5, GZMB-LIKE, IFN-γ and TGFB1. The developed RT qPCR assay revealed that the immune markers for protection were differentially expressed at all time points. Although an increase in expression of the immune markers in all vaccinated animals at least one of the two time points post vaccination, it does not predict survival. Therefore, increase in expression of the immune markers post vaccination cannot be used to indicate protection.

Key words

RT qPCR, vaccine development, housekeeping genes, immune markers, inactivated vaccine

4.2 Introduction

Heartwater is a tick-transmissible infection caused by *E*. *ruminantium* and spread by *Amblyomma* genus ticks. Heartwater is a disease of goats, cattle and sheep and some wild ruminants in sub-Saharan Africa, Madagascar, Reunion, Mauritius, Zanzibar, the Comoros Islands, and Sao Tomé and three Caribbean islands: Guadeloupe, Marie-Galante, and Antigua (Spickler, 2015). The disease is indicated by fever, heavy breathing, loss of appetite, diarrhea, nervousness (inco-ordination and convulsion), lateral recumbency position with leg paddling and the buildup of liquid in the lungs, brain, thoracic cavity and pericardial sac (Bezuidenhout, 2009). Mortality of susceptible animals include: 6% in Persian or Afrikander sheep, 60% in cattle, 80% in Merino sheep and Angora goats (Mahan et al., 1992). Heartwater causes a notable decline in farmers productivity in areas where it is widespread and poses a significant threat in non-indigenous livestock that are moved into heartwater areas (Meltzer et al., 1996).

Heartwater is controlled by acaricides, antibiotic treatment of sick animals and vaccination of animals. Using a blood vaccine, vaccination employs the infection and treatment method. However, the infection and treatment vaccination method is laborious as mandatory daily monitoring of rectal temperature of the animal after vaccination is required, the necessary treatment of reacting animals with antibiotics, and the need for maintaining a cold chain for the distribution of the vaccine. Also, the vaccine isolate is live which present a risk of transmitting blood pathogens; and does not cross protect against all field isolates. The disadvantages of the infection and treatment vaccination method has led to investigations of developing more efficient vaccines.

The development of *in vitro* culture of *E*. *ruminantium* provided means of modifying the virulence of an isolate and supply antigens to be used for various vaccines (Jongejan, 1991a). Martinez et al. (1994) used cell culture derived inactivated elementary bodies mixed with Freund's adjuvant to immunize nine goats of which six survived the homologous challenge. Mahan et al. (1995b) reported similar results where 100% of

sheep immunized with inactivated elementary bodies mixed with Freund's adjuvant were protected and survived the challenge. The inactivated vaccine has been successfully used to immunize cattle and protected 100% of cattle against a homologous challenge (Totté et al., 1997). However, these promising results were regrettably not reproducible when tests were performed in a natural tick field situation, where varying genotypes with different immunogenicities are present.

It has been established that humoral immune response possibly contributes a minor role in the fight against *E*. *ruminantium* infection (Stewart, 1987). Protective immunity against heartwater is mediated by cellular immune responses (Du Plessis et al., 1991). Mwangi et al. (1998) reported that the cellular immune response to heartwater is characterized by the proliferation of $CD4^+$, $CD8^+$, γδ T cells associated with T helper 1 (Th1) type. The T cells secret high levels of IFN-γ, interleukin (IL)-2 receptor alpha and IL-6 (Byrom et al., 2000). Th1 cells are responsible for immunity mediated by cells, including delayed-type hypersensitivity reactions, recruitment and stimulation of inflammatory macrophages and leukocytes, cytolytic responses, leading to protection against intracellular pathogens (Esteves et al., 2004a).

It is important that a vaccine elicit a protective immune response. A protective immune response could be indicated by regulation of immune biomarkers of protection. Immune biomarkers are biological immune indicators that can be measured to determine responses to a therapeutic intervention (Llibre and Duffy, 2018). The efficacy and effectiveness of a vaccine must be tested by challenging the animals with the disease-causing pathogen, further subjecting the animal to suffer from the symptoms of the disease. It is necessary to investigate and develop alternative methods to monitor immune responses induced in animals by determining the immune markers for protection and/or markers associated with a severe outcome. Therefore, the current study is aimed at developing an RT qPCR assay for the evaluation of a multivalent inactivated vaccine by monitoring the immune biomarkers for protection.

4.3 Materials and Methods

4.3.1 Selection of housekeeping genes as reference for a RT qPCR assay using RNA sequences obtained from sheep PBMC infected with *E. ruminantium*

Housekeeping genes were selected from literature as the most used for RT qPCR analyses. The RNA-seq analysis of these selected housekeeping genes were done using previous RNA-seq data sets prepared from peripheral blood mononuclear cells (PBMC) of animals infected and challenged with ticks infected experimentally with *E. ruminantium* Welgevonden using the methods by Nefefe et al. (2017). The data sets are considered confidential by the ARC and are not publicly available.

The RNA sequences were mapped to the sheep housekeeping homologues sequences selected from GenBank and are listed in table 4.1. The longest isoform (transcript variant X1, -X2, etc. indicated in the table), for a gene sequence was selected for RNA-seq mapping from GenBank, allowing complementarity to other transcripts of the same gene within the coding sequence.

Table 4.1: Summary of the candidate ovine housekeeping genes used in the RNA-seq analyses and evaluated in this study. The Gene symbol, Gene ID (the number allocated to the gene in the ovine genome sequence), Official name, Transcript variant (where applicable) and gene size are shown.

Gene symbol	Gene ID	Transcript variant	Protein name	Length (nt)
ACTB	443052	-	actin, beta	1839
ATP5F1B	101106318	-	ATP synthase, H + transporting, mitochondrial F0 complex, subunit B1	1775
B2M	443295	-	beta-2 microglobulin	385
CANX	101122273	-	calnexin	4047
EEF1A1	100101228	-	eukaryotic translation elongation factor 1 alpha 1	1744
GAPDH	443005	-	glyceraldehyde-3-phosphate dehydrogenase	1285
GUSB	101108802	-	glucuronidase, beta	4384
HPRT	100188975	-	hypoxanthine guanine phosphoribosyl transferase	1348
PGK1	100216440	-	phosphoglycerate kinase 1	1775
POLR2A	100144762	-	DNA-directed RNA polymerase II subunit RPB1	6736
PPIA	443258	X1	peptidyl-prolyl cis-trans isomerase A	815
PPIB	554319	-	peptidyl-prolyl cis-trans isomerase B	908
RPL13	443072	-	60S ribosomal protein L13	706
RPL22	101122293	-	60S ribosomal protein L22	463
RPL30	106990098	-	60S ribosomal protein L30	468
RPL31	101104219	-	60S ribosomal protein L31	438
RPL37	101103841	-	60S ribosomal protein L37	369
RPS18	100036761	X1	ribosomal protein S18	602
SDHA	100036762	-	succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondriA	665
TBP	100144760		TATA box binding protein	1968
TFRC	100885768	X1	transferrin receptor	5882
YWHAZ	780452	X1	3 tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	3429

From the animal trial described by Nefefe et al. (2017), four biological replicates taken as averages of three technical replicates, taken at eight time points from sheep PBMC isolated from heartwater infected animals were compared for this analysis. The RNA-seq data was analysed using the CLC Genomics Workbench 9.5. Data for each sample was imported and adapters and low-quality base reads were trimmed. To specifically focus on immune modulation by these cells, the reads were mapped, using the RNA-seq analysis tool, to the preselected ovine immune housekeeping reference gene sequences listed in table 4.2 obtained from NCBI (https://www.ncbi.nlm.nih.gov) using gene annotation and ID numbers updated on 16-August-2022. The RNA-seq mapping option settings were Mismatch cost (3); Insertion cost (3); Deletion cost (3); Length fraction (0.7) and Similarity fraction (0.8). The mapping data sets were further analysed using the transcriptomics analysis set up experiment tool of CLC. The RNA-seq data sets at each time point were collectively compared using the paired multi-comparison setting and normalised. Comparative transcriptomic experiments allowed for appropriate statistical analysis including Baggerley's test on proportions, T-test and the empirical analysis of digital gene editing (EDGE) tool.

Candidate housekeeping genes were selected by eliminating genes with a mean TPM of less than 100 and was filtered by determining the CV of TPM values for all data sets included in this analysis. The CV was calculated by dividing the standard deviation by the mean for each selected gene and the genes with the lowest CV values were selected as possible housekeeping genes. All qPCR primers were designed for selected genes using the latest sequence updates (August 2022) using the primer design software, OligoPerfect Primer Designer from ThermoFisher Scientific, (https://apps.thermofisher.com/apps/oligoperfect). Tm of these primers are all 60 °C and the length of PCR products vary from 96 bp to 150 bp. Some of the housekeeping genes are from Schulze et al. (2017) and were double checked using the latest sequence version if applicable. The primer sequences for amplification of the candidate housekeeping genes are listed in table 4.3 below (not highlighted).

4.3.2 Selection of immune markers using transcriptome sequence data

For the selection of immune markers, the same data sets as described by Nefefe et al. (2017) were used and the markers were selected by Dr Pretorius at the ARC-OVR (manuscript in preparation). Briefly, four sheep were vaccinated using a modified version of the infection and treatment method, where animals were infected with Welgevonden infected ticks and treated with tetracycline when a febrile reaction developed. These animals were challenged 30 days after they recovered from antibiotic treatment with experimentally infected ticks and all four survived the challenge without symptoms, while naïve animals (challenge controls) included in the study succumbed to heartwater.

The infection was monitored, and samples were collected at various time points after confirmed attachment of the male tick: (D1, D12 and D16 post infection [PI]) and (D1, D4 and D6 post challenge [PC]). These animals were challenged with experimentally infected ticks 30 days after they recovered from antibiotic treatment and all four survived the challenge without symptoms, while naïve animals (challenge controls) included in the study succumbed to heartwater. The ticks used to infect the sheep were tested with the pCS20 qPCR designed by Steyn et al. (2008) to determine infectivity, all ticks tested were infected with *E. ruminantium* Welgevonden. The presence of *E. ruminantium* in the blood of infected animals was also confirmed using the pCS20 qPCR where *E. ruminantium* DNA was detected on D12PI, and D16PI as well as in two animals on D1PC (Nefefe et al., 2017). Genes that were only upregulated post challenge and not during infection were identified and randomly selected for this assay. The selected markers are highlighted in table 4.2.

Table 4.2: Selected housekeeping genes (not highlighted) and immune biomarkers (highlighted in blue)

Gene ID	Gene Symbol	Accession number	Primer sequences
		XM_042236905.1	F:5' AGGTGGAACGTGTGATCACC 3′
			R:5' GTTGTCTAGACCGTTGGCCA 3'
100036761	RPS18		
		XM_027975714.2	F:5' CTCTGGACTGTACCCACCCT 3′
101122293	RPL22		R:5' CGATTGTTACAACACCGCCA 3′
		XM_027972283.2	F:5' GCCATGGTTCAAGGGATGGA 3′
100101228	EEF1A1		R:5' CGCAAGGGTTTGTCAGTTGG 3′
		XM_027980212.2	F:5' GAGTTCGTGCAGTTCCACCC 3′
100036762	SDHA		R:5' CTCTCACCCTGGCTGTTGAT 3'
		NM_001190390.1	F:5' ACAGTCAAGGCAGAGAACGG 3′
443005	GAPDH		R:5' CCAGCATCACCCCACTTGAT 3'
		NM_001267887.1	F:5' GATGAAGCCATTGCTGAACTTGA 3′
780452	YWHAZ		R:5' CAGCTTCGTCTCCTTGGGTA 3'
		XM_015105023.3	F:5' TTCTTTGCCGACCTGTTGGA 3′
100188975	HPRT1		R:5' TCACCTGTTGACTGGTCGTT 3'
		NM_001009284.2	F:5' CCTTGGTCCTTCTCGGGCTG 3′
443295	B2M		R:5' TCTGGCGGGTGTCTTGAGTAT 3'
		NM_001142516.1	F:5' CCTCTGGCATACCTGTTGGC 3′
100216440	PGK1		R:5' CACCCACAGGTCCATTCCAC 3'
		XM_004003001.4	F:5' ACCTCAAATCAGCGCTGTCA 3′
100885768	TFRC		R:5' CAGCCTCACGTGGGACATAA 3'
		NM_001009784.3	F:5' GCAGATGTGGATCAGCAAGC 3′
443052	ACTB		R:5' GGGTGTAACGCAGCTAACAG 3'
		XM_004018407.5	F:5' CACTGCCCCAGTCACATCAT 3′
101112853	ALAS1		R:5' GGGCACTGTGGGGTAATTGA 3'
		XM_004012836.4	F:5' AGCCTGTGACTGTCCATTCC 3′
100270789	RPL19		R:5' ACGTTACCTTCTCGGGCATT 3'
		XM_012178077.4	F:5' GTTGTGAAGGCCTACCTCCC 3′
101114105	EEF2		R:5' GCCAGTGGTCAAACACACAC 3'
	CD41 –	XM_004012986.5	F:5' CGAGATGAGACCGACTTCCG 3'
101115719	ITGA2B		R:5' ATGTGGGTGTCTCCATGCAG 3'
	CD156A-	XM_042238593.1	F:5' AAAGCAGGTCATCAAGCCGA 3'
105605819	ADAM8		R:5' CTTCGGGTCAACAGCTTCCT 3'
		XM_042232717.1	F:5' GACAGCTATCTCTCCGTGGC 3'
101104174	CXCR5		R:5' CTGGGGAAGAACTGGCAGAG 3'
		XM_004007264.5	T F:5' TGGGCTGGGAACTTTGTGT 3'
101103712	CD8B		R:5' CCAAGGGGACGCATCCTATC 3'
		XM_012101941.4	F:5' AACTCATCTGTGCAGCCCTC 3'
443328	CD5		R:5' GCTCCAGGGAGTTGCATCTT 3'
	GZMB-	XM_027957506.2	F:5' AGACATCAGCTGTGAAGCCC 3'
101108034	LIKE		R:5' CTCCTGCACGGTCAGCTTTA 3'
		NM_001009803.1	F:5' CTCCGGCCTAACTCTCTCCT 3'
443396	IFNG		R:5' AAGAGGCCCACCCTTAGCTA 3'
		NM_001009400.2	F:5' GAGCCCTGGACACCAACTAC 3'
443417	TGFB1		R:5' GTAGCCCTTGGGTTCGTGAA 3'

4.3.3 Validation of the housekeeping genes and immune markers selected for the RT-qPCR using RNA isolated from a current inactivated vaccine trial

4.3.3.1 Propagation of *E. ruminantium*

The infection of Bovine Aorta endothelial cells (BA886) with the *E. ruminantium* Welgevonden isolate was done according to Zweygarth et al. (1997) and Tjale et al. (2018). The *E. ruminantium* Welgevonden isolate was originally collected on the Welgevonden farm in the Northern Transvaal (Limpopo province) in South Africa from an *A. hebraeum* male tick (Du Plessis, 1985). For the current study the *E. ruminantium* Welgevonden isolate used was kept in liquid nitrogen and stored in 500 µl sucrose-potassium phosphate glutamate medium (SPG) composed of 0.218 M sucrose, 3.8 mM KH_2PO_4, 7.1 mM K_2HPO_4, 4.9 mM $C_5H_8NO_4K$. The cells were grown in Dulbecco's modified eagle's medium/Ham's nutrient mixture: F12 containing L-glutamine, 15 mM HEPES and sodium bicarbonate (DME/F-12). The medium was further supplemented with 10% heat inactivated fetal bovine serum, 1.2 g/L sodium bicarbonate and 100 IU penicillin and 100 µg/ml streptomycin. The monolayer of the BA886 cells in 25 cm^2 or 75 cm^2 flasks were incubated at 37 ºC with CO_2. The infective material used to inoculate the BA886 cells in the current study was *E. ruminantium* Welgevonden elementary bodies at passage 179. Cytopathic effect was observed after 6 days, and the elementary bodies were harvested from cells that were 90% infected and could be visualized with Rapi-Diff II stain. The elementary bodies were purified according to the method provided by CIRAD. Briefly, the cells were gently scraped, and the lysate transferred into a 50 ml Falcon tube and lysed by aspiration with a 25 G needle. The lysate is then centrifuge for 15 minutes at 1800 x g at 4°C. The supernatant was transferred to a PPCO Nalgene tube and centrifuge for 30 minutes at 20 000 x g at 4°C. The supernatant was discarded, and pellet (EB) resuspended with approximately 1 ml of physiological saline.

4.3.3.2 Preparation of the inactivated vaccine

Elementary bodies were inactivated for 24 hours at 4 °C with 0.1% sodium azide, and a 10 µl aliquot was collected before and after inactivation to test efficiency using the Live/Dead bacterial kit from Invitrogen. The Live/Dead bacterial kit from Invitrogen makes use of a combination of two nucleic acid stains, green fluorescent SYTO® 9 dye and red-fluorescent propidium iodide for viability determinations, and a calibrated suspension of microspheres for precise sample volume measurements. The samples were diluted 1:100 to 1:1000 in saline and 1 µl of each dye was added. The elementary body viability was assessed, and concentration determined after an incubation time of 15 minutes at 37 °C. Protein concentration was assessed using the BSA method using the Pierce™ BCA Protein Assay Kit according to the manufacturer's instruction. The protein was mixed 40:60 with the Montanide ISA 61VG (Seppic) adjuvant using an ULTRA-TURRAX® Tube Drive mixer.

4.3.3.3 Infection of ticks with *E. ruminantium* Welgevonden

Uninfected *A. hebraeum* nymphs from the naïve tick colony at the ARC-OVR, were infected by feeding uninfected *A. hebraeum* nymphs on sheep infected intravenously with *E. ruminantium* Welgevonden (Tjale et al., 2018). The nymphs were placed into an acaridarium where they moulted into adults. A portion of adult ticks were tested for the presence of *E. ruminantium* by pCS20 qPCR to approximate the infectivity of the ticks. Screening for *E. ruminantium* infection was performed on individual tick.

4.3.3.4 Animal trial and collection of samples

An animal trial was done at ARC-OVR, the trial consisted of four groups of 7 animals as outlined in table 4.3, Group 1 (G1), G2 and two control groups (G3 and G4). Group 1 and G2 were vaccinated subcutaneously with inactivated *E. ruminantium* Welgevonden vaccine. The animals were challenged 2 months after the second boost (G1) with 10 ticks experimentally infected with the *E. ruminantium* Welgevonden strain and were monitored

for onset of disease by measuring their temperatures daily and monitoring for typical heartwater symptoms. Once an animal has an increased temperature of 41.5 °C for about 2 days, it was treated with 100 (1 ml/10 kg)Terramycin® and considered dead.

Table 4.3: Animal groups for animal trial

Group number	Immunized with	No of animals	Challenged with
Group 1 (trial group)	2x with 100 µg inactivated E. *ruminantium* Welgevonden in adjuvant	7	Welgevonden infected ticks
Group 2 (trial group)	1x with 200 µg inactivated *E. ruminantium* Welgevonden in adjuvant	7	Welgevonden infected ticks
Group 3 (control group)	1x Adjuvant	7	Welgevonden infected ticks
Group 4 (control group)	None, Naive	7	Welgevonden infected ticks

4.3.3.5 Blood collection, RNA extraction

Whole blood samples for RT qPCR were collected from the animal trial on D0 (before vaccination); D1; D4 and D7 post vaccination (PV) and D10 post challenge (PC). Blood (10 ml) was collected in BD Vacutainer® EDTA tubes (Becton, Dickinson). A total of 1 mL blood was centrifuged at 1500 x g for 5 min and the plasma was removed. To reduce the volume of the cell pellet that should not exceed 25% of the total volume of Trizol added (i.e 250 µl if 1 ml Trizol is added), most of the remaining red blood cells were lysed by adding 1 ml 1x BD lyse buffer and incubated for 10 minutes at room temperature. The cells were pelleted at 1500 x g for 5 min. The supernatant was removed, and the remaining pellet were mixed with 750 µL Trizol and stored at -20 °C until processed according to the instructions of the manufacturer. After total RNA isolation, the genomic DNA was removed using a DNA-free kit (Ambion, Invitrogen) according to the instructions of the manufacturer and stored in 75% Ethanol at -20 °C or in Diethyl pyrocarbonate (DEPC) treated water at -70 °C until used for RT qPCR.

4.3.3.6 Validation of housekeeping genes

Three different statistical algorithms (Ct mean, CV of RPKM values and CT values and the ΔCt method) were applied to estimate the expression stability of each housekeeping gene from the isolated RNA and an online tool, RefFinder was used to rank the identified housekeeping genes for RT qPCR data normalization (Schulze et al., 2017).

4.3.3.7 Reverse transcription quantitative real-time qPCR

Relative changes in gene expression of immune markers were measured by RT qPCR according to Livak and Schmittgen (2001). Each reaction was performed in a final volume of 20 µl containing 1X QuantiTect SYBR Green RT PCR Mix, a final concentration 0.5 µM of each primer (primers highlighted in table 4.3), 0.25 µl QuantiTect RT mix, 1 µl template RNA (pools) (see supplementary table 4.3 and 4.4 for how the pools for template RNA were achieved). The fold change at which it is considered that a gene is upregulated or downregulated is at least >1.5 and significant with the T-test.

4.4 Results

4.4.1 Identification of candidate housekeeping genes using transcriptome data

Based on Nefefe et al. (2017), the analysis of RPKM values revealed that there are a number of genes that are expressed at different expression levels with CANX and POLR2A having the highest levels of variation between the 24 data sets analysed as shown in the box-and-whisker plots indicating the range of normalized RPKM values calculated in CLC of the selected candidate reference genes (Figure 4.1). Fourteen housekeeping genes that had stable expression were determined using the CV method calculated by dividing the TPM average for each gene, by the standard deviation between all 24 data sets (table 4.5) were used for validation using the RNA extracted from samples obtained from the inactivated vaccine animal trial.

Figure 4.1. Box-and-whisker plots indicating the range of normalised RPKM values calculated in CLC of the selected candidate reference genes throughout *E. ruminantium* infection. The whiskers represent standard deviation of n samples (n = 24).

4.4.2 Selection of immune markers for protection

Using RNA-seq data from a previous study (Nefefe et al., 2017), potential genes differentially expressed were identified (Figure 4.2). For the RT qPCR, functional markers expressed on the surface of cells or cytokines produced by the cells were selected and the intracellular markers of transcription were excluded. The intracellular transcription factor regulation markers can be expressed by non-immune cells as well.

CD8B, CD5 and CXCR5 were also downregulated at D1PC in positive control animals. One of the reasons for this is that the cells are homing to the site of infection, although further analysis must be done at the infection sites to confirm this in future. CD8B and Granzyme B (GZMB) are markers specific to $CD8^+$ T cells (cytotoxic T cells) while CD5 is expressed by most T cells and a subset of B cells. A subset of $CD4^+$ T cells expressing CXCR5 is similar to Thf cells and exist in blood. Thf cells expressing the CXCR5 receptor

could activate B cells to produce antibodies. CD41 is expressed on blood platelets and the immune response induced by these cells in response to heartwater infection has not been investigated since most assays used purified PBMCs and not whole blood. CD156A is expressed on antigen presenting cells and could suggest that the *E*. *ruminantium* is processed and assimilated. IFN-γ was included even though it was expressed at all time points since it has long been considered a biomarker for protection.

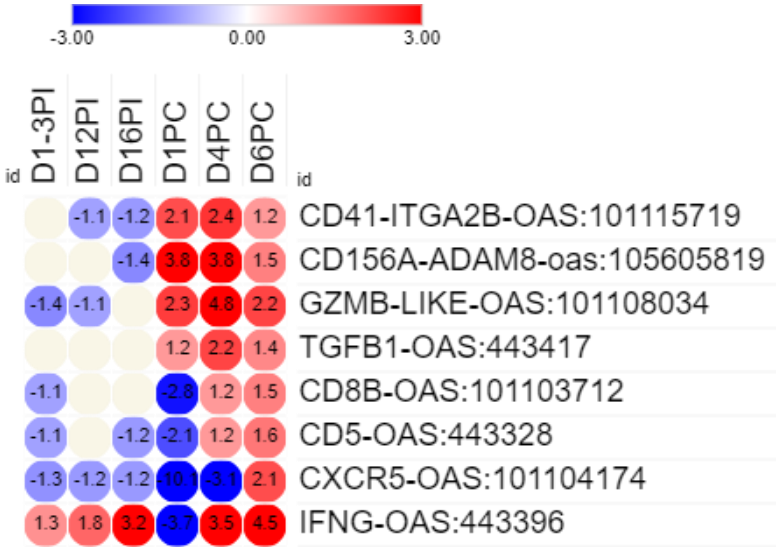

Figure 4.2: Markers selected that were upregulated in animals that survived heartwater challenge. This shows the heat map of markers that were upregulated at the post challenge time points (PC) but not during infection.

4.4.3 Animal trial

All the animals were treated except one sheep (nr 15), in group 2. It was observed that some of the animals in G1 (sheep 5, 9 and 10) and sheep 18 in G2 had less severe symptoms and survived on average longer than the rest of the animals (29-35 days) while the controls were treated within 26 days. These animals also recovered faster than the control group animals after antibiotic treatment. Similar results were obtained between the group of animals that were boosted and the group receiving a single shot.

Figure 4.3: Survival plot of animals in group 1, 2, 3, and 4

4.4.4 qPCR validation of housekeeping genes to use using total RNA samples collected during animal trial

Total RNA was isolated from whole blood collected D0; D1; D4 and D7PV and D10PC as described above (animal trial), and using the survival outcome results from the trial, the RNA samples were pooled as described in table 4.4 to determine the best housekeeping genes to include in the final assay using Ct, STDEV, CV and the delta Ct methods to rank the housekeeping genes in a pairwise comparison.

Table 4.4 Animal groups for the animal trial pooled for qPCR screening.

Group number	Immunized with		Time points and pools			
			D0	**D1PV**	**D7PV**	**D10PC**
Group 1	2x with 100 µg Welgevonden, adjuvant	Lived longer [Sheep 4, 5, 9 and 10]	Pool 1	Pool 2	Pool 3	Pool 4
		Treated [Sheep 6, 7, 11]	Pool 5	Pool 6	Pool 7	Pool 8
Group 2	1x with 200 µg Welgevonden, adjuvant	Lived longer [Sheep 15 and 18]	Pool 9	Pool 10	Pool 11	Pool 12
		Treated [12, 13, 14, 16 and 17]	Pool 13	Pool 14	Pool 15	Pool 16
Group 3	1x Adjuvant	Treated [19, 20, 21, 22, 23, 25 and 26]	Pool 17	Pool 18	Pool 19	Pool 20

To test the expression stability of the 14 selected housekeeping genes, five pools were randomly selected for the qPCR and the methods above were done to determine the top 4 genes to be included in the final RT qPCR assay. After total RNA extraction was completed, the samples were suspended in 50 µl DEPC treated water and concentration determined using the Nano drop. For each group listed in table 4.2, 1 µg total RNA of each animal/group was pooled prior to the first qPCR screening. Only those that had a positive fold change in the group were further tested to determine if each individual animal reacted similarly. SDHA, RPL22, YWHAZ and GAPDH was detected at similar Ct points, had the most stable expression between samples (STDEV and CV methods) and the least variation with the Delta Ct method. The qPCR results thus also correlated with the transcriptome data sets where the CV were calculated based on the TPM values generated in CLC (Table 4.3).

According to the ranking of the selected housekeeping genes (table 4.5), it was identified that SDHA, RPL22, YWHAZ and GAPDH are the most suitable choice combination of housekeeping genes for normalization of RT qPCR results for mRNA-levels of target

markers. These four housekeeping genes showed the least expression variation throughout the experimental conditions at any given time point.

Table: 4.5: The ranking of the selected housekeeping genes

				(Determined the CV from the mean Ct and STEV in column one and two)		(Determined the CV from the mean TPM and STDEV From original sequencing data)		Mean STDEV as determined by the Delta Ct method (next sheet) in a pairwise comparisson)		Combination of the four methods used to determine final ranking	
	mean Ct values	STDEV	Rank	Ct CV (stdev/av	Rank	TPM CV	Rank	Delta CT mean SD	Rank	Overall	Rank
SDHA	13,836	0,13	1	0,10	1	0,31	4	1,6	3	2,3	1
RPL22	13,051	1,73	3	0,13	3	0,28	2	2,5	12	5,0	2
RPS18	14,635	2,31	8	0,22	8	0,17	1	1,3	1	4,5	3
YWHAZ	13,567	2,45	5	0,18	5	0,43	6	2,3	9	6,3	4
GAPDH	13,064	2,60	7	0,20	7	0,37	5	2,3	8	6,8	5
EEF1A1	14,987	6,39	12	0,43	12	0,31	3	1,5	2	7,3	6
ALAS1	15,246	1,83	2	0,12	2		12	2,6	13	7,3	7
B2M	23,567	3,93	4	0,17	4	0,53	8	2,6	14	7,5	8
TFRC	15,396	2,96	6	0,19	6	0,69	10	2,3	10	8,0	9
PGK1	14,845	3,43	9	0,23	9	0,56	9	2,1	7	8,5	10
ACTB	12,403	6,17	13	0,50	13	1,30	11	1,6	4	10,3	11
RPL19	13,613	4,03	11	0,30	11		14	1,9	5	10,3	12
HPRT1	18,026	10,67	14	0,59	14	0,52	7	2,1	6	10,3	13
EEF2	14,741	3,80	10	0,26	10		13	2,4	11	11,0	14

4.4.5 Expression levels of the selected markers for protection using RT-qPCR with the validated housekeeping genes using RNA from the animal trial

All the markers were upregulated at D1PV for all the pools except for animals that died in group G1 (pool [P] 6) and in group G3 (P18) where the animals were immunized with the adjuvant. However, at D7PV transcripts in P11 and P15 from animals in G2 that received one dose of vaccine were detected (table 4.6). CD41, Granzyme and IFN-γ were upregulated in the adjuvant only group at D7PV. At D10PC, upregulated transcripts for all genes were detected in P12 except CD5 and TGFB1, the animals that lived longer during the experimental tick challenge. Overall, significant differences in the expression of markers in vaccinated animals in comparison with the adjuvant only group were detected. The detection of these transcripts can be considered as indicative of immunological memory that was activated after vaccination and the expression patterns depended on the treatment that the animals received.

However, these selected markers were detected in both animals that survived and those that succumbed to infection. For instance, the expression of the CD156A marker was detected in 4 of the 6 pools that represents G2 and was upregulated in both animals that survived and those that died. Similarly, IFN-γ and GZMB at D7PV were upregulated for G2 animals that survived longer (pool 10) and that died (pool 15). Both markers were also upregulated at D7PV in the adjuvant only animal group that were severely infected and required treatment.

Marker TGFB1 was downregulated at all time points in G3 (adjuvant only) and upregulated in vaccinated animals in pools 2, 8, 10 14 and 15.

IFN-γ was upregulated at D1PV or D7PV for all animal groups including the control animal group (adjuvant only). The expression of IFN-γ decreased at D7PV for animal G1 but were increased in pool 15 and the control animal group (P19). However, at D10PC, IFN-γ was downregulated in animal G1, but upregulated in both animals that survived and those that died in G2.

Table 4.6: $2^{-\Delta\Delta CT}$ to analyze the significant relative fold changes in gene expression of the immune markers.

Fold change (the shading indicate upregulation that is statistically significant)

Gene (transcript)	G1: 2x 100 µg Inactivated Welgevonden vaccine						G2: 1x 200 µg Inactivated Welgevonden vaccine						G3: 1x Adjuvant		
	Lived longer			Died			Lived longer			Died			Died		
	D1PV	D7PV	D10PC	D1PV	D7PV	D10PC	D1PV	D7PV	D10PC	D1PV	D7PV	D10PC	D1PV	D7PV	D10PC
	P2	P3	P4	P6	P7	P8	P10	P11	P12	P14	P15	P16	P18	P19	P20
CD41	10.6	0.6	0.4	0.0	0.0	0.0	1.4	0.5	94.0	1.3	14.9	0.3	0.0	3.2	3.0
CD156A	5.7	0.8	0.2	0.2	0.0	0.1	2.6	1.0	3.7	9.2	26.0	0.6	0.0	0.3	1.2
CXCR5	5.2	0.5	0.1	0.2	0.1	0.1	2.5	0.9	33.4	6.7	14.7	6.4	0.1	0.8	0.0
CD8B	3.0	0.8	0.2	0.1	0.0	0.1	2.7	1.0	7.3	5.8	15.5	1.5	0.0	0.0	0.0
CD5	1.7	0.3	0.2	0.7	0.4	0.7	2.5	0.9	0.2	2.2	4.1	1.7	0.1	0.6	0.0
GZMB	7.0	0.6	0.5	0.0	0.0	0.1	10.4	3.9	171.8	0.8	21.6	3.2	0.1	1.6	0.0
IFNG	14.0	0.5	0.3	0.0	0.0	0.0	4.7	10.0	108.0	0.0	18.2	0.0	0.1	8.2	0.0
TGFB1	2.9	1.2	1.2	0.0	0.0	107.3	1.9	0.7	0.0	15.7	3.0	1.8	0.0	0.0	0.0

4.5 Discussion

When investigating a specific experimental model, it is important to validate the expression of housekeeping genes to obtain reproducible and reliable results for relative quantification of mRNA-levels in target genes of interest (Kozera and and Rapacz, 2013). A housekeeping gene should have a similar expression in all tissues and experimental conditions (Garrido et al., 2020). Prediction using RPKM values correlated with the stability detected using the delta CT method after RT-qPCR for the markers selected for use in the RT qPCR assay. Therefore, RNA-seq can be used to select housekeeping genes.

The markers selected for use in the RT qPCR assay were based on data from animals that were immunized by infection and treatment; the markers were only detected after a subsequent challenge and not during the initial infection (Nefefe et al., 2017). However, these were regarded as markers for protection of sheep with immunity acquired after natural *E. ruminantium* infection transmitted by ticks after treatment. In the present inactivated heartwater vaccine trial, these markers were also upregulated in at least one of the two time-points after vaccination of sheep and these were downregulated at the same time points in the adjuvant only group except for CD41, IFN-γ and GZMB at D7PV. In the present inactivated vaccine trial, these markers were also up regulated after vaccination of sheep. In contrast, these markers were down regulated both post vaccination and post challenge in the adjuvant only negative control group (except for CD41, IFN-γ and GZMB at D7PV and CD41 at D10PC), thus confirming that these markers are not detected during primary infection of heartwater in naïve animals.

However, if these markers were to be used to predict if animals will be protected from heartwater after vaccination, the prediction will be dependent on the time point of sample collection. In G1 animals, a difference in expression could only be seen at D1PV where upregulated genes were detected in animals that lived longer but not in animals that died.

The fact that these genes were not upregulated after challenge may indicated that despite living longer, sufficient memory and adaptive immune responses were not activated PC and these animals would not survive. In G2, all animals reacted at some point PV, but only the animals that survived/lived longer had a high response at D10PC (P12) as well, and perhaps the detection of these transcripts (except CD5 and TGFB1) after challenge can be used to predict if an animal will survive the challenge or not. None of the selected markers could predict protection after vaccination and new or additional markers that will be an early indication of protection PV will have to be selected in future using transcriptome studies.

In addition to the primary role as regulators of haemostasis and thrombosis, platelets have been found to play an important role in both innate and adaptive immune responses (Ali et al., 2015). Among other cells, platelets are the first cells to detect endothelial injury and invasion of pathogens to the bloodstream or tissue through their receptors resulting in platelet activation. Once a pathogen successfully invades the host, chemokines and cytokines are secreted by activated platelets and direct lymphocytes, neutrophils and monocytes to site of inflammation (Portier and Campbell, 2021). The chemokines and cytokines target the pathogen, preventing the infection from spreading. Expression of platelets due to heartwater infection has not been previously reported and in the current study, CD41 was upregulated D1PV. However, its expression decreased on D7PV and D10PC in G1 animals. The first exposure to antigen could elicit a radical immune response hence the high expression of the markers at D1PV in G1 animals after the second boost.

A fluctuation in the expression of CD156A between D7PV and D10PC was observed between immunized animal group 1 and 2. CD156A is expressed on antigen presenting cells and may be indicative of *E*. *ruminantium* antigen uptake. Also, the marker is expressed in neutrophils which are first responders to invaders and produce antimicrobial enzymes that attack the pathogen. When the antigen is introduced for the first time to the

host, the attack on the antigen is expected to be high hence the high level of expression of the marker at D1PV in G1. However, at D10PC the expression of the marker decreased.

CXCR5 is a receptor for the chemokine CXCL13 that is expressed in lymphatic tissues, such as follicles in lymph nodes as well as in spleen. The CXCR5 is expressed in mature B lymphocytes and by a CD4 T helper cell subset, T follicular B helper T cells (T_{FH}) (Chevalier et al., 2011). CXCR5 is essential for directing B cells to lymphoid tissues and in humoral response (Han, 2014). *Ehrlichia ruminantium* in mammalian host replicate first within the regional lymph nodes and is found in spleen of infected animals. The upregulation of CXCR5 in P2, P10 and P12-15 in the animal experiment could be the result of activation of immune cells in the lymph nodes and spleen to fight the infection.

CD8B and Granzyme B (GZMB) are markers specific to $CD8^+$ T cells (cytotoxic T cells) that induce lysis of their target. The current study observed that both markers are expressed in both severely infected and protected animals. However, for the negative control group the markers were hardly expressed. IFN-γ been regarded as an immune marker for protection. The current study shows that it is expressed in both severely infected and treated animals. More data is needed to certify IFN-γ as a biomarker for protection in heartwater infection. Though some animals lived longer than other animals, post tick challenge all animals showed heartwater symptoms and were treated except for S15.

4.6 Conclusion

The housekeeping genes were selected and validated for the purpose of developing a RT qPCR to relatively quantify changes in gene expression of immune markers for protection. The RT qPCR assay was developed to evaluate a multivalent inactivated vaccine against heartwater. The assay was successfully developed and can be validated.

Using a total dosage of 200 µg antigen, the vaccine did not protect the animals against heartwater. The expression levels of the immune markers for protection did not indicate the protective immunity against the disease post vaccination. More research on optimizing antigen dosage is required to comprehend the protective immunity against heartwater. Additional markers that indicate protection post-immunization must be identified and a larger pool of animals that survived challenge need to be included in the analysis.

4.7 References

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4.8 Supplementary data

Supplementary Table 4.1: Animal groups and individual animals combined to make RNA pools at different time points to be tested for immune markers

Group number	Immunized with		Time points and pools			
			D0	D1PV	D7PV	D10PC
Group 1A	2X 100 µg inactivated *E. ruminantium* Welgevonden in adjuvant	Lived longer [sheep 4, 5, 9 and 10]	Pool 1	Pool 2	Pool 3	Pool 4
Group 1B		Treated [sheep 6, 7, 11]	Pool 5	Pool 6	Pool 7	Pool 8
Group 2A	1X 200 µg inactivated *E. ruminantium* Welgevonden in adjuvant	Lived longer [15 and 18]	Pool 9	Pool 10	Pool 11	Pool 12
Group 2B		Treated [12, 13, 14, 16 and 17]	Pool 13	Pool 14	Pool 15	Pool 16
Group 3	1X Adjuvant	Treated [19, 20, 21, 22, 23, 25 and 26]	Pool 17	Pool 18	Pool 19	Pool 20

Supplementary Table 4.2: Sheep numbers with their RNA concentrations

Sheep no	D0 ng/µl	D1PI ng/µl	D7PI ng/µl	D10PC ng/µl
4	315.2	441.6	324.2	154.8
5	471.2	372.7	229.5	200.5
9	679.8	627.8	184.5	170.9
10	843.8	310.9	358.5	238.6
6	138.6	177.4	103.3	137.4
7	374.5	250.0	100.2	249.3
11	306.5	153.6	196.6	390.5
15	337.8	1043.8	500.3	504.8
18	284.9	225.6	866.0	279.1
12	174.0	NA	613.5	427.2
13	132.7	378.6	373.6	420.3
14	437.6	468.1	814.6	832.7
16	154.5	438.8	464.4	128.8
17	490.1	536.1	464.1	546.4
19	364.9	938.2	203.1	596.6
20	266.9	405.5	428.3	256.1
21	431.0	42.2	89.7	585.3
22	532.9	91.7	92.9	220.2
23	1414.2	54.0	107.6	183.2
25	608.6	89.4	302.6	455.4
26	610.0	49.0	388.0	426.1

NA: not available

Supplementary Table 4.3: Volume of RNA for each sheep needed to make the pools.

Sheep no	D0 µl	D1PI µl	D7PI µl	D10PC µl
4	3.17	2.26	3.08	6.45
5	2.12	2.68	4.35	4.98
9	1.47	1.59	5.42	5.85
10	1.18	3.21	2.78	4.19
6	7.21	5.63	9.68	7.27
7	2.67	4.00	9.98	4.01
11	3.26	6.51	5.08	2.56
15	2.96	0.95	1.99	1.98
18	3.51	4.43	1.15	3.58
12	5.74	NA	1.62	2.34
13	7.53	2.64	2.67	2.37
14	2.28	2.13	1.22	1.20
16	6.47	2.27	2.15	7.76
17	2.04	1.86	2.15	1.83
19	2.74	1.06	4.92	1.67
20	3.74	2.46	2.33	3.90
21	2.30	23.69	11.14	1.70
22	1.87	10.90	10.76	4.54
23	0.70	18.51	9.29	5.45
25	1.64	11.18	3.30	2.19
26	1.63	20.40	2.57	2.34

NA: not available

Total concentration must be 1000 ng in each pool

Example: Sheep 4 D0 RNA concentration is 315 ng/µl

315 ng in 1 µl

1000 ng in X µl

[315 ng] [X] = [1000 ng] [1 µl]

X = [1000 ng] [1 µl] / [315 ng]

X = 3.17 µl

Supplementary Table 4.4: Volume of RNA, buffer and H_2O needed for making each pool

Sheep	D0	D1PI	D7PI	D10PC
4	**Pool 1**	**Pool 2**	**Pool 3**	**Pool 4**
5	7.94 µl RNA	9.74 µl RNA	15.63 µl RNA	21.47 µl RNA
9	5 µl buffer	5 µl buffer	5 µl buffer	5 µl buffer
10	37.06 µl H_2O	35.26 µl H_2O	29.37 µl H_2O	23.53 µl H_2O
6	**Pool 5**	**Pool 6**	**Pool 7**	**Pool 8**
7	13.14 µl RNA	16.14 µl	24.74 µl RNA	13.84 µl RNA
11	5 µl buffer	5 µl buffer	5 µl buffer	5 µl buffer
	31.86 µl H_2O	28.86 µl H_2O	20.26 µl H_2O	31.16µl H_2O
15	**Pool 9**	**Pool 10**	**Pool 11**	**Pool 12**
18	6.47 µl RNA	5.38 µl RNA	3.14 µl RNA	5.56 µl RNA
	5 µl buffer	5 µl buffer	5 µl buffer	5 µl buffer
	38.53 µl H_2O	39.61 µl H_2O	41.86 µl H_2O	39.44 µl H_2O
12	**Pool 13**	**Pool 14**	**Pool 15**	**Pool 16**
13	24.06 µl RNA	8.9 µl RNA	9.81 µl RNA	15.5 µl RNA
14	5 µl buffer	5 µl buffer	5 µl buffer	5 µl buffer
16	20.94 µl H_2O	36.1 µl H_2O	35.19 µl H_2O	29.5 µl H_2O
17				
19	**Pool 17**	**Pool 18**	**Pool 19**	**Pool 20**
20	14.62 µl RNA	88.2/2 =44,1 µl	44.31 µl RNA	27.31 µl RNA
21	5 µl buffer	RNA (pool split)	5 µl buffer	5 µl buffer
22	30.38 µl H_2O	5 µl buffer	0.7 µl H_2O	17.69 µl H_2O
23		0.9 µl H_2O		
25				
26				

Chapter 5

General Discussion, Recommendations and Conclusion

Heartwater, a disease caused by *E*. *ruminantium* in some wild and domestic ruminants still poses a threat to farmers even many centuries after its discovery. The heartwater causing agent, *E*. *ruminantium* has diverse immunogenicity and evidence of the occurrence of recombination between strains resulting in new strains has been reported (Allsopp and Allsopp, 2007, Cangi et al., 2016). The only vaccine currently in use is made up of a live Ball3 strain in sheep blood and employs the infection and treatment method (Van der Merwe, 1987). This vaccination method is laborious and limiting to some rural small holder farmers as the vaccine requires cold chain storage. Also, the vaccine lacks cross protection against some virulent and field isolates (Allsopp, 2010). An inactivated vaccine with broad protective ability is of importance. Some requirements for developing an effective vaccine are to isolate field strains in *in vitro* culture to provide the antigen required for the vaccine, and to genetically characterize the isolates to determine the possibility of their use in an inactivated cocktail vaccine. Then, the vaccine can be developed and tested.

MLST is a useful tool to use for genotypic characterization of *E*. *ruminantium* as it reveals genetic diversity among tested field isolates (Adakal et al., 2009). The MLST method has been extensively explored using different structural genes (Adakal et al., 2010, Nakao et al., 2011). The success of the method is not limited to the genotypic characterization of *E*. *ruminantium* but other pathogens such as *Neisseria meningitidis* and "*Candidatus* Neoehrlichia mikurensis", a tickborne agent of neoehrlichiosis (Jolley et al., 2000, Grankvist et al., 2015).

In the current study, the isolates were successfully genetically characterized by MLST. Genetic diversity in the sequenced regions was informed by SNPs observed. The same observations were reported by Allsopp et al. (2003) and Adakal et al. (2009). An obvious observation is distinction of West African isolates from other geographic isolates (Cangi et al., 2016). Based on the regularly observed distinction of West African isolates, it is pivotal to genotype circulating field isolates in West Africa using different genes to determine if the distinction remains. Additionally, researchers must investigate the phenotypic features of the West Africa isolates for the purpose of cross protection against the diverse southern Africa isolates.

MLST is based on structural genes and does not inform virulence or immunogenicity of isolates. A gene that could be used to extrapolate phenotypic features of isolates and was used as a DNA vaccine that elicited protective immunity in mice is *map1 (*Nyika *et al.,* 2002*)*. The use of the gene to genetically characterize diversity of *E*. *ruminantium* isolates has been reported (Raliniaina et al., 2010). However, *map1* gene is highly polymorphic which presents a challenge for designing primers that can detect all genotypes (Allsopp et al., 2001). The polymorphic nature of *map*1 gene disqualifies it as a candidate for genotypic characterization. It is evident that there is a need for extensive research to ascertain characterization genes that can reveal phenotypic features of *E*. *ruminantium* isolates, such as immunogenicity and virulence. That will alleviate the persistent challenges of determining best vaccine isolates and establish improved treatment protocols.

If a genotypic characterization method that inform virulence and immunogenicity of isolates could be developed, it would be beneficial if these *E*. *ruminantium* isolates could be propagated *in vitro* using salivary glands and gut from ticks directly from the field. The conventional method of isolating *E*. *ruminantium* involves the use of blood stabilate and other infected cell lines as inoculum (Bell-Sakyi et al., 2000, Logan et al., 1987). Isolating

E. *ruminantium* from tick material compared to using blood would be cost effective, less time consuming since there is no need for infecting live animals and waiting for incubation period of the disease and considers the welfare of animals as it eliminates the use of blood donor animals. Although *in vitro* isolation of *E*. *ruminantium* from tick material has potential for successful application, the culture system needs to be optimized. The system is not feasible when large sample size is required. DNA from tick is attained by cutting two tick legs close to the body of tick to get the hemolymph and that requires precision, ticks must be kept alive while testing for *E*. *ruminantium* positivity.

It is recommended that a smaller sample size be used, and both antifungal and antibiotics are included in the culture medium. Also, observing the cultures for more than 30 days without any contamination can be considered to conclusively decide a negative result as ticks could have low copy number of *E*. *ruminantium*. Furthermore, it could be of interest to test the contamination in the cultures so that when cell culture is repeated an antimicrobial drug effective against that specific contaminating agent is included in the culture medium.

For subsequent propagation of vaccine isolates and development of vaccine, methods to determine success of the vaccine must be considered. In this study, an animal trial was conducted to test the effectiveness of the developed inactivated vaccine using a total of 200 µg of antigen to immunize sheep. Moreover, a RT qPCR to relatively quantify changes in gene expression of immune markers for protection was developed. The results showed conclusively that immunization with the antigen at the dose of 200 µg did not protect sheep against a homologous tick challenge as all sheep except one sheep were treated with antibiotic post challenge. The immune markers for protection were differentially expressed throughout the experiment and upregulation of the markers did not correlate to protection as both experimental and control group animals died.

It is recommended that more research to determine optimum dosage of the vaccine be conducted before concluding that the vaccine does not offer protective immunity. Also, additional immune markers should be included and tested for any developed heartwater vaccine. The above-mentioned recommendations could be a breakthrough to understanding specific protective immunity against heartwater.

In conclusion, the study showed genetic diversity among *E*. *ruminantium* field isolates from ticks collected from cattle in three provinces of South Africa. The RT qPCR assay developed to evaluate a multivalent inactivated vaccine against heartwater can be validated and applied in further studies. Also, there is a need for alternate genotypic genes that reveal immunogenicity of isolates and optimization of *E*. *ruminantium in vitro* culture systems, using tick salivary gland and gut as inoculum is of paramount importance to be able to establish a pan-African biobank of *E*. *ruminantium* isolates.

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Appendices

Appendix 1

CLUSTAL multiple sequence alignment of concatenated nucleotide sequences of housekeeping genes (*sodB*, *secY*, *lipB*, and *lipA*) partial sequences. The asterisk at the end of the alignment represent a conserved region.

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E67             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
W47             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
U66             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
W36             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
Welg            TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
Ball3           TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
Blaauw          TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
Kwany           TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
Nonile          TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
SBF4            TACCTTATGATGAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
SBF7            TACCTTATGATGAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
UmBanein        TACCTTATGATGAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
Mara            TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
Gardel          TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTAGAGG-GTATTGCTAATAAG
Senegal         TACCTTATGATAAAGCTATGTGTTTTATGCAACAGAGGGTAGAGG-GTATTGCTAATAAG
Sankat          TACCTTATGATAAAGCTATGTGTTTTATGCAACAGAGGGTAGAGG-GTATTGCTAATAAG
L52             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
U11             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
U89             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
W25             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
W102            TACCTTATGATGAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
W26             TACCTTATGATGAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
W54             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
L60             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
L53             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
W91             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
Kumm2           TACCTTATGAGCAGGCTGTAAGTTTGATGCAACAAAGAGTAGAGG-GTATAGCTAGTAAG
River           TACCTTATGAGCAGGCTGTAAGTTTGATGCAACAAAGAGTAGAGG-GTATAGCTAGTAAG
Omatjenne       TACCTTATGAGCAGGCTGTAAGTTTGATGCAACAAAGAGTAGAGG-GTATAGCTAGTAAG
C25             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
C38             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
C49             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
C52             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
C69             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
C87             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
C97             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
C98             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
I34             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
I55             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
I56             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
I70             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
I77             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
I81             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
M3              TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
M11             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
M19             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
M22             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
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M56             TACCTTATGATAAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
C14             TACCTTATGATGAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
C57             TACCTTATGATGAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
I72             TACCTTATGATGAAGCTATGTGTTTTATGCAACAAAGGGTCGAGG-GTATTGCTAATAAG
                **********  * *** *  **** ******** ** ** **** **** **** ****

E67             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
W47             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
U66             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
W36             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
Welg            ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
Ball3           ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
Blaauw          ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
Kwany           ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
Nonile          ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
SBF4            ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
SBF7            ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
UmBanein        ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
Mara            ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
Gardel          ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
Senegal         ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCATTATATACAGCTGGTACTAGT
Sankat          ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCATTATATACAGCTGGTACTAGT
L52             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
U11             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
U89             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
W25             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
W102            ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
W26             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
W54             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCATTATATACAGCTGGTACTAGT
L60             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
L53             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
W91             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
Kumm2           TTGCAGGATGAGCTAGTGTGGTTGCTTGAACATTTTCCATTGTATACAGCTGGTACTAGT
River           TTGCAGGATGAGCTAGTGTGGTTGCTTGAACATTTTCCATTGTATACAGCTGGTACTAGT
Omatjenne       TTGCAGGATGAGCTAGTGTGGTTGCTTGAACATTTTCCATTGTATACAGCTGGTACTAGT
C25             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
C38             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
C49             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
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C69             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
C87             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
C97             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
C98             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
I34             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
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I81             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
M3              ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
M11             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
M19             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
M22             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
M56             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
C14             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
C57             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
I72             ACACAAGATGAACTAGTATGGTTACTTGAACATTTTCCGTTATATACGGCTGGTACTAGT
                   ** ***** ***** ***** ************** ** ***** ************

E67             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
W47             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
U66             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
W36             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
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Welg            GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
Ball3           GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
Blaauw          GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
Kwany           GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
Nonile          GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
SBF4            GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
SBF7            GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
UmBanein        GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
Mara            GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
Gardel          GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
Senegal         GCAAGGAGTGAGGAATTACTAACTGATAGTTTGTTTCCTATATATTCTACAGGTAGAGGT
Sankat          GCAAGGAGTGAGGAATTACTAACTGATAGTTTGTTTCCTATATATTCTACAGGTAGAGGT
L52             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
U11             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
U89             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
W25             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
W102            GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
W26             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
W54             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
L60             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
L53             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
W91             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
Kumm2           GCAAAAGGTGAAGAGTTATTAACTGATAGTTTATTTCCTGTATATACTACAGGTAGGGGT
River           GCAAAAGGTGAAGAGTTATTAACTGATAGTTTATTTCCTGTATATACTACAGGTAGGGGT
Omatjenne       GCAAAAGGTGAAGAGTTATTAACTGATAGTTTATTTCCTGTATATACTACAGGTAGGGGT
C25             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
C38             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
C49             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
C52             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
C69             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
C87             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
C97             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
C98             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
I34             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
I55             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
I56             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
I70             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
I77             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
I81             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
M3              GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
M11             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
M19             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
M22             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
M56             GCAAGGAGTGAGGAATTACTAACCGATAGTTTATTTCCTGTATATTCTACAGGTAGAGGT
C14             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
C57             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
I72             GCAAGGAGTGAGGAATTACTAACCGATAGTTTGTTTCCTGTGTATTCTACAGGTAGAGGT
                ****   **** ** *** **** ******** ****** * *** ********** ***

E67             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
W47             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
U66             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
W36             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
Welg            GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
Ball3           GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
Blaauw          GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
Kwany           GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
Nonile          GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
SBF4            GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
SBF7            GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
UmBanein        GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
Mara            GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
Gardel          GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
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Senegal         GGTAAATACACTTATCATGGTCCTGGTCAAAGAGTTGCTTATGTGATGATGGATTTAAAA
Sankat          GGTAAATACACTTATCATGGTCCTGGTCAAAGAGTTGCTTATGTGATGATGGATTTAAAA
L52             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
U11             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
U89             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
W25             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
W102            GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
W26             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
W54             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
L60             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
L53             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
W91             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
Kumm2           GGTAAATATACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTAATGATGGATTTAAAA
River           GGTAAATATACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTAATGATGGATTTAAAA
Omatjenne       GGTAAATATACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTAATGATGGATTTAAAA
C25             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
C38             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
C49             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
C52             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
C69             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
C87             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
C97             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
C98             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
I34             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
I55             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
I56             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
I70             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
I77             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
I81             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
M3              GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
M11             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
M19             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
M22             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
M56             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
C14             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
C57             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
I72             GGTAAATACACTTATCATGGTCCTGGTCAAAGAATTGCTTATGTGATGATGGATTTAAAA
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E67             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
W47             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
U66             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
W36             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
Welg            GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
Ball3           GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
Blaauw          GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
Kwany           GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
Nonile          GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
SBF4            GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
SBF7            GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
UmBanein        GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
Mara            GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
Gardel          GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
Senegal         GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTGAA
Sankat          GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTGAA
L52             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
U11             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
U89             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
W25             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
W102            GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
W26             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
W54             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
L60             GCAAGAGATAAATGTAATGTTAGGTTGTATATTGAAACTTTGGGTGAGTGGATTGTTAAA
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L53             GCAAGAGATAAATGTAATGTTAGGTTGTATATTGAAACTTTGGGTGAGTGGATTGTTAAA
W91             GCAAGAGATAAATGTAATGTTAGGTTGTATATTGAAACTTTGGGTGAGTGGATTGTTAAA
Kumm2           GTAAGAAATATATGTAATGTTAAATTATATATTGAGAGTTTGGGGAAATGGATTGTTGAA
River           GTAAGAAATATATGTAATGTTAAATTATATATTGAGAGTTTGGGGAAATGGATTGTTGAA
Omatjenne       GTAAGAAATATATGTAATGTTAAATTATATATTGAGAGTTTGGGGAAATGGATTGTTGAA
C25             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
C38             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
C49             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
C52             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
C69             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
C87             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
C97             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
C98             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
I34             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
I55             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
I56             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
I70             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
I77             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
I81             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
M3              GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
M11             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
M19             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
M22             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
M56             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
C14             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
C57             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
I72             GCAAGAGATAAATGTAATGTTAGGTTGTATGTTGAAACTTTGGGTGAGTGGATTGTTAAA
                * **** *** ***********  ** *** **** * ******  * ********* **

E67             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
W47             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
U66             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
W36             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Welg            ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Ball3           ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Blaauw          ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Kwany           ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Nonile          ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
SBF4            ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
SBF7            ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
UmBanein        ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Mara            ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Gardel          ACTTTAAAACATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Senegal         ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Sankat          ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
L52             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
U11             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
U89             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
W25             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
W102            ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
W26             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
W54             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
L60             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
L53             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
W91             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
Kumm2           ACATTGAAGCATTTTTCAATACAATCATATTTTAATCC-TAACTTAATAGGGG--TGTGG
River           ACATTGAAGCATTTTTCAATACAATCATATTTTAATCC-TAACTTAATAGGGG--TGTGG
Omatjenne       ACATTGAAGCATTTTTCAATACAATCATATTTTAATCC-TAACTTAATAGGGG--TGTGG
C25             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
C38             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
C49             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
C52             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
C69             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
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C87             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
C97             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
C98             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
I34             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
I55             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
I56             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
I70             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
I77             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
I81             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
M3              ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
M11             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
M19             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
M22             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
M56             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
C14             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
C57             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
I72             ACTTTAAAGCATTTTTCAATACGATCATATTTTAATCC-TAATTTAATAGGGG--TATGG
                ** ** ** ************* *************** *** **********  * ***

E67             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
W47             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
U66             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
W36             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Welg            GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Ball3           GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Blaauw          GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Kwany           GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Nonile          GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
SBF4            GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
SBF7            GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
UmBanein        GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Mara            GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Gardel          GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Senegal         GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Sankat          GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
L52             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
U11             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
U89             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
W25             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
W102            GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
W26             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
W54             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
L60             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
L53             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
W91             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
Kumm2           ATAGACC-ATAATGGAGTTAAAGAAAAAATTGCTGCATTTGGTATTAGAATAAAAAAGAT
River           ATAGACC-ATAATGGAGTTAAAGAAAAAATTGCTGCATTTGGTATTAGAATAAAAAAGAT
Omatjenne       ATAGACC-ATAATGGAGTTAAAGAAAAAATTGCTGCATTTGGTATTAGAATAAAAAAGAT
C25             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
C38             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
C49             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
C52             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
C69             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
C87             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
C97             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
C98             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
I34             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
I55             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
I56             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
I70             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
I77             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
I81             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
M3              GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
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M11             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
M19             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
M22             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
M56             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
C14             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
C57             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
I72             GTTAACC-ATAATGGTAGTGAAAAAAAAATTGCTGCATTTGGCATTAGAATAAAAAAGAT
                 *  *** *******   * ** ******************** *****************

E67             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
W47             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
U66             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
W36             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
Welg            GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
Ball3           GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
Blaauw          GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
Kwany           GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
Nonile          GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
SBF4            GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
SBF7            GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
UmBanein        GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
Mara            GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
Gardel          GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
Senegal         GATTTAGCCCCATATTTGAGTGCTAATGTGTTAAATTATCACTA-TGGAAAGCATCATCA
Sankat          GATTTAGCCCCATATTTGAGTGCTAATGTGTTAAATTATCACTA-TGGAAAGCATCATCA
L52             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
U11             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
U89             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
W25             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
W102            GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
W26             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
W54             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
L60             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
L53             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
W91             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
Kumm2           GATTTAGTACCATATTTAAGTGCTAGTGTGTTAGACTATCATTA-TGGTAAACATCATCA
River           GATTTAGTACCATATTTAAGTGCTAGTGTGTTAGACTATCATTA-TGGTAAACATCATCA
Omatjenne       GATTTAGTACCATATTTAAGTGCTAGTGTGTTAGACTATCATTA-TGGTAAACATCATCA
C25             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
C38             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
C49             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
C52             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
C69             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
C87             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
C97             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
C98             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
I34             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
I55             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
I56             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
I70             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
I77             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
I81             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
M3              GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
M11             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
M19             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
M22             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
M56             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
C14             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
C57             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
I72             GATTTAGCCCCATATTTGAGTGCTAATGTGTTGAATTATCACTA-TGGAAAGCATCATCA
                ******* ******** ******* ******  * ***** ** *** ** ********

E67             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
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W47             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
U66             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
W36             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
Welg            AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
Ball3           AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
Blaauw          AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
Kwany           AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
Nonile          AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
SBF4            AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
SBF7            AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
UmBanein        AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
Mara            AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
Gardel          AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
Senegal         AGGTTATGTGAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
Sankat          AGGTTATGTGAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
L52             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
U11             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
U89             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
W25             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
W102            AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
W26             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
W54             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
L60             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
L53             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
W91             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
Kumm2           AGGTTATGTTAATGCTTTAAA-CAATCTTGTTGCAGGGACTGATTTTTGTACTTATACAA
River           AGGTTATGTTAATGCTTTAAA-CAATCTTGTTGCAGGGACTGATTTTTGTACTTATACAA
Omatjenne       AGGTTATGTTAATGCTTTAAA-CAATCTTGTTGCAGGGACTGATTTTTGTACTTATACAA
C25             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
C38             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
C49             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
C52             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
C69             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
C87             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
C97             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
C98             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
I34             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
I55             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
I56             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
I70             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
I77             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
I81             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
M3              AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
M11             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
M19             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
M22             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
M56             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
C14             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACATGTACGA
C57             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
I72             AGGTTATGTAAATGCTTTAAA-TAATCTTGTTGCAGGGACTGATTTCTGTACCTGTACGA
                ********* **********  ********************** ***** * *** *

E67             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
W47             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
U66             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
W36             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Welg            ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Ball3           ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Blaauw          ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Kwany           ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Nonile          ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
SBF4            ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
SBF7            ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
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UmBanein        ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Mara            ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Gardel          ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Senegal         ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Sankat          ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
L52             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
U11             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
U89             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
W25             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
W102            ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
W26             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
W54             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
L60             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
L53             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
W91             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
Kumm2           ATGAAGATTTACCTACAGTTATTAAGGCTACGCATGGTAATTTAGCAACCAGATCAATAT
River           ATGAAGATTTACCTACAGTTATTAAGGCTACGCATGGTAATTTAGCAACCAGATCAATAT
Omatjenne       ATGAAGATTTACCTACAGTTATTAAGGCTACGCATGGTAATTTAGCAACCAGATCAATAT
C25             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
C38             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
C49             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
C52             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
C69             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
C87             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAAATCAATAT
C97             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
C98             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
I34             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
I55             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
I56             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
I70             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAAATCAATAT
I77             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
I81             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
M3              ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
M11             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
M19             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
M22             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
M56             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
C14             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
C57             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
I72             ATGAAGATTTACCTGCGGTTATTAAAGCTACACATAGTGATTTAGCAACTAGATCAATAT
                ************** * ******** ***** *** ** ********** * ********

E67             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
W47             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
U66             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
W36             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
Welg            TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
Ball3           TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
Blaauw          TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAGTCTATAACAAAGAATG
Kwany           TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAGTCTATAACAAAGAATG
Nonile          TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
SBF4            TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
SBF7            TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
UmBanein        TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
Mara            TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
Gardel          TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
Senegal         TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
Sankat          TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
L52             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
U11             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
U89             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
W25             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
W102            TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
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W26             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
W54             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
L60             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAGTCTATAACAAAGAATG
L53             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAGTCTATAACAAAGAATG
W91             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAGTCTATAACAAAGAATG
Kumm2           TTAATAATGCTGGTCAAGTATGGAATCATAACTTTTATTGGCAGTCTATCAAAAAGAATG
River           TTAATAATGCTGGTCAAGTATGGAATCATAACTTTTATTGGCAGTCTATCAAAAAGAATG
Omatjenne       TTAATAATGCTGGTCAAGTATGGAATCATAACTTTTATTGGCAGTCTATCAAAAAGAATG
C25             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
C38             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
C49             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
C52             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
C69             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
C87             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
C97             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
C98             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
I34             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
I55             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
I56             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
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I77             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
I81             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
M3              TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
M11             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAGTCTATAACAAAGAATG
M19             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
M22             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
M56             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
C14             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
C57             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
I72             TTAATAATGCTGGTCAAGTATGGAATCATAATTTTTATTGGCAATCTATAACAAAGAATG
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E67             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
W47             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
U66             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
W36             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
Welg            GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
Ball3           GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
Blaauw          GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
Kwany           GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
Nonile          GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
SBF4            GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
SBF7            GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
UmBanein        GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
Mara            GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
Gardel          GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
Senegal         GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTATTG
Sankat          GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTATTG
L52             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
U11             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
U89             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
W25             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
W102            GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
W26             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
W54             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
L60             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
L53             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
W91             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
Kumm2           GAGGTATAGCTCCTACAGGAGAATTACTCAATAGAATTAATAAAGATTTTGGAAGTATTG
River           GAGGTATAGCTCCTACAGGAGAATTACTCAATAGAATTAATAAAGATTTTGGAAGTATTG
Omatjenne       GAGGTATAGCTCCTACAGGAGAATTACTCAATAGAATTAATAAAGATTTTGGAAGTATTG
C25             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
C38             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
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C49             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
C52             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
C69             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
C87             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
C97             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
C98             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
I34             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
I55             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
I56             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
I70             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
I77             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
I81             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
M3              GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
M11             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
M19             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
M22             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
M56             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
C14             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
C57             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
I72             GTGTTGTTACGCCTAAAGGAGAGTTATTAGATAGAATAAATAAAGATTTTGAAAGTGTTG
                * * * *  * **** ****** *** *  ******* ************* **** ***

E67             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
W47             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
U66             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
W36             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Welg            AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Ball3           AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Blaauw          AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Kwany           AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Nonile          AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
SBF4            AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
SBF7            AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
UmBanein        AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Mara            AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Gardel          AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Senegal         AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Sankat          AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
L52             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
U11             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
U89             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
W25             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
W102            AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
W26             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
W54             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
L60             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
L53             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
W91             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
Kumm2           ACAAGTTTAATCATGCTTTTACTGAAGCTGGAAAGAGTCATTTTGGTAGTGGTTGGATAT
River           ACAAGTTTAATCATGCTTTTACTGAAGCTGGAAAGAGTCATTTTGGTAGTGGTTGGATAT
Omatjenne       ACAAGTTTAATCATGCTTTTACTGAAGCTGGAAAGAGTCATTTTGGTAGTGGTTGGATAT
C25             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
C38             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
C49             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
C52             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
C69             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
C87             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
C97             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
C98             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
I34             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
I55             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
I56             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
I70             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
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I77             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
I81             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
M3              AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
M11             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
M19             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
M22             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
M56             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
C14             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
C57             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
I72             AAAAGTTTAATAATGCTTTTACTGAAGCTGGAAAAGGTCATTTTGGTAGTGGATGGGTAT
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E67             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
W47             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
U66             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
W36             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Welg            GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Ball3           GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Blaauw          GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Kwany           GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Nonile          GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
SBF4            GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
SBF7            GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
UmBanein        GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Mara            GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Gardel          GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Senegal         GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Sankat          GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
L52             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
U11             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
U89             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
W25             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
W102            GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
W26             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
W54             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
L60             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
L53             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
W91             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
Kumm2           GGTTAGTTTTTGATATTACAGAACAAAAACTTAAGATTCTTTGTACAGC-TAATGGTGAT
River           GGTTAGTTTTTGATATTACAGAACAAAAACTTAAGATTCTTTGTACAGC-TAATGGTGAT
Omatjenne       GGTTAGTTTTTGATATTACAGAACAAAAACTTAAGATTCTTTGTACAGC-TAATGGTGAT
C25             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
C38             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
C49             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
C52             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
C69             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
C87             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
C97             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
C98             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
I34             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
I55             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
I56             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
I70             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
I77             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
I81             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
M3              GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
M11             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
M19             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
M22             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
M56             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
C14             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
C57             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
I72             GGTTGGTTTTTGATACTGTAGAGCAAAAACTTAAAATTTTGTGTACAGC-TAATGGTGAT
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E67             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
W47             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
U66             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
W36             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
Welg            ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
Ball3           ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
Blaauw          ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
Kwany           ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
Nonile          ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
SBF4            ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
SBF7            ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
UmBanein        ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
Mara            ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
Gardel          ACTCCTATTACT-CAATATCCTGATACATATCCATTACTGACTATGGATGTAGTTTGGCT
Senegal         ACTCCTATTACT-CAATATCCTGATACATATCCATTACTGACTATGGATGTAGTTTAGCT
Sankat          ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTAGCT
L52             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
U11             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
U89             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
W25             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
W102            ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
W26             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
W54             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
L60             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
L53             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
W91             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
Kumm2           ACTCCTATTACT-CAGTATCCTGATACATATCCATTATTGACTATGGATGTAGCCTAGCT
River           ACTCCTATTACT-CAGTATCCTGATACATATCCATTATTGACTATGGATGTAGCCTAGCT
Omatjenne       ACTCCTATTACT-CAGTATCCTGATACATATCCATTATTGACTATGGATGTAGCCTAGCT
C25             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
C38             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
C49             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
C52             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
C69             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
C87             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
C97             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
C98             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
I34             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
I55             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
I56             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
I70             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
I77             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
I81             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
M3              ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
M11             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
M19             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
M22             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
M56             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
C14             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
C57             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
I72             ACTCCTATTACT-CAATATCCTGATACATACCCATTACTGACTATGGATGTAGTTTGGCT
                ************ ** ************** ****** ***************  * ***

E67             AAAGCTGTTAGTTCTTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
W47             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
U66             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
W36             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
Welg            AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
Ball3           AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
Blaauw          AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
Kwany           AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
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Nonile          AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
SBF4            AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
SBF7            AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
UmBanein        AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
Mara            AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
Gardel          AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
Senegal         AAAGCTGTTAGTTTGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
Sankat          AAAGCTGTTAGTTTGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
L52             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
U11             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
U89             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
W25             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
W102            AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
W26             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
W54             AAAGCTGTTAGTTCTTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
L60             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
L53             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
W91             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
Kumm2           AAGGCTGTAAGTTTACTAAAGTTACAGCATGTTGTTATCACATCTGTAGATAGGGATGAT
River           AAGGCTGTAAGTTTACTAAAGTTACAGCATGTTGTTATCACATCTGTAGATAGGGATGAT
Omatjenne       AAGGCTGTAAGTTTACTAAAGTTACAGCATGTTGTTATCACATCTGTAGATAGGGATGAT
C25             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
C38             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
C49             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
C52             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
C69             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
C87             AAAGCTGTTAGTTCTTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
C97             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
C98             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
I34             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
I55             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
I56             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
I70             AAAGCTGTTAGTTCTTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
I77             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
I81             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
M3              AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
M11             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
M19             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
M22             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
M56             AAAGCTGTTAGTTCGTTAAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
C14             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
C57             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
I72             AAAGCTGTTAGTTCGTTGAAGTTACAGCATGTTGTAATTACATCTGTGGATCGAGATGAC
                ** ***** ****   * ***************** ** ******** *** * *****

E67             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
W47             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
U66             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
W36             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
Welg            TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
Ball3           TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
Blaauw          TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
Kwany           TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
Nonile          TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
SBF4            TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
SBF7            TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
UmBanein        TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
Mara            TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
Gardel          TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
Senegal         TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAGATACGCAAAAGAGAT
Sankat          TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAGATACGCAAAAGAGAT
L52             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
U11             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
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U89             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
W25             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
W102            TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
W26             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
W54             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
L60             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
L53             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
W91             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
Kumm2           TTAGAAGATGGTGGTGCTGGGCATTTTGTGGAATGTATAGAGGAAATTCGAAAAAGAGAT
River           TTAGAAGATGGTGGTGCTGGGCATTTTGTGGAATGTATAGAGGAAATTCGAAAAAGAGAT
Omatjenne       TTAGAAGATGGTGGTGCTGGGCATTTTGTGGAATGTATAGAGGAAATTCGAAAAAGAGAT
C25             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
C38             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
C49             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
C52             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
C69             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
C87             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
C97             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
C98             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
I34             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
I55             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
I56             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
I70             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
I77             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
I81             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
M3              TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
M11             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
M19             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
M22             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
M56             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGTAAAAGGGAT
C14             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
C57             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
I72             TTAGAAGATGGTGGTGCTGGGCATTTTGTAGAATGTATAGAAGAAATACGCAAAAGGGAT
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E67             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
W47             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
U66             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
W36             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
Welg            AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
Ball3           AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
Blaauw          AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
Kwany           AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
Nonile          AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
SBF4            AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
SBF7            AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
UmBanein        AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
Mara            AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
Gardel          AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
Senegal         AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
Sankat          AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
L52             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
U11             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
U89             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
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W102            AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
W26             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
W54             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
L60             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
L53             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
W91             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
Kumm2           AACAATGTTACTATTGAAATATTAACTCCAGATTTTTTAAATAAGCATAGTGCAATTGAT
River           AACAATGTTACTATTGAAATATTAACTCCAGATTTTTTAAATAAGCATAGTGCAATTGAT
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Omatjenne       AACAATGTTACTATTGAAATATTAACTCCAGATTTTTTAAATAAGCATAGTGCAATTGAT
C25             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
C38             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
C49             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
C52             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
C69             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
C87             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
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I77             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
I81             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
M3              AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
M11             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACGTGATGCAATTGAT
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C14             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
C57             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
I72             AGTAATGTCACTATTGAAATATTAACTCCAGATTTTTTAAATAAACATGATGCAATTGAT
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E67             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
W47             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
U66             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
W36             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
Welg            AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
Ball3           AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
Blaauw          AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
Kwany           AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
Nonile          AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
SBF4            AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
SBF7            AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
UmBanein        AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
Mara            AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGTTA
Gardel          AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
Senegal         AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
Sankat          AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
L52             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGTTA
U11             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
U89             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
W25             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGTTA
W102            AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
W26             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
W54             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
L60             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
L53             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
W91             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
Kumm2           AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACTGTACCAAGGTTA
River           AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACTGTACCAAGGTTA
Omatjenne       AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACTGTACCAAGGTTA
C25             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
C38             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGTTA
C49             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
C52             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
C69             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
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C97             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
C98             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
I34             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
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I55             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
I56             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
I70             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
I77             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
I81             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
M3              AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
M11             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
M19             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
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M56             AAAATTGCTAAAGCATTTCCTGATGTGTATAACCATAATGTAGAAACAGTACCAAGGCTA
C14             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
C57             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
I72             AAAATTGCTAAAGCATTTCCTGATGTGTATAATCATAATGTAGAAACAGTACCAAGGCTG
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W47             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
U66             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
W36             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Welg            TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Ball3           TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Blaauw          TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Kwany           TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Nonile          TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
SBF4            TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
SBF7            TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
UmBanein        TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Mara            TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Gardel          TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Senegal         TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Sankat          TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
L52             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
U11             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
U89             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
W25             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
W102            TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
W26             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
W54             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
L60             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
L53             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
W91             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
Kumm2           TATGCCAAAATTAGACCAAGGGCACGATATTTTCATTCTTTATATTTGCTTAAAACGATC
River           TATGCCAAAATTAGACCAAGGGCACGATATTTTCATTCTTTATATTTGCTTAAAACGATC
Omatjenne       TATGCCAAAATTAGACCAAGGGCACGATATTTTCATTCTTTATATTTGCTTAAAACGATC
C25             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
C38             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
C49             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
C52             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
C69             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
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I56             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
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I77             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
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M11             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
M19             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
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M56             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
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C14             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
C57             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
I72             TATGCTAAGATTAGACCAAAAGCACGTTATTTTCATTCTTTATATTTGCTAAAAACAATA
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E67             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
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U66             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
W36             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
Welg            AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
Ball3           AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
Blaauw          AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
Kwany           AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
Nonile          AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
SBF4            AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
SBF7            AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
UmBanein        AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
Mara            AAGCAGAAAAACCCTAGGATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
Gardel          AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
Senegal         AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
Sankat          AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
L52             AAGCAGAAAAACCCTAGGATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
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W91             AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
Kumm2           AAACAAAAAAAATCCTCAAATATTTACTAAATCTGGAATAATGTTGGGCTTAGGGGAAATG
River           AAACAAAAAAAATCCTCAAATATTTACTAAATCTGGAATAATGTTGGGCTTAGGGGAAATG
Omatjenne       AAACAAAAAAAATCCTCAAATATTTACTAAATCTGGAATAATGTTGGGCTTAGGGGAAATG
C25             AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
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C69             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
C87             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
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C98             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
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M11             AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
M19             AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
M22             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
M56             AAGCAGAAAAACCCTAGAATATTTACTAAGTCCGGTATAATGGTAGGATTGGGAGAACTA
C14             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
C57             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
I72             AAGCAGAAAAACCCTAGAATATTTACTAAGTCAGGTATAATGGTAGGATTGGGAGAACTA
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E67             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
W47             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
U66             AAAGAAGAGATATACCAAGATAGATGCAGTTATATCAAAACATTCAGTGGGTGTTTTAGG
W36             AAAGAAGAGATATACCAAGATAGATGCAGTTATATCAAAACATTCAGTGGGTGTTTTAGG
Welg            AAAGAAGAGATATACCAAGATAGATGCAGTTATATCAAAACATTCAGTGGGTGTTTTAGG
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Ball3           AAAGAAGAGATATACCAAGATAGATGCAGTTATATCAAAACATTCAGTGGGTGTTTTAGG
Blaauw          AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
Kwany           AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
Nonile          AAAGAAGAGATATACCAAGATAGATGCAGTTATATCAAAACATTCAGTGGGTGTTTTAGG
SBF4            AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
SBF7            AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
UmBanein        AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
Mara            AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
Gardel          AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
Senegal         AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAGCATTCAGTTGGTGTTTTAGG
Sankat          AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAGCATTCAGTTGGTGTTTTAGG
L52             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
U11             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
U89             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
W25             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
W102            AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
W26             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
W54             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
L60             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
L53             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
W91             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
Kumm2           AAAGAGGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTATTTTAGG
River           AAAGAGGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTATTTTAGG
Omatjenne       AAAGAGGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTATTTTAGG
C25             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
C38             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
C49             AAAGAAGAGATATACCAAGATAGATGCAGTTATATCAAAACATTCAGTGGGTGTTTTAGG
C52             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
C69             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
C87             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
C97             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
C98             AAAGAAGAGATATACCAAGATAGATGCAGTTATATCAAAACATTCAGTGGGTGTTTTAGG
I34             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
I55             AAAGAAGAGATATACCAAGATAGATGCAGTTATATCAAAACATTCAGTGGGTGTTTTAGG
I56             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
I70             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
I77             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
I81             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
M3              AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
M11             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
M19             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
M22             AAAGAAGAGATATACCAAGATAGATGCAGTTATATCAAAACATTCAGTGGGTGTTTTAGG
M56             AAAGAAGAGATATATCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
C14             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
C57             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
I72             AAAGAAGAGATATACCAAGATAGATGCAGTTATCTCAAAACATTCAGTGGGTGTTTTAGG
                ***** ******** ****************** ***** ******** *** *******

E67             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
W47             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
U66             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
W36             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
Welg            TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
Ball3           TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
Blaauw          TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
Kwany           TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
Nonile          TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
SBF4            TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
SBF7            TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
UmBanein        TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
Mara            TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
Gardel          TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
Senegal         TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCTCTTAATGT
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Sankat          TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCTCTTAATGT
L52             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
U11             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
U89             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
W25             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
W102            TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
W26             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
W54             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
L60             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
L53             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
W91             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
Kumm2           TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGGAGAATGACAATTTTTGCTCTAAATGT
River           TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGGAGAATGACAATTTTTGCTCTAAATGT
Omatjenne       TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGGAGAATGACAATTTTTGCTCTAAATGT
C25             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
C38             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
C49             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
C52             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
C69             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
C87             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
C97             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
C98             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
I34             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
I55             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
I56             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
I70             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
I77             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
I81             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
M3              TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
M11             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
M19             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
M22             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
M56             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
C14             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
C57             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
I72             TATTTTTAATGTATTTTCTGGTGGTGCTTTAGGTAGAATGACAATTTTTGCCCTTAATGT
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E67             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
W47             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
U66             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
W36             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
Welg            TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
Ball3           TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
Blaauw          TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
Kwany           TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
Nonile          TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
SBF4            TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
SBF7            TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
UmBanein        TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
Mara            TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
Gardel          TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
Senegal         TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAGTTCCAAGATT
Sankat          TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAGTTCCAAGATT
L52             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
U11             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
U89             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
W25             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
W102            TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
W26             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
W54             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
L60             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
L53             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
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W91             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
Kumm2           TATGCCATATATAGTATCTTCTATAATTATGCAGTTGTTATCAGTGACAATTCCAAAATT
River           TATGCCATATATAGTATCTTCTATAATTATGCAGTTGTTATCAGTGACAATTCCAAAATT
Omatjenne       TATGCCATATATAGTATCTTCTATAATTATGCAGTTGTTATCAGTGACAATTCCAAAATT
C25             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
C38             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
C49             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
C52             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
C69             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
C87             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
C97             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
C98             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
I34             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
I55             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
I56             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
I70             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
I77             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
I81             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
M3              TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
M11             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
M19             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
M22             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
M56             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
C14             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
C57             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
I72             TATGCCATATATAGTTTCTTCTATAATTATGCAGTTACTTTCAGTTACAATTCCAAGATT
                *************** ********************  * ***** *** ****** ***

E67             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
W47             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
U66             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
W36             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
Welg            GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
Ball3           GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
Blaauw          AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
Kwany           AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
Nonile          GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
SBF4            GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
SBF7            GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
UmBanein        GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
Mara            AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
Gardel          GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
Senegal         AAATGAGATGAGGCAAAGTGGTGAATTAGGTAGAGTAAAGATTAATACTTATGTACGATA
Sankat          AAATGAGATGAGGCAAAGTGGTGAATTAGGTAGAGTAAAGATTAATACTTATGTACGATA
L52             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
U11             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
U89             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
W25             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
W102            GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
W26             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
W54             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
L60             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
L53             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
W91             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
Kumm2           GAATGAAATGAGGCAAAGTGGTGAGCTAGGTAGGGTAAAAATTAATACTTATGTACGGTA
River           GAATGAAATGAGGCAAAGTGGTGAGCTAGGTAGGGTAAAAATTAATACTTATGTACGGTA
Omatjenne       GAATGAAATGAGGCAAAGTGGTGAGCTAGGTAGGGTAAAAATTAATACTTATGTACGGTA
C25             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
C38             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
C49             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
C52             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
C69             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
C87             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
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C97             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
C98             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
I34             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
I55             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
I56             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
I70             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
I77             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
I81             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
M3              GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
M11             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
M19             AAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
M22             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTAAGATA
M56             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
C14             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
C57             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
I72             GAATGAGATGAGACAAAGTGGTGAATTAGGTAGGGTAAAGATTAATACTTATGTACGATA
                 ***** ***** **********  ******* ***** *************** * **

E67             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
W47             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
U66             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
W36             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
Welg            TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
Ball3           TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
Blaauw          TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
Kwany           TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
Nonile          TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
SBF4            TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
SBF7            TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
UmBanein        TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
Mara            TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
Gardel          TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
Senegal         TATAACTATTGTTTTCTGTTTAATTCAAGGGTCTATTATCTTGCTTGGGTTAGAGAAAAT
Sankat          TATAACTATTGTTTTCTGTTTAATTCAAGGGTCTATTATCTTGCTTGGGTTAGAGAAAAT
L52             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
U11             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
U89             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
W25             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
W102            TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
W26             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
W54             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
L60             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
L53             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
W91             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
Kumm2           TATAACTATTCTGTTTTGTTTAATTCAAGGTTCTATTATCTTACTTGGATTAGAAAAAAT
River           TATAACTATTCTGTTTTGTTTAATTCAAGGTTCTATTATCTTACTTGGATTAGAAAAAAT
Omatjenne       TATAACTATTCTGTTTTGTTTAATTCAAGGTTCTATTATCTTACTTGGATTAGAAAAAAT
C25             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
C38             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
C49             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
C52             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
C69             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
C87             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
C97             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
C98             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
I34             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
I55             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
I56             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
I70             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
I77             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
I81             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
M3              TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
M11             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
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M19             TATAACTATTGGTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
M22             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
M56             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
C14             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
C57             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
I72             TATAACTATTGTTTTCTGTTTAATTCAAGGTTCTATTATCTTGCTTGGGTTAGAAAAAAT
                **********   ** ************** *********** ***** ***** *****

E67             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
W47             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
U66             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
W36             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Welg            GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Ball3           GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Blaauw          GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Kwany           GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Nonile          GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
SBF4            GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
SBF7            GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
UmBanein        GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Mara            GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Gardel          GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Senegal         GAATACACAGACTAGTATTGTAGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Sankat          GAATACACAGACTAGTATTGTAGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
L52             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
U11             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
U89             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
W25             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
W102            GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
W26             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
W54             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
L60             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
L53             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
W91             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
Kumm2           GAATACAGAAGCTAGTATTGTAGTGATTGATCCTGGTTTATTATTTAGAGTAGTAGGTAT
River           GAATACAGAAGCTAGTATTGTAGTGATTGATCCTGGTTTATTATTTAGAGTAGTAGGTAT
Omatjenne       GAATACAGAAGCTAGTATTGTAGTGATTGATCCTGGTTTATTATTTAGAGTAGTAGGTAT
C25             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
C38             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
C49             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
C52             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
C69             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
C87             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
C97             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
C98             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
I34             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
I55             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
I56             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
I70             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
I77             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
I81             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
M3              GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
M11             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
M19             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
M22             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
M56             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
C14             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
C57             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
I72             GAATACACAGACTAGTATTGTGGTAATTGATCCTGGTTTGTTATTTAGGGTAGTAGGTAT
                ******* *  ********** ** ************** ******** ***********

E67             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
W47             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
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U66             TAGTAGCTTGTTAGGTGGTACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
W36             TAGTAGCTTGTTAGGTGGTACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
Welg            TAGTAGCTTGTTAGGTGGTACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
Ball3           TAGTAGCTTGTTAGGTGGTACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
Blaauw          TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
Kwany           TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
Nonile          TAGTAGCTTGTTAGGTGGTACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
SBF4            TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
SBF7            TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
UmBanein        TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
Mara            TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
Gardel          TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
Senegal         TAGTAGCTTGTTAGGCGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
Sankat          TAGTAGCTTGTTAGGCGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
L52             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
U11             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
U89             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
W25             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
W102            TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
W26             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
W54             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
L60             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
L53             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
W91             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
Kumm2           TAGTAGCTTACTAGGTGGGACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAA
River           TAGTAGCTTACTAGGTGGGACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAA
Omatjenne       TAGTAGCTTACTAGGTGGGACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAA
C25             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
C38             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
C49             TAGTAGCTTGTTAGGTGGTACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
C52             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
C69             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
C87             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
C97             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
C98             TAGTAGCTTGTTAGGTGGTACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
I34             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
I55             TAGTAGCTTGTTAGGTGGTACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
I56             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
I70             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
I77             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
I81             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
M3              TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
M11             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
M19             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
M22             TAGTAGCTTGTTAGGTGGTACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
M56             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTCTGGTTAGGTGATAGTATAAATAAAAG
C14             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
C57             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
I72             TAGTAGCTTGTTAGGTGGCACAATGTTTCTACTTTGGTTAGGTGATAGTATAAATAAAAG
                *********  **** ** ************** *************************

E67             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
W47             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
U66             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
W36             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
Welg            GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
Ball3           GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
Blaauw          GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
Kwany           GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
Nonile          GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
SBF4            GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
SBF7            GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
UmBanein        GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
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Mara            GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
Gardel          GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATTGCAGAGTTACCAGG
Senegal         GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATTGCAGAATTACCAGG
Sankat          GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATTGCAGAGTTACCAGG
L52             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
U11             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
U89             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
W25             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
W102            GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
W26             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
W54             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
L60             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
L53             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
W91             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
Kumm2           GGGGATTGGTAATGGAGTCTCCATGATTATATTTACAGGAATAATTGCAGAGTTACCAGG
River           GGGGATTGGTAATGGAGTCTCCATGATTATATTTACAGGAATAATTGCAGAGTTACCAGG
Omatjenne       GGGGATTGGTAATGGAGTCTCCATGATTATATTTACAGGAATAATTGCAGAGTTACCAGG
C25             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
C38             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
C49             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
C52             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
C69             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
C87             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
C97             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
C98             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
I34             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
I55             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
I56             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
I70             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
I77             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
I81             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
M3              GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
M11             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
M19             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
M22             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
M56             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
C14             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
C57             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
I72             GGGTATTGGTAATGGGATTTCTCTAATTATATTTACAGGAATAATCGCAGAGTTACCAGG
                *** ***********  * **  * ******************** ***** ********

E67             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
W47             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
U66             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
W36             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
Welg            TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
Ball3           TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
Blaauw          TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
Kwany           TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
Nonile          TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
SBF4            TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
SBF7            TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
UmBanein        TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
Mara            TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
Gardel          TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
Senegal         TTCTTTTTCTGCCATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
Sankat          TTCTTTTTCTGCCATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
L52             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
U11             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
U89             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
W25             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
W102            TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
W26             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
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W54             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
L60             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
L53             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
W91             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
Kumm2           TTCTTTCTCTTCTATGTTTTTATTAGGTAAAAATGGTAATGTACCAATCTTTGTTATCTT
River           TTCTTTCTCTTCTATGTTTTTATTAGGTAAAAATGGTAATGTACCAATCTTTGTTATCTT
Omatjenne       TTCTTTCTCTTCTATGTTTTTATTAGGTAAAAATGGTAATGTACCAATCTTTGTTATCTT
C25             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
C38             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
C49             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
C52             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
C69             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
C87             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
C97             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
C98             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
I34             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
I55             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
I56             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
I70             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
I77             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
I81             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
M3              TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
M11             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
M19             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTTATTTT
M22             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
M56             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
C14             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
C57             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
I72             TTCTTTTTCTGCTATGTTTTTATTAGGTAAAAATGGCAATGTGCCAATATTTGTGATTTT
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E67             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
W47             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
U66             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
W36             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
Welg            ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
Ball3           ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
Blaauw          ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
Kwany           ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
Nonile          ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
SBF4            ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
SBF7            ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
UmBanein        ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
Mara            ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
Gardel          ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
Senegal         ATTAATGATATTAATATTTTTTGCATTACTGTTATTAATCATTTTTGTTGAAAAATCTTA
Sankat          ATTAATGATATTAATATTTTTTGCATTACTGTTATTAATCATTTTTGTTGAAAAATCTTA
L52             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
U11             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
U89             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
W25             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
W102            ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
W26             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
W54             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
L60             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
L53             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
W91             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
Kumm2           ATTAATGATATTGGTATTTTTTGCGTTATTGTTACTAATTATTTTTATTGAAAAATCTTA
River           ATTAATGATATTGGTATTTTTTGCGTTATTGTTACTAATTATTTTTATTGAAAAATCTTA
Omatjenne       ATTAATGATATTGGTATTTTTTGCGTTATTGTTACTAATTATTTTTATTGAAAAATCTTA
C25             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
C38             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
C49             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
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C52             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
C69             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
C87             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
C97             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
C98             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
I34             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
I55             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
I56             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
I70             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
I77             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
I81             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
M3              ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
M11             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
M19             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
M22             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
M56             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
C14             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
C57             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
I72             ATTAATGATATTAATATTTTTTGCATTACTATTGTTAATCATTTTCGTTGAAAAATCTTA
                ************  ********** *** * **  **** *****  ************

E67             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
W47             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
U66             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
W36             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Welg            TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Ball3           TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Blaauw          TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Kwany           TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Nonile          TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
SBF4            TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
SBF7            TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
UmBanein        TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Mara            TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Gardel          TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Senegal         TCGAAAAATTTTGGTACAATATCCAAAAAGGCAGGTTAAAAATAAGTTATATAAT
Sankat          TCGAAAAATTTTGGTACAATATCCAAAAAGGCAGGTTAAAAATAAGTTATATAAT
L52             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
U11             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
U89             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
W25             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
W102            TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
W26             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
W54             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
L60             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
L53             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
W91             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Kumm2           TCGTAAGATTTTAGTACAGTACCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
River          TCGTAAGATTTTAGTACAGTACCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
Omatjenne       TCGTAAGATTTTAGTACAGTACCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C25             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C38             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C49             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C52             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C69             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C87             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C97             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C98             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
I34             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
I55             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
I56             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
I70             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
I77             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
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I81             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
M3              TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
M11             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
M19             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
M22             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
M56             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C14             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
C57             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
I72             TAGAAAAATTTTAGTACAATATCCAAAAAGGCAGGTTAAAAATAAATTATATAAT
                * * ** ***** ***** ** ********************** *********
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Appendix 2

Approval Documents

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Assessment of an inactivated multivalent heartwater vaccine under field conditions**
Project Number	**REC205-19**
Researcher / Principal Investigator	**Ms Z Lukanji**

Dissertation / Thesis submitted for	**Doctoral**

Supervisor	**Prof LCBGD Neves**

Conditions	**Please address the following:** **An informed consent document should be uploaded for the sampling of animals and collection of ticks.** **DAFF section 20 approval is required prior to tick sampling - uploaded approval only corresponds to the collection of blood from HW affected animals.** **The DAFF section 20 requires that only FMD PCR negative samples may be moved from HH to DVTD. This portion / testing should be inserted into the M&M section of the application.**

CONDITIONALLY APPROVED	Date: **2019-11-08**
CHAIRMAN: UP Research Ethics Committee	Signature:

Office of the Chairman: Research Ethics Committee
Room 2-24, Pathology Building, Onderstepoort
University of Pretoria, Private Bag X04
Onderstepoort 0110, South Africa
Tel +27 (0)12 529 8052
Email
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

5 December 2019

Approval Certificate with Conditions
New Application

AEC Reference No.: REC205-19
Title: Assessment of an inactivated multivalent heartwater vaccine under field conditions
Researcher: Ms Z Lukanji
Student's Supervisor: Prof LCBGD Neves

Dear Ms Z Lukanji,

The **New Application** as supported by documents received between 2019-09-10 and 2019-11-25 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-11-25.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Cattle (for tick collection only)	144 max
Goats (blood collection only)	54 max
Sheep (blood collection only)	54 max
Samples Blood collection (sheep, goats)	54 per species (Total 108 samples max)
Ticks (A. hebraeum)	432 max (total to be collected from Cattle)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-12-05.
3. Please remember to use your protocol number (REC205-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. Condition of approval: CAN NOT collect samples above what is approved by AEC.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

12 October 2021

Approval Certificate
Annual Renewal
(EXT2)

AEC Reference No.:	REC205-19
Title:	Assessment of an inactivated multivalent heartwater vaccine under field conditions
Researcher:	Ms Z Lukanji
Student's Supervisor:	Dr D Morar-Leather

Dear Ms Z Lukanji,

The **Annual Renewal** as supported by documents received between 2021-08-16 and 2021-10-01 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-10-01.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number Available**
Cattle (blood collection only)	144 max
Goats (blood collection only)	54 max
Sheep (blood collection only)	54 max
Samples Blood collection (sheep, goats)	54 per species (Total 108 samples max)
Ticks (A. hebraeum)	432 max (total to be collected from Cattle)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-10-12.
3. Please remember to use your protocol number (REC205-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

29 May 2023

Approval Certificate
Amendment 1

AEC Reference No.:	REC205-19 Line 4
Title:	Molecular characterization of South African Ehrlichia ruminantium field isolates and development of a qPCR assay for the evaluation of a multivalent inactivated vaccine
Researcher:	Ms Z Lukanji
Student's Supervisor:	Prof LCBGD Neves

Dear Ms Z Lukanji,

The **Amendment** as supported by documents received between 2023-03-28 and 2023-05-29 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-05-29.

Please note the following about your ethics approval:

1. The change in the Title is approved: (new title) Molecular characterization of South African Ehrlichia ruminantium field isolates and development of a qPCR assay for the evaluation of a multivalent inactivated vaccine
2. The change in species/animals/ samples is approved

Species	No Approved	Additional Required	Total Approved
Cattle - N/A	144	108	252
Samples	**No Approved**	**Additional Required**	**Total Approved**
A. hebraeum - Tick DNA - Bushbuckri - Stored	427	0	427
A. hebraeum - Ticks - South Afri - Live	292	1152	1444
Blood - Bushbuckri - Live	0	0	0

3. Please note that the approved date(s) from the original application certificate / annual renewal certificate will be applicable to this amendment.
4. Please remember to use your protocol number (REC205-19) on any documents or correspondence with the AEC regarding your research.
5. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
6. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
7. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
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Fax +27 12 529 8321
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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
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Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6 (1210)

Ms Zinathi Lukanji
University of Pretoria
Tel: 012 529 8268
E-mail: zinathi.lukanji@up.ac.za

Dear Ms Lukanji

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application dated 27 June 2019 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. This permit is valid only for the collection and analysis of blood collected from cases of Heartwater in animals, the collection and analysis of ticks and the culturing of *E. ruminantium*. A separate Section 20 permit must be obtained prior to the manufacturing or testing of any Heartwater vaccine. Please apply in writing to HerryG@daff.gov.za;
3. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;

4. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
5. No part of this research project may begin until the valid ethical approval has been obtained in writing from the relevant South African authority;
6. The following samples may be collected:
   6.1. Blood may be collected from ruminants showing clinical signs of Heartwater at the Hluvukani Animal Health Centre;
   6.2. Ticks may be collected from the following areas in the Foot and mouth disease (FMD) free zone: Ekuphindisweni, Ocilwane and Mvutshini diptanks in Kwa Zulu Natal Province, Makhuduthamaga Municipality in Limpopo Province;
   6.3. Ticks may be collected from the following areas in the FMD controlled area: Utah, Welverdiend, Hluvukani and Hlavekisa in Bushbuckridge, Mpumalanga Province;
7. It is the responsibility of the researcher to consult with the relevant state veterinarian to ensure no restrictions have been placed on the above mentioned areas prior to collection and to facilitate the issuing of movement permits as may be required;
8. All ticks and blood samples collected from the FMD controlled area must be transported to the Hans Hoheisen Wildlife Research Station (HHWRS) BSL 2+ facility;
9. With regard to the removal of samples from the HHWRS:
   9.1. Extracted DNA may be moved from HHWRS to the Department of Veterinary Tropical Diseases (DVTD). Please contact the Skukuza state veterinary office to arrange for the necessary veterinary movement permits;
   9.2. Only cultures of *E. ruminantium* that test negative on PCR for FMD virus at the DAFF approved Transboundary Animal Diseases Programme (TAD-P) facility, may be moved to the DVTD. Please contact the Skukuza state veterinary office to arrange for the necessary veterinary movement permits;
10. Ticks collected in the FMD free zone may be moved directly to DVDT;
11. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84), must be reported immediately to the state veterinarian of the area;
12. All ticks and samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and the National Road

Traffic Act, 1996 (Act No. 93 of 1996). Live ticks must be packaged in such a manner as to prevent escape during transport;

13. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the research project. Only a registered waste disposal company may be used for the removal of all potentially infectious waste from the research project;
14. Records must be kept for five years for auditing purposes;
15. A dispensation on point 13 above is attached;

Title of research/study: Assessment of an inactivated multivalent heartwater vaccine in South Africa
Researcher: Ms Zinathi Lukanji
Institution: BSL 2+ Laboratory, Hans Hoheisen Wildlife Research Station; Research and Training Laboratories, Department of Veterinary Tropical Diseases, Onderstepoort Faculty of Veterinary Science; Hluvukani Animal Health Centre, Bushbuckridge, Mpumalanga Province; Transboundary Animal Diseases Programme, Onderstepoort.
Permit Expiry date: 31 July 2020
Our ref Number: 12/11/1/1/6 (1210)
Your ref: None Provided.

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -07- 30

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@dalrrd.gov.za
Reference: 12/11/1/1/6A (1210) (JD)

Responsible person: Ms Zinathi Lukanji
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria
Email: zinathi.lukanji@up.ac.za

Dear Ms Lukanji

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) – EXTENSION OF THE EXPIRY DATE

Title of research project / study: "Assessment of an inactivated multivalent heartwater vaccine in South Africa"

An amendment is hereby granted on the attached Section 20 approval that was issued for the above mentioned study on 2019-07-30.

i) As requested, the validity of the section 20 approval is extended to 31 December 2021;
ii) All other conditions as specified in the Section 20 approval of 2019-07-30 remain in full effect. This includes the validity of laboratory approvals in terms of SANAS and DALRRD.

Kind regards,

Dr Mpho Maja
DIRECTOR: ANIMAL HEALTH
Date: 2020 -10- 13

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Ms Marna Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1/6A (1210) (JD)

Responsible person: Ms Zinathi Lukanji
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria
Email: zinathi.lukanji@up.ac.za

Dear Ms Lukanji,

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) – EXTENSION OF THE EXPIRY DATE

Title of research project / study: "Assessment of an inactivated multivalent heartwater vaccine in South Africa"

An amendment is hereby granted on the Section 20 approval that was issued for the above mentioned study on 2019-07-30.

i) As requested, the validity of the section 20 approval is extended to 31 December 2022;

ii) All other conditions as specified in the Section 20 approval of 2019-07-30 remain in full effect. This includes the validity of laboratory approvals in terms of SANAS and DALRRD.

Kind regards,

Mauce pp.

Dr Mpho Maja
DIRECTOR: ANIMAL HEALTH
Date: 2021 -10- 12