PREVALENCE AND MOLECULAR SUSCEPTIBILITY TESTING OF *MYCOPLASMA GENITALIUM* IN KWAZULU-NATAL POPULATION

By

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Submitted in partial fulfilment for the degree of

MASTER OF MEDICAL SCIENCE

In the
Department of Medical Microbiology at
School of Laboratory Medicine and Medical Sciences
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South Africa

2021

PREFACE

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Medical Microbiology, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Prof. K.P Mlisana and Dr. R Singh.

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DECLARATION

I, **Londeka Mvuna** declare that:

(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

(ii) This dissertation has not been submitted for any degree or examination at any other university.

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Signed:  Date: 02 February 2021

DEDICATION

I dedicate this thesis to my mother, Mrs. Mvuna (OkaMagaye), and my entire family am genuinely grateful for everything. Your prayers are what kept me this far.

FUNDING

Funding for this project was received from:

- DST/NRF Centre of Excellence in HIV Prevention Fellowship (CAPRISA).

ACKNOWLEDGMENTS

I want to express my sincere appreciation to the following people who have assisted in making this research project a success:

- My supervisor Prof. K.P Mlisana for her guidance and for allowing me to pursue my Master's degree.
- Sincere appreciation to my co-supervisor, Dr. R. Singh, for the effort, guidance, and patience you have given me. Thank you for working tirelessly in teaching me everything. I have indeed learned a lot from you.
- Thanks also to Meleshni Naicker and the whole Clinical Medicine Laboratory team from the University of KwaZulu-Natal for assisting me with part of the lab work.
- Thankful to Prof. A. Kharsany, Cherie Cawood, and David Khanyile for allowing me to use the samples collected from CAPRISA HIPPS cohort Study.
- Great gratitude to Dr. V. Ramsuran for financial assistance for sequencing, and there are no words that can describe my appreciation.
- Appreciation to Mrs. Fikile Nkwanyana for her assistance with statistical work.
- I am thankful to CAPRISA Centre of Excellence (CoE) for funding the project.
- Lastly, to God for giving me the strength to complete this project. Phillippians 4v13

TABLE OF CONTENT

LIST OF FIGURES

Chapter 2

Chapter 4

LIST OF TABLES

Chapter 3

Chapter 4

ACRONYMS AND ABBREVIATIONS

AMR	Anti-macrolide resistance
AZM	Azithromycin
BREC	Biomedical Research Ethics Committee
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CDC	Centre for Disease Control and Prevention
CO_2	Carbon dioxide
CP	Cycle number
C. trachomatis	*Chlamydia trachomatis*
C_T	Cycling threshold
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
et al.	And others
Etc.	*Et cetera*
FRET	Fluorescence resonance energy transfer
FSW	Female sex workers
GIT	Genital tract infections
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GyrA	DNA gyrase subunit A
GyrB	DNA gyrase subunit B
HIPPS	HIV Incidence Provincial Surveillance System
HIV	Human immuno-deficiency virus
HRM	High resolution melting
HSV	Herpes simplex virus
IL	Interleukin
kDa	Kilo daltons
KRISP	KwaZulu-Natal Research Innovation and Sequencing Platform
KZN	KwaZulu-Natal
MCP-1	Monocyte chemotactic protein 1
MDR	Multidrug resistance

MIC	Minimum inhibitory concentration
M. genitalium	*Mycoplasma genitalium*
$MgCl_2$	Magnesium chloride
MgPa	*Mycoplasma genitalium* adhesion gene
M. pneumoniae	*Mycoplasma pneumoniae*
MRM	Macrolide resistance mutation
MSA	Multiple sequence alignment
MSM	Men who have sex with men
MsrA	Methionine sulfoxide reductase
MSW	Men who have sex with women
MUS	Male urethritis syndrome
NAAT	Nucleic acid amplification test
NCGU	Non-chlamydial gonococcal urethritis
N. gonorrhoeae	*Neisseria gonorrhoeae*
NGU	Non-gonococcal urethritis
ParC	Topoisomerase subunit A
Par-E	Topoisomerase subunit B
PCR	Polymerase chain reaction
PHC	Primary health care service
PID	Pelvic inflammatory disease
qPCR	quantitative real-time PCR
QRDR	Quinolone resistance determining region
RAM	Resistant associated mutations
Rec-A	DNA recombination protein enzyme
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
STIs	Sexually transmitted infections
TBE	Tris borate EDTA
TEM	Transmission electron microscopy
Tet-(M)	Tetracycline resistance protein
TLR	Toll-like receptors
TMA	Transcription mediated amplification
TOP	Termination of pregnancy

tRNA	Transfer ribonucleic acid
T. *vaginalis*	*Trichomonas vaginalis*
UGA	Uracil guanine adenine
UK	United Kingdom
UKZN	University of KwaZulu-Natal
USA	United States of America
UV	Ultraviolet
VDS	Vaginal discharge syndrome
WHO	World Health Organization

Symbols

&	And
~	Approximately
β	Beta
bp	Base pair
ºC	Degree Celsius
g	Gram
>	Greater than
<	Less than
µL	Microlitre
mg	Milligram
mM	Millimolar
mL	Millilitre
- ve	Negative
%	Percentage
pmol	Picomoles
+ ve	Positive

ABSTRACT

Background: *Mycoplasma genitalium* is a recently classified sexually transmitted pathogen associated with causing urethritis and cervicitis, potentially causing reproductive complications. *Mycoplasma genitalium* has been shown to develop resistance to the currently recommended drug regimens, namely, azithromycin used as first-line therapy and moxifloxacin (second-line). The resistance prevalence of *M. genitalium* to the current treatment is diverse across the world. In South Africa, few studies have evaluated the prevalence of resistance to azithromycin and other fluoroquinolones. This study was conducted to evaluate the presence of macrolide and fluoroquinolone-resistant gene mutations in a KwaZulu-Natal population infected with *M. genitalium*.

Methods: Samples from the CAPRISA HIPPS cohort study were used for this analysis. Deoxyribonucleic acid was extracted from 100 stored *M. genitalium* positive self-collected vaginal swab samples. Real-time PCR was performed to confirm *M. genitalium* positivity. Genes associated with resistance to macrolides (23S rRNA, L4, L22) and fluoroquinolones (*gryA*) were sequenced and analysed.

Results: All 100 samples were confirmed genotypically to be *M. genitalium* positive and were sequenced. From the 100 samples tested for *M. genitalium*, 73 were successful for 23S rRNA, 99 for L4, 91 for L22, and 80 for *gryA*. Of the seventy-three 23S rRNA sequences, five samples carried mutations associated with macrolide resistance. A total of 12 mutations coding for macrolide resistance (5 for 23S, 4 for L4, and 3 for L22) were identified following sequencing. The prevalence of resistance mutations to macrolides was thus 7% (5 of 73) for 23 S rRNA, 4% (4of 99) for L4, and 3% (3 of 91) for L22 protein. In the five samples that harboured 23S rRNA gene mutation one had: A2071T, A2072C, A2072T, and two with A2072G mutation. L4 and L22 harboured silent mutations at positions: T327C, G429A, C438T, C438A, G81A, and C351T. In the 80 samples successful for *gryA*, six carried mutations with a prevalence of 8% (6/80 x100%).

Conclusion: Ongoing antimicrobial surveillance needs to be performed in our local populations as in our study we have seen the presence of mutations to macrolides and fluoroquinolones. This study was performed on samples collected during the early stages of introducing azithromycin as the first-line regimen for *M. genitalium* infection. More current studies need to be performed at a later time point to evaluate the antimicrobial resistance burden in *M. genitalium*.

CHAPTER 1

1.1 Introduction

Sexually transmitted infections (STIs) are infections that are mostly caused by having unprotected sexual intercourse (World Health Organization, 2018). STIs are one of the leading health challenges worldwide, as they are associated with a burden of different diseases that lead to severe effects if left untreated (World Health Organization, 2018). According to the WHO, there is an estimated 376 million new infections that are acquired every year (World Health Organization, 2018) The most common causes of leading STI's worldwide are *Chlamydia trachomatis, Neisseria gonorrhoeae*, *Trichomonas vaginalis,* and *Mycoplasma genitalium* (World Health Organization, 2018). These sexually transmitted pathogens affect both genders (females and males), different age groups, and some can be transmitted to children during pregnancy and birth (World Health Organization, 2016). They are also linked with causing various STIs such as vaginal or penile discharge, dysuria, itching, rash, etc (World Health Organization, 2016, World Health Organization, 2018). These infections are curable if properly treated using approved treatment guidelines after correct diagnosis. However, in low and middle-income countries, diagnosis is entirely dependent on the syndromic management approach, which treats infections based on the symptoms presented by an individual (Lewis and Maruma, 2009; Kularatne *et al.*, 2017).

South Africa has a high burden of STIs together with a high prevalence of human immunodeficiency virus (HIV), and the STIs are associated with an increase in HIV susceptibility and transmission, which may complicate the control of infections in the population (Ending, 2017; Johnson *et al.*, 2017; Wand *et al.*, 2020). KwaZulu-Natal (KZN) province is also known to have a high STI and HIV burden, and this was witnessed in a recent study conducted in KZN, which showed an increase of 20% STIs prevalence in 2019 from women compared to the previously 13% observed by Naidoo and colleagues (Naidoo *et al.*, 2014; Wand *et al.*, 2020). Therefore, STI management is one of the challenges that need attention in South Africa.

1.2 *Mycoplasma genitalium*

Mycoplasma genitalium is a sexually transmitted pathogen isolated in both women and men. It is associated with causing sexual infections such as urethritis, non-chlamydial gonococcal urethritis (NCGU) in men (Taylor-Robinson and Jensen, 2011). In contrast, in women, it mostly causes infections like cervicitis, pelvic inflammatory disease (PID), and endometritis (Taylor-Robinson and Furr, 1981; Jensen *et al.*, 2016; Tabrizi *et al.*, 2017). There is evidence to support the correlation of *M. genitalium* and HIV positive status, as high *M. genitalium* has been detected in HIV-infected individuals compared to uninfected individuals (Mahlangu *et al.*, 2019). *Mycoplasma genitalium* causes similar symptoms to other STIs, and therefore co-

infections are not uncommon (Ona *et al.*, 2016; Harrison *et al.*, 2019). The *M. genitalium* infections maybe masked by these other STIs and at times left untreated, which can lead to adverse reproduction effects such as infertility (Manhart, 2013).

The highly fastidious nature of *M. genitalium* has led to its difficulty in culturing that results in less usage of the culturing method (Kirkconnell *et al.*, 2019). Therefore, *M. genitalium* is diagnosed using different molecular methods, i.e nucleic acid amplification test (NAAT), which are known to be highly sensitive, and referred to as the current "gold standard" for its diagnosis (Waites *et al.*, 2012). The prevalence of *M. genitalium* varies globally and in different population groups female sex workers, women, heterosexual men, and homosexual men (men who have sex with men). Various studies in Western Europe, North America, and Australia, show an estimated prevalence of *M. genitalium* in men ranging between 1% to 3.3% (Mezzini *et al.*, 2013; Salado-Rasmussen and Jensen, 2014; Horner and Martin, 2017). In women it is between 1% to 6.4% (Walker *et al.*, 2013, Mahlangu *et al.*, 2019). Studies conducted in the sub-Saharan region in countries like Uganda and Kenya, the prevalence was 10% to 14%, 12.9% to 26.3%, respectively in female sex workers (FSWs), respectively (Pepin *et al.*, 2005; Cohen *et al.*, 2007; Gomih-Alakija *et al.*, 2014; Lokken *et al.*, 2017). In South Africa, *M. genitalium* prevalence ranges from 2.1% to 24%, which demands attention and focus on appropriate treatment and management (Mhlongo *et al.*, 2010; Hay *et al.*, 2015; Le Roux *et al.*, 2018; Mahlangu *et al.*, 2019; Smullin *et al.*, 2020) .

In most STI treatment guidelines throughout the developing world, routine testing is not recommended for *M. genitalium*. This is because most STI treatment guidelines use syndromic management approach, which treats infections based on symptoms without laboratory screening (Kularatne *et al.*, 2017). The South African treatment STI guidelines recommend 1 g of azithromycin (AZM), first-line regimen that is recommended to treat *M. genitalium* infections syndromically (Department of Health, 2015). The fluoroquinolone, moxifloxacin, is recommended as a second-line drug to treat complicated *M. genitalium* infections with a dosage of 400 mg for 7 days (Murray *et al.*, 2017). Current and previously conducted studies are showing a rapid decline in curing *M. genitalium* infections using the recommended regimens. This rapid decline observed in different parts of the world is complicating the control of infections caused by this pathogen due to resistance acquisition (Anderson *et al.*, 2017; Murray *et al.*, 2017; Muller *et al.*, 2019; Pitt *et al.*, 2020). *M. genitalium* is likely to affect the morbidity of life as it now causes infections resistant to the current drug regimen that are available for its treatment in the public sector (Read *et al.*, 2019a; Couldwell and Lewis, 2015; Sethi *et al.*, 2017). *Mycoplasma genitalium* is likely to develop into being a superbug due to the increase of resistance and may become unmanageable/untreatable in future (Jensen and Bradshaw, 2015; Getman *et al.*, 2016; Munoz and Goje, 2016; Wiesenfeld and Manhart, 2017).

The prevalence of *M. getalium* drug resistance to both moxifloxacin and azithromycin varies across the world. In previous studies where sequencing-based technology was used, the prevalence of resistance to azithromycin the first-line regimen was 100% in Greenland (Gesink *et al.*, 2012), 57% in Japan (Shimada *et al.*, 2011) , 43% in Sydney (Tagg *et al.*, 2013), 38% in Denmark (Salado-Rasmussen and Jensen, 2014), and 41% in the United Kingdom (Pond *et al.*, 2014). In the sub-Saharan region, a few studies have been conducted to detect *Mycoplasma genitalium* mutations. A study conducted on women in the rural areas of Limpopo, showed 9.8% macrolide resistance prevalence, while 25% of macrolide prevalence was detected in termination of pregnancy (TOP), Pretoria (Hay *et al.*, 2015; Le Roux *et al.*, 2018).

Few studies have evaluated the prevalence of mutations in South Africa. In KZN province, no study has been conducted so far to determine the mutation rate of macrolide and fluoroquinolone, while KZN has a high rate of STIs (Wand *et al.*, 2020). Hence, this prompted a need to evaluate the percentage prevalence of macrolide and fluoroquinolone in this province. In light of the above, we sort to screen for resistance determinants to the current regimens in a cohort of young South African women previously diagnosed with *M. genitalium* infection. The prevalence of *M. genitalium* detected in this population group of women between 15 to 25 years was 5.5 % (Kharsany *et al.*, 2020). In this KZN population, a retrospective approach was used to investigate the prevalence of macrolide and fluoroquinolone resistance in *M. genitalium,* as it is an established cause of various infections in both women and men.

1.3 Aim of the study:

- We aim to confirm the presence of *M. genitalium* using TaqMan real time PCR and the detection of mutations associated with the first and second line drugs using Sanger sequencing.

1.4 Objectives of the study:

- Confirm the presence of *M. genitalium* positive samples using TaqMan real time PCR.
- To evaluate *Mycoplasma genitalium* positive samples for macrolide (23S rRNA, L4, and L22) and fluoroquinolone (*gryA*) mutations using, Sanger sequencing.

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristics of *Mycoplasma genitalium*

Classifying or grouping different microorganisms according to their physical structure (morphology), taxonomy, and the genome is essential for better understanding how it acquires energy for survival purposes, developing a resistant mechanism, evolving, and differs from other species of the same genus (Gupta, 2016). The sub-sections in this section will describe taxonomy, genome, morphology, and pathogenesis of *Mycoplasma genitalium*.

2.1.1 Taxonomy of *Mycoplasma genitalium*

Mycoplasma genitalium are microorganisms that belong to the family of *Mycoplasmataceae*, order *Terenicutes* under the class of *Mollicutes* [mollis=soft, cutis=skin] (Taylor-Robinson and Jensen, 2011). It is divided into two genera *Ureaplasma* and *Mycoplasma* (Tully *et al*., 1981). They are known to lack a cell wall and have self-replicating properties (binary fission) (Fraser *et al.*, 1995).

It is highly fastidious as it require a complex medium supplemented with amino acid, yeast extract, and horse serum and grow very slowly (Taylor-Robinson and Furr, 1981; Tully *et al.*, 1981). The class of the *Mollicutes* is known to evolve from the genera of Gram-positive bacterial class such as *Lactobacillus* and *Clostridia* by the process called degenerative evolution (Woese *et al.*, 1980; Sasaki *et al.*, 1989; Taylor-Robinson and Jensen, 2011; Thompson *et al.*, 2011). Degenerative evolution has been implicated in the loss of the cell wall of *M. genitalium* and a reduction in its genome, hence referred to as the naked bacteria (Fraser *et al.*, 1995; Thompson *et al.*, 2011).

The first report of *Mycoplasma genitalium* was discovered in 1980 by Tully *et al,* in a study of 13 men who were infected with non-gonococcal urethritis (NGU), and on culture analysis, they observed two strains that were different from the other known *Mycoplasma* (Tully *et al.*, 1981). Therefore, the new *Mycoplasma* strains were G-37 and M-30, later named *Mycoplasma genitalium* (Tully *et al.*, 1981; Taylor-Robinson and Jensen, 2011). *Mycoplasma genitalium* is distinctly different from the other *Mycoplasma* because it ferments glucose, not urea or arginine (Thompson *et al.*, 2011).

2.1.2. Genome of *Mycoplasma genitalium*

In 1995 the first full genome of *M. genitalium* was first sequenced by Fraser and colleagues and this led to a better understanding of the genome (Fraser *et al.*, 1995; Fookes *et al.*, 2017). *Mycoplasma genitalium* has a circular double-stranded DNA, and with the smallest known genome size (580 kb) compared to any free-living organism (Fraser *et al.*, 1995; Su and Baseman, 1990; Himmelreich *et al.*, 1997). It contains low 32% of guanine and cytosine content distribution in its genome which was shown to assist with continuous DNA rearrangement (Razin, 2006; Ma *et al.*, 2007; Taylor-Robinson and Jensen, 2011; Roachford *et al.*, 2019).

The small genome size leads to a minimal size metabolic activity, limiting enzymes, and amino acid biosynthesis (Fraser *et al.*, 1995; Fookes *et al.*, 2017). An increase to 518 protein-coding genes for *M. genitalium* has recently been reported compared to the 480 *M. genitalium* protein-coding genes previously reported of these 518 protein-coding genes 52% were of unknown function (NCBI, 2016; Roachford *et al.*, 2019). The terminal structure of *M. genitalium* has a repetitive chromosomal sequence called *Mycoplasma genitalium* repetitive sequences (MPars), which are divided into nine segments (Fraser *et al.*, 1995; McGowin and Totten, 2017). The MgPars are associated to be the reservoir for genome variation, and as it recombines with *M. genitalium* major immunodominant-adhesion gene called MgPa, which is associated with generating a more variable sequences that have an impact in controlling *M. genitalium* infections (McGowin and Totten, 2017). Therefore, this is associated with creating variable mutants.

Mycoplasma genitalium is different from the other mycoplasmas because its shown to use UGA (uracil guanine, and adenine) that codes for tryptophan and not a stop codon (Taylor-Robinson and Jensen, 2011). In the sequencing study done by Richard Herrmann and Berta Reiner in 1997, it was shown that *Mycoplasma pneumoniae* is the only microorganism that is closely related to *M. genitalium* as they share many genes and proteins such as the cytadherence protein P1 in *M. pneumoniae* and MgPa in *M. genitalium* (Himmelreich *et al.*, 1997). Therefore, serological testing of *Mycoplasma genitalium* is hampered by cross-reactivity with *Mycoplasma pneumoniae* (Gnanadurai and Fifer, 2020).

2.1.3. *Mycoplasma genitalium* morphology

Mycoplasma genitalium has a flask or pear-like shape of which consists of a protruded terminal structure, which has advantages. Some of the advantages include attachment on surfaces and motility (Taylor-Robinson and Furr, 1981; Tully *et al.*, 1981; Taylor-Robinson and Jensen, 2011). Transmission electron microscopy (TEM) has shown that the *M. genitalium* structure contains a terminal tip organelle which consists of repetitive (triple) layers of proteins that are responsible for several functions such as gliding motility into different surfaces and adherence (Bredt, 1983; Girón *et al.*, 1996; Roachford *et al.*, 2019). These layers were observed to be 7.5 to 10 nm wide and comprising of a middle layer that is less electron-dense than the other layers (Taylor-Robinson, 1995; Taylor-Robinson and Jensen, 2011). The internal structure of *M. genitalium* was observed to be rod-like shaped and responsible for the circular motility (Taylor-Robinson *et al.*, 2000). *Mycoplasma genitalium* is coccoid shaped under a dark field microscope and has sizes ranging from 0.6 to 0.7 μm in length, widths 0.3 to 0.4 μm, and 0.06 to 0.07 μm a tip which is smallest compared to the other mycoplasmas (Tully *et al.*, 1983; Taylor-Robinson and Jensen, 2011).

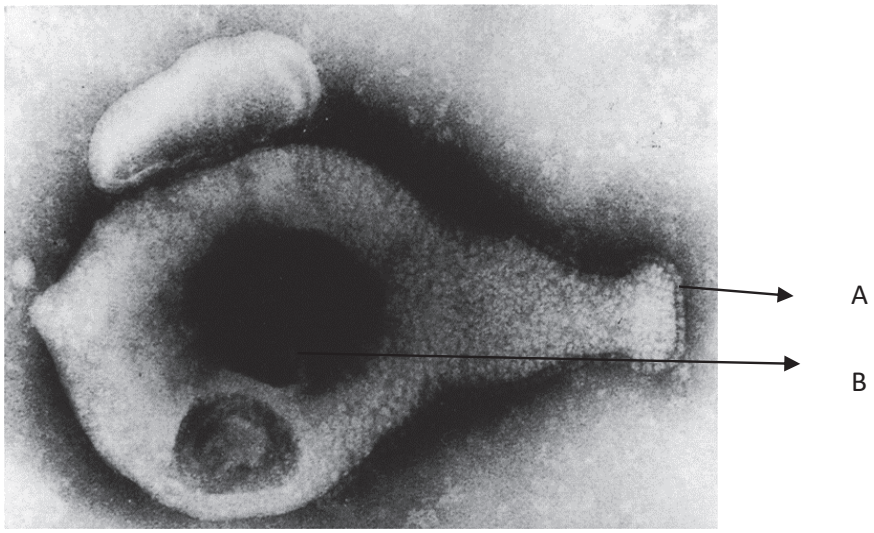


Figure 2.1.3.1 Representation of *M. genitalium* image under transmission electron microscopy (TEM) (Tully *et al.*, 1981)

Figure 2.1.3.1 Shows the pear/ bottle neck structure of *M. genitalium* when negatively stained with ammonium molybdate. A- Shows the protruded structure of *M. genitalium* which it uses for adhesion. B- Shows the electron dense core structure which is important for motility in *M. genitalium.*

2.2 Pathogenesis of *Mycoplasma genitalium*

Understanding the different pathogenic processes that microorganisms use to cause different sexually transmitted infections is necessary for the better understanding of its pathogenicity and the required treatment for eradication of these infections that they cause.

2.2.1 Pathogenicity and Virulence

Direct attachment to the surface of the host mucosal membranes is the first step for the pathogenicity of *M. genitalium* (Burgos *et al.*, 2006; Taylor-Robinson and Jensen, 2011). It adheres well to surfaces like epithelial cells, fallopian tubes, spermatozoa, erythrocytes, and Vero cells (Collier, 1990; Svenstrup *et al.*, 2003; Burgos *et al.*, 2006; Taylor-Robinson and Jensen, 2011). The adherence is facilitated by the mobility of its terminal tip organelle structure (Taylor-Robinson and Jensen, 2011) The major *M. genitalium* immunodominant protein MgPa is necessary for the attachments and interaction with different MgPars (repetition sequences) found on the external surface of *M. genitalium* is associated with increasing virulence (Himmelreich *et al.*, 1997; Szczepanek *et al.*, 2012). This interaction is useful for the sustainable structure development of *M. genitalium*, development of cell proliferation, and cellular maintenance (Das *et al.*, 2014; Ma *et al.*, 2016; McGowin and Totten, 2017). During infection, the recombinant interaction elicits inflammation on the different epithelial cells, which results in the different disease presentations. These diseases include urethritis, non-chlamydial gonococcal urethritis (NCGU) in men, while in women it mostly causes infections like cervicitis, pelvic inflammatory disease (PID), and endometritis (Taylor-Robinson and Furr, 1981; Jensen *et al.*, 2016; Tabrizi *et al.*, 2017).

2.2.2 Virulence of *Mycoplasma genitalium*

There are different virulent factors, described below, which are used by *M. genitalium* to assist with its survival and adherence which increases its pathogenicity.

2.2.2.1 Escaping the Immune system response

Like all other pathogenic microorganisms, *Mycoplasma genitalium* has it on survival mechanisms of escaping recognition by the immune system response of the host, such that it survives well within the host. This includes antigenic variation, enzymes, and localization of *M. genitalium* (Sethi *et al*., 2012).

2.2.2.2 Antigenic variation

The external structure of *Mycoplasma genitalium* contains the MgPa operon, which is composed of two major adhesion protein genes, MgpB (P140) and MgpC (P110). The external structure is composed of other repetitive segments in its genome chromosomal repetitive elements called MgPars, which are required by adhesion operon to encode with surface proteins (Das *et al.*, 2014). The interaction of MgPa gene and MgPar sequences is hypothesized to be associated with recombination, and this is linked with causing antigenic variation of this microorganism (Das *et al.*, 2014; McGowin and Totten, 2017).

In previous studies conducted on animals and humans, it was observed that individuals infected with *M. genitalium* produced different types of strains that evolved whenever recombination occurred (Iverson-Cabral *et al.*, 2007; Ma *et al.*, 2016; McGowin and Totten, 2017). This was observed in an *in-vivo* study where chimpanzees were inoculated with the G-37 wild type strain of *M. genitalium* and they produced different types of *M. genitalium* strains (Taylor-Robinson *et al.*, 1985; Ma *et al.*, 2016). The repetitive chromosomal elements (MgPars) in the structural complex is associated with giving *M. genitalium* an ability to survive different environmental factors (Criss *et al.*, 2005; Das *et al.*, 2014; Ma *et al.*, 2014). This process is associated with an increase to persistence infections in both animal models and humans.

2.2.2.3 Enzymes that assist *Mycoplasma genitalium* with its pathogenicity

Different enzymes are found on the external surface of *M. genitalium,* which facilitates its survival. Methionine sulfoxide reductase (MsrA) is an oxidation inhibitor that enables the restoration/repair of proteins that are being lost by the bacteria preventing bacterial damage (Dhandayuthapani *et al.*, 2001; Sethi *et al.*, 2012; Roachford *et al.*, 2019). Deoxyribonucleotide (DNA) recombination protein enzyme (RecA) is associated with minimal restoration of DNA damage during genomic recombination with the *M. genitalium* genes MgpB (MG191) and MgpC (MG192) locus (Iverson-Cabral *et al.*, 2007; Burgos *et al.*, 2012; Roachford *et al.*, 2019). Genomic recombination of mgpB (MG191 gene) and mgpC (MG192 gene) adhesins with noncoding MgPar regions exemplifies how *M. genitalium* has adapted to a persistent lifestyle in humans since this antigenic variability is likely in response to host immune pressure.

2.2.2.4 Intracellular localization of *Mycoplasma genitalium*

The survival of this pathogen inside the mucosal membrane creates the persistence of infections in both human and animal models (Tully *et al.*, 1986). McGowin and colleagues showed in an *in vitro* study carried out in Vero cells that the intracellular survival of this pathogen, is associated with escaping the direct opsonization process through phagocytosis, thus allowing its intracellular survival (McGowin *et al.*, 2009). This is associated with the persistence of infections and is postulated to be the major cause of upper urogenital tract outcomes, which include infertility (men and women), pelvic inflammatory disease (PID), and adverse complications during pregnancy (Seña *et al.*, 2018).

2.2.3 Acute immune response towards infection and *M. genitalium* association with human immunodeficiency virus (HIV)

The development of acute infection due to the strong attachment of *M. genitalium* to urogenital epithelial cells triggers the immune system response (McGowin *et al.*, 2013). McGowin and colleagues (2013), showed that *Mycoplasma genitalium* infection damages tissues leading to cytokine production. The most cytokines that get produced are interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein 1 (MCP-1), and causes activation of toll-like receptors (TLRs) especially toll like receptor 2/6 (TLR2/6) to respond to the infection activating the innate immune response (McGowin *et al.*, 2009; McGowin *et al.*, 2012; McGowin *et al.*, 2013).

These cytokines are mostly associated with the recruitment of human immunodeficiency virus (HIV) susceptible cells (Mavedzenge *et al.*, 2012; McGowin *et al.*, 2013; Madsen *et al.*, 2017). This is associated with an increase of HIV replication with higher chances of transmission (Sasaki *et al.*, 1993; Soni *et al.*, 2010; Mavedzenge *et al.*, 2012). Therefore, in *M. genitalium* positive individuals, it has been observed that it infects the epithelial layer decreasing the integrity, activating HIV targets cells (macrophages, dendritic cells), and results in shedding of the mucosal surfaces (Manhart *et al.*, 2008; Mavedzenge *et al.*, 2012; Vandepitte *et al.*, 2013; Napierala Mavedzenge *et al.*, 2015; Madsen *et al.*, 2017). Das *et al* (2014), in an *in vitro* study, demonstrated that *M. genitalium* infection facilitates the crossing of HIV in the mucosa.

More, *M. genitalium* infections have been observed in HIV-positive individuals, and with chances of the infection persisting longer (Zhao *et al.*, 2019). In a longitudinal study conducted in Zimbabwe, a low

bacterial load of *M. genitalium* was associated with a 2.7 fold increase of HIV-1 RNA load compared to the individuals who were *M. genitalium* uninfected (Napierala Mavedzenge *et al.*, 2015). *Mycoplasma genitalium* infection was associated with being an independent factor for the acquisition of HIV upon the participants (Napierala Mavedzenge *et al.*, 2015). A similar study conducted in Uganda showed that individuals diagnosed to be *M. genitalium* positive had a 2 to 3 fold increase of HIV seroconversion (Vandepitte *et al.*, 2013). The association of *M. genitalium* with the increase to HIV acquisition has been increasing in different studies (Lewis *et al.*, 2012; Mahlangu *et al.*, 2019).

2.3 People at risk, risk factors, and transmission

Determining the risk of *M. genitalium* acquisition will assist in prevention and decreasing the infection rate. Knowing these factors will also show the need to test for *M. genitalium* as it is likely to be misdiagnosed with other common STIs, as they have similar symptoms (Ajani *et al.*, 2017).

2.3.1 People at risk and risk factors

Female sex workers (FSWs), pregnant women, heterosexual women, and homosexual men (MSM) are shown to be at a higher risk of being infected with *M. genitalium* compared to heterosexual men (Xiang *et al.*, 2012; Slifirski *et al.*, 2017). Women were observed to be more susceptible to STIs due to the susceptibility of females' genital tract (Kaushic *et al.*, 2011; Yi *et al.*, 2013; Slifirski *et al.*, 2017). The authors suggested that this could be ascribed to the fact that women have larger surface area in the cervical vaginal mucosa compared to the urethral mucosa in the male counter parts (Kaushic *et al.*, 2011; Yi *et al.*, 2013; Slifirski *et al.*, 2017). The usage of protection, the number of sexual partners, reproductive age and the nature of sex are associated with increased risk factors for *M. genitalium* acquisition in the different population groups (Slifirski *et al.*, 2017). Manhart and colleagues showed an increase of 10% acquisition of *M. genitalium* for each new partner (Manhart *et al.*, 2007).

Mycoplasma genitalium is transmitted through having unprotected sexual intercourse in both heterosexual and homosexual partners (Anagrius *et al.*, 2005). There is supporting evidence that those individuals with multiple sexual partners are at a higher risk of contracting *M. genitalium* infection (Manhart, 2013). Oral sex is not a suitable mode of transmitting *M. genitalium* infection, as only one pharyngeal sample tested positive for *M. genitalium* infection in a study of MSM and FSW (Bradshaw *et al.*, 2009; Deguchi *et al.*, 2009).

2.4 Diagnosis of *Mycoplasma genitalium*

Accurate diagnosis of pathogenic microorganism is essential for better understanding of its pathogenicity, and conditions which favors its growth for survival. Its antimicrobial susceptibility such that adequate treatment is administered.

2.4.1 Culturing of *Mycoplasma genitalium*

Mycoplasma genitalium cannot be detected using the Gram stain as it lack the cell wall (Le Roux and Hoosen, 2010). Culturing is considered the gold standard for the detection of *Mycoplasma genitalium* (Baseman *et al*., 2004). Cultures of *M. genitalium* were observed to be egg fried shaped under anaerobic conditions, in an atmosphere of nitrogen with 5% of CO_2 (Tully *et al.*, 1983; Taylor-Robinson and Jensen, 2011). The challenge is to grow these microorganisms in the lab as they have a slow growth rate and are highly fastidious (Sethi *et al.*, 2012). It growth *in vitro* requires a complex medium that is supplemented with sterols and amino acids, fatty acids etc, and it is usually grown in sucrose phosphate media (SP4) (Blaylock *et al.*, 2004). According to the Centre for Disease Control and Prevention (CDC) 2015, its growth can take several months, and very few laboratories in the world have been able to isolate these microorganisms successfully (Control and Prevention, 2015). Growing, *M. genitalium* using cell culture method (Vero cells) has been observed to be successful, but it was still time consuming (Mondeja *et al.*, 2016). Recent studies have shown more detection of mutations resistance genes in *M. genitalium* to be more advantageous compared to phenotypic analysis (Hay *et al.*, 2015; Nijhuis *et al.*, 2015; Le Roux *et al.*, 2018; Mahlangu *et al.*, 2019).

2.4.2 Molecular assays

Mycoplasma genitalium was first detected through molecular methods by Jensen *et al* in 1991 and this was thought to be the breaking point to research for this micro-organism (Jensen *et al.*, 1991). This method has enabled the study of prevalence, mutations, and diagnosis in the different populations (Sethi *et al.*, 2017). Therefore, the nucleic acid amplification test (NAAT) usage has increased the ability to study this microorganism, as the molecular methods are found to be fast and more sensitive (Taylor-Robinson and Jensen, 2011; Waites *et al.*, 2012; Tabrizi *et al.*, 2017). The quantitative real-time PCR developed by Yoshida *et al* in 2002, has enabled the quantification of *M. genitalium* (Yoshida *et al.*, 2002). These methods include testing the DNA of *M. genitalium*.

There are different types of NAAT methods that have been used to study *M. genitalium.* These methods include: high resolution melting (HRM) analysis, sequencing, fluorescence resonance energy transfer probes (FRET) with melting curve analysis, labeled hydrolysis probes (Twin *et al.*, 2012; Touati *et al.*, 2014; Gossé *et al.*, 2016; Kristiansen *et al.*, 2016; Braam *et al.*, 2018). All of the above assays are commonly used for detection of mutations in *M. genitalium*, while hydrolysis probes (TaqMan) assay is used for screening of *M. genitalium*. It works by targeting a specific DNA product using fluorogenic dye probes, which allows amplification of the targeted DNA (oligonucleotides) as the PCR reaction proceeds (Liu *et al.*, 2006). Sanger sequencing allows the detection of variation in a single nucleotide change (Kristiansen *et al.*, 2016).

In recent studies conducted across the world, lack of commercially available assays for rapid detection of *M. genitalium* in clinical settings is highlighted. Most of these countries used the syndromic management approach, where clinical symptoms are treated without laboratory testing for a specific STI. Certain countries have different guidelines that are used to test and treat (Workowski *et al.*, 2015; Jensen *et al.*, 2016; Soni *et al.*, 2018; Pitt *et al.*, 2020). In these countries such as Australia and Europe the testing of *M. genitalium* in symptomatic individuals using both the DNA and RNA based assays has been approved (Workowski *et al.*, 2015; Jensen *et al.*, 2016; Soni *et al.*, 2018; Pitt *et al.*, 2020). These commercial assays are observed to have high specificity and sensitivity of 98% to 100% with internal control and have been tested for cross-reactivity, examples of these assays includes: MG AmpliSens PCR, Resistance Plus MG assay (SpeeDx), SpeeDX *MG* (resistance plus) assay (Gaydos, 2017; Gaydos *et al.*, 2019). The AmpliSens works by screening *M. genitalium*, while the other assays detect mutations and the resistance genes these include the following: Resistance Plus MG assay (SpeeDx), SpeeDX *MG* (resistance plus) assay (Gaydos 2017).

These techniques are essential for the correct treatment of the infected individuals, which will help prevent the spread of *M. genitalium* infection, resistance strains, and its management. Therefore, this leads to this pathogen lying undetected for longer periods, which is likely to increase chances of transmission if it not adequately treated (McGowin *et al.*, 2012).

The increasing resistance of *M. genitalium* across the globe, which is associated more with macrolides compared to fluoroquinolones, has led to the need of developing assays that will simultaneously detect *Mycoplasma genitalium* and mutations (Le Roy *et al.*, 2017). These assays are fast, sensitive, and can be used in clinical settings. In Australia, an approved assay that simultaneously detects *Mycoplasma genitalium* and resistance was developed (SpeeDx *MG* resistance Plus), (Tabrizi *et al.*, 2017; Read *et al.*, 2019a). This assay can detect the most common resistance genes found in the 23S rRNA region in position 2058 and 2059 (A2058G, A2059G, A2058T, A2058C, and A2059C) (*E. coli numbering*), (Tabrizi *et al.*,

2016; Braam *et al.*, 2018). Commercial assays that are used for fluoroquinolone detection have been developed, but they are still limited. However, newer approaches are required for the detection/diagnosis of *M. genitalium* especially in the developing countries.

2.5 The treatment for *M. genitalium* infection

The lack of a rigid cell wall of the class of *Mollicutes* species limits the ability to use many antibiotics. Therefore, treatment of these infections pre-excludes the antibiotics that inhibit the cell wall synthesis, namely: β-lactams (penicillin, cephalosporin, or polypeptide), and glycopeptides antibiotics (vancomycin), (Redelinghuys *et al.*, 2014; Fernández *et al.*, 2016). Tetracyclines, macrolides, and fluoroquinolones are the only currently antibiotics used as they inhibit protein synthesis and DNA replication (Ahmadi *et al.*, 2016; Fernández *et al.*, 2016).

2.5.1 Tetracycline

The tetracyclines are a group of antibiotics that hinder or inhibit ribosomal protein synthesis for its effectiveness and are therefore bacteriostatic (Chopra *et al.*, 1992; Chopra and Roberts, 2001; Nguyen *et al.*, 2014). This is because its bind reversibly to the 30S subunit of the bacterial chromosome, preventing the interaction of the aminoacyl- tRNA with the acceptor site, thus inhibiting protein synthesis (Sohmen *et al.*, 2009; Nguyen *et al.*, 2014). The predominant drug that was used to treat *M. genilium* is doxycycline (Chopra and Roberts, 2001). However, studies have shown that it does not completely eradicate *M. genitalium* infections (Falk *et al.*, 2003; Anagrius *et al.*, 2013).

2.5.2 Macrolides mode of action and used as treatment

Macrolides mode of action is through binding reversibly to the 50S ribosomal subunit, which separates the index of peptidyl tRNA from the ribosome inhibiting peptide chain for protein synthesis (Leclercq, 2002). The recommended STI management treatment guidelines to treat *M. genitalium* infections are different in the world. Macrolide (azithromycin) is usually the primary drug of choice, recommended by some STI management health guidelines like South African STI management, and Centre for Disease Control and Prevention (Control and Prevention, 2015; Department of Health, 2015). Azithromycin (AZM) is known to have high mucosal cell penetration with only a single dose (1 g), therefore this maybe the cause of its

being superior in treating *M. genitalium* infections compared to doxycycline (Taylor-Robinson and Jensen, 2011; Sethi *et al.*, 2017).

2.5.3 Fluoroquinolones are second line group of antibiotics that are used to treat *M. genitalium* infections

Moxifloxacin is used as the second-line antibiotic used to treat individuals with persistent *M. genitalium* infections. The efficacy of moxifloxacin was observed to be higher compared to azithromycin when clearing of infection was used as the outcome. Moxifloxacin is the fourth group of fluoroquinolones, which is effective through inhibiting DNA replication by binding to DNA gyrase and DNA topoisomerase IV inhibiting DNA replication (Alekshun and Levy, 2007; Fàbrega *et al.*, 2009). A dosage of 100 mg twice daily for seven days is the recommended treatment to treat infections that have failed the first line recommended dosage of azithromycin (Couldwell and Lewis, 2015). This was due to the better treatment of eradicating *M. genitalium* infections (Couldwell and Lewis, 2015; Bradshaw *et al.*, 2017).

In Japan, sitafloxacin is the second-line drug that is used in treating *M. genitalium* infection as moxifloxacin is not approved for usage. In a surveillance study conducted in Japan, sitafloxacin was shown to be superior in eradicating *M. genitalium* infections compared to moxifloxacin (Deguchi *et al.*, 2018). In an *in vitro* study conducted in 2015 by Jensen and Bradshaw, sitafloxacin was observed to have lower inhibitory concentrations in comparison to moxifloxacin , and therefore sitafloxacin was recommended for usage with a dosage of 100 mg twice for seven days (Jensen and Bradshaw, 2015; Read *et al.*, 2019a).

2.6. Resistance mechanisms and treatment failure of antibiotics in *Mycoplasma genitalium*

The ability of pathogenic microorganisms to develop resistance to the current antibiotics hinders the effectiveness of treatment. This allows it to survive longer because of the limited uptake of a drug, changing of the targeted site by the antibiotic, and inactivation of the antibiotic (Reygaert, 2018).

2.6.1 Acquisition of resistance in the current drugs that are used

The alteration of the targeted sites of the different antibiotics that are used to treat *Mycoplasma genitalium* infections is the first step that allows *M. genitalium* to develop resistance to the drugs. Therefore, each group of antibiotics used to treat *M. genitalium* infections have been seen to be less effective, and the causes of the decrease antibiotic effectiveness has brought the need to carry out resistance studies. Alekshun and

Levy, in 2007, associated resistance to azithromycin with the blockage of antibiotic efflux into the cell, causing inactivation of antibiotics in the cell (Alekshun and Levy, 2007). Waites *et al*., in 2014 showed structural changes in the large ribosomal RNA subunit (the 50S), and Lysnyansky *et al*., in 2015 showed changes in the central loop of the V domain of the 23S rRNA, corroborating results that show a change in the large ribosome which leads to infectiveness of antibiotics (Waites *et al.*, 2014; Lysnyansky *et al.*, 2015). Hence, changes in the V- region of 23S rRNA is associated with conferring resistance to macrolides (Jensen *et al.*, 2008; Gesink *et al.*, 2012).

The tetracyclines have a bacteriostatic action because it bind reversibly to the 30S subunit of the bacterial chromosome, preventing the aminoacyl-tRNA's interaction with the acceptor site inhibiting protein synthesis (Nguyen *et al.*, 2014). Resistance towards the tetracyclines is associated with the acquisition of the tetracycline resistance gene *tet*(M) gene (Waites *et al.*, 2014; Chernova *et al.*, 2016), which is located on a Tn*916* transposon (Taraskina *et al.*, 2002). This transposon encodes the tetracycline resistance protein Tet(M), which protects the ribosome from tetracycline's action. This mutation creates a confirmation change in the small ribosomal subunit (30S). Cross-resistance of the *mycoplasma* spp. to the other antibiotics is observed after the acquisition of the *tet*(M) gene (Waites *et al.*, 2014; Fernández *et al.*, 2016).

Fluoroquinolones bind to DNA gyrase and DNA topoisomerase IV, thereby inhibiting DNA replication (Alekshun and Levy, 2007; Fàbrega *et al.*, 2009). The acquisition of resistance is associated with mutations in the quinolone resistance determining region (QRDR), which results in the alteration of the targeted sites (Chernova *et al.*, 2016). The targeted genes sites are DNA gyrase subunit A (*gyr*A), DNA gyrase subunit B (*gyr*B), topoisomerase subunit A (*par*C) and topoisomerase subunit B (*par*E) (Chernova *et al.*, 2016; Fernández *et al.*, 2016; Sethi *et al.*, 2017). Mutations are also associated with causing minimum drug accumulation in the cell because of influx suppression (Redgrave *et al.*, 2014).

In 2006 Bradshaw *et al*, linked the first-line treatment failure to azithromycin resistance phenotypically (Bradshaw *et al.*, 2006). In the study they showed the presence of *M. genitalium* in the test of cure and was further evaluated phenotypically by performing minimum inhibitory concentrations MIC of azithromycin strains isolated from the test of cure visit (Bradshaw *et al.*, 2006). Other studies conducted have shown similar results (Jensen *et al.*, 2008; Falk *et al.*, 2015). This is because the infected individuals remained persistently positive, implying poor eradication of infection.

Ribosomal gene proteins L4 and L22 are also implicated with macrolide resistance, although associated with low-grade resistance (Muller *et al.*, 2019). These ribosomal gene proteins are found on the extended loop of L4 and L22, which are adjacent to the critical binding site for macrolides (Diner and Hayes, 2009; Ito *et al.*, 2011). In studies where resistance was investigated, its showed that mutations in the ribosomal gene proteins usually accompanying the 23S rRNA mutation either at 2058 or 2059 position (*E. coli*) numbering (Jensen et al., 2008; Ito et al., 2011; Shimada et al., 2011; Chrisment et al., 2012). This was supported by a study conducted by Ito *et al* 2011, where mutation in L4 ribosomal gene protein accompanied 23S rRNA mutation at 2058 position (*E. coli* numbering) (Ito *et al.*, 2011). Mutations detected at positions 2058 and 2059 (*Escherichia coli* numbering) are mostly considered as the "hot spot'' for macrolides resistance (Jensen *et al.*, 2016; Deguchi *et al.*, 2017; Murray *et al.*, 2017). A high transition of the single base change of adenine to guanine is being observed to be dominant compared to the other base changes, which sometimes may lead to transversion (Jensen *et al.*, 2008, Gesink *et al.*, 2012).

The persistence of *M. genitalium* has been associated with the ability to invade the cell and persist intracellularly, which is associated with causing the infections to persist longer (Herrmann and Reiner, 1998). Therefore, the 2016 European guidelines recommended the use of azithromycin but with an extended dosage (Jensen *et al.*, 2016). The dosage became 500 mg on the first day orally followed by 250 mg for 4 to 5 days to clear the infection, and azithromycin was regarded as the primary drug of choice (Couldwell *et al.*, 2013; Ma *et al.*, 2016). However, continuous failure of azithromycin chemotherapy treatment is being observed in different individuals, and areas especially in Australia, Japan, and United Kingdom (Gesink *et al.*, 2012; Twin *et al.*, 2012; Kikuchi *et al.*, 2014; Pond *et al.*, 2014).

Fluoroquinolone (moxifloxacin) group of antibiotics are the only drugs that are observed to be able to eradicate *M. genitalium* infection after azithromycin treatment failure (Couldwell *et al.*, 2013; Manhart *et al.*, 2013b). Moxifloxacin is used as the second-line drug of choice to treat *M. genitalium* infection with the dosage of 400 mg daily for 7 to 10 days, in most countries such as the USA, Australia, Europe, Greenland except for Japan as 100 mg twice a day for seven days of sitafloxacin is the recommended regimen (Kikuchi *et al.*, 2014). However, a study in Australia conducted by Couldwel *et al* in 2013, reported the failure of fluoroquinolone (moxifloxacin) drug in a study of *M. genitalium* infected men (Couldwell *et al.*, 2013; Manhart *et al.*, 2013a; Murray *et al.*, 2017). Therefore, this increased level of resistance to both first-line and second-line drugs has brought a concern for the treatment and management of *M. genitalium* infection worldwide.

2.6.3 Alternative drugs of choice towards curing resistant *M. genitalium* infections

Mutations in 23S rRNA, L4, L22 is associated with macrolide (azithromycin) resistance and *gryA* gene is associated with resistance fluroquinolone (moxifloxacin) (Couldwell *et al.*, 2013). Braam *et al*. (2017), reported multidrug resistance of *M. genitalium* in countries such as Australia and Japan (Braam *et al.*, 2017). According to the World Health Organization (WHO), for an antibiotic to be considered effective, it needs to eradicate > 95% of the *M. genitalium* infection (Walker and Failey. 2013).

In the many conducted studies an escalating increase of resistance mutations associated with macrolide (azithromycin) and fluoroquinolone (moxifloxacin) resistance has been identified leading to treatment failure. This has led to the need to try new treatment options for *M. genitalium* infections (Couldwell and Lewis, 2015; Sethi *et al.*, 2017). Therefore, drugs like pristinamycin a streptogramin, which acts by binding to 50S of the ribosome and blocking protein synthesis, seem to be promising in the few studies that have been conducted in eliminating multi-drug resistant strains of *M. genitalium* (Couldwell and Lewis, 2015; Sethi *et al.*, 2017). Few studies have been conducted to evaluate its effectiveness, and pristinamycin has been observed to have an efficacy of >85% in individuals with multidrug-resistant infections (Read *et al.*, 2018).

Salado-Rasmussen and Jensen suggested the use of combined therapy rather than monotherapy to reduce persisting infections of *M. genitalium* (Salado-Rasmussen and Jensen, 2014). Sethi *et al*. 2017 suggested the use of the following drugs to assist in the eradication of persisting *M. genitalium* infection: josamycin, lefamulin, sitafloxacin, solithromycin, and zoliflodacin (Sethi *et al.*, 2017). Most of these drugs are known to inhibit DNA and protein synthesis (Sethi *et al.*, 2017). However, not many studies have been published that evaluate the efficacy and side effects of these drugs. Therefore, this limits the availability of drugs that can be used to eliminate this multi-drug resistant pathogen.

2.7 Prevalence of *Mycoplasma genitalium* in South Africa

In South Africa, the prevalence of *Mycoplasma genitalium* ranges between 2.1% to 24%, and this includes individuals who are symptomatic and asymptomatic, HIV positive or negative (Mhlongo *et al.*, 2010; Hay *et al.*, 2015; Le Roux *et al.*, 2018; Mahlangu *et al.*, 2019; Smullin *et al.*, 2020). There is limited mutational prevalence percentage in our population. In a small study populations where resistance mutation was tested a prevalence of 9.8% macrolide resistance was detected in a cross-sectional study of symptomatic women in Mopani District, Limpopo, South Africa (Hay *et al.*, 2015). A study conducted in the termination of pregnancy (TOP) Pretoria, South Africa detected a prevalence of 25% macrolide resistance in 2 016 samples with a change of A2059G in 23S rRNA (Le Roux *et al.*, 2018). In this study 12.5% of fluoroquinolone resistance was detected in *parC* gene (Le Roux *et al.*, 2018)

In a case report study of a patient with male urethritis syndrome (MUS) treatment failure to azithromycin and ceftriaxone had been reported (Maduna *et al.*, 2019). Genetic analysis showed mutations in 23S rRNA and *parC* gene (Maduna *et al.*, 2019). Therefore, resistance is more likely to increase in the future because 1 g of AZM was made as to the primary drug of choice in the South African treatment guidelines to treat STI infections according to the guidelines of the syndromic method (Department of Health, 2015). The incorporation of azithromycin into the treatment guidelines was because of resistance that emerged in drugs that were previously used to treat STI infections. Azithromycin was shown to have higher oral absorption, good tissue penetration, a broader spectrum of activity (Bignell and Garley, 2010). Therefore, azithromycin was shown to be more likely to eradicate STI infections (Falk *et al.*, 2003).

However, very few studies have been carried out in South Africa regarding the effectiveness of the treatment, resistance rate, and co-infection of *M. genitalium* with other STIs, impact on HIV infection, and overall prevalence of *M. genitalium.* In the few *M. genitalium* studies conducted in South Africa with small population groups, mutations to both macrolides and fluoroquinolones have been observed. Whilst low prevalence of mutations is observed, the variation of different mutations brings a concern as they are more likely to perpetuate the spread of emerging resistant strains associated with azithromycin (Laumen *et al.*, 2020). The observation of the mutations associated with the macrolide and fluoroquinolone-resistance prompted the need for this study in KwaZulu-Natal.

CHAPTER 3

Materials and Methods

3.1 Ethical Clearance: The protocol for the study was approved by the University of KwaZulu-Natal (UKZN) Biomedical Research Ethics Committee (BREC) reference no. BE685/18 (Appendix D).

3.2 Study setting: This is a sub-study from Centre for the AIDS Programme of Research in South Africa (CAPRISA) HIV Incidence Provincial Surveillance System (HIPPS) cross-sectional community-based study. The parental study was designed to evaluated and measure the prevalence of human immunodeficiency virus (HIV) and incidence association scale up with HIV prevention and treatment efforts. In this study they screened for sexually transmitted infections (STIs) such as *Chlamydia trachomatis (C. trachomatis), Neisseria gonorrheae (N. gonorrhoeae), Trichomonas vaginalis (T. vaginalis),* syphilis*,* herpes simplex virus (HSV2) and *Mycoplasma genitalium (M. genitalium)*. Briefly, the study was carried out randomly chosen households in Greater Edendale (peri-urban/urban) and Vulindlela (rural) sub-districts of uMgungundlovu District Municipality, KwaZulu-Natal (KZN), South Africa (Kharsany *et al.*, 2015; Kharsany *et al.*, 2018). The cohort enrolled 9 812 female and male participants between the ages of 15 to 49 years, who were from randomly chosen households during 2014 and 2015. One individual per household was allowed to participate in the study. Women provided self-collected vulvovaginal swabs, while men provided urine and blood samples collected after obtaining written informed consent from each participant in the parental study. Patients, categorical risk factor data (sociodemographic, behavioral, and sexual behavior) included: age, education, marital status, alcohol consumption, other STIs, STI diagnosis, condom usage, HIV status, and STI treatment, which was extrapolated from the participants consenting from this study. The STIs were tested in the parental study using a multiplex PCR testing for the following microorganisms: *C. trachomatis, N. gonorrhoeae, T. vaginalis,* syphilis*,* herpes simplex virus (HSV2) *and M. genitalium.* This was conducted by the NCID (Kharsany *et al.*, 2020). The main objective of the study was to assess the impact of programmatic intervention efforts in a "real world", non-trial setting on HIV incidence (Kharsany *et al.*, 2015).

3.3 Sample size

One hundred *Mycoplasma genitalium* positive samples from females that had been stored from the above parent study were used for this study. The samples were chosen based on similar ages and sample availability.

3.4 Study population

Samples were from a female population between the ages of 15 to 25 years from each randomly chosen household at either Greater Edendale or Vulindlela communities at uMgungundlovu District Municipality, KZN, South Africa. Each participant was given a unique study number (barcode), which connected them to the sample, chosen household, and fingerprints (Kharsany *et al.*, 2018). Informed consent forms and questionnaires were given to each participant of the study, which informed them about the confidentiality and voluntary participation in the parental study. Individuals suspected of symptoms of STIs were given symptom-specific referral notes to access primary health care services (PHC). Inclusion criteria: Male and females between the ages of 15 to 49 years who agree to participate and provide samples for the study were enrolled to participate. Exclusion criteria: In the parental study individuals who were non-residents of the selected household, who were unable to consent, who refuse to participate or give samples, mentally disabled and who were planning to move were excluded from the study (Kharsany *et al.*, 2015).

3.5 Sample collection

One hundred self-collected stored vulvovaginal swab samples were collected from Centre for the AIDS Programme of Research in South Africa (CAPRISA) HIV Incidence Provincial Surveillance System (HIPPS) household survey cross-sectional study, University of KwaZulu-Natal (UKZN) Biomedical Research Ethics Committee (BREC) BREC reference number (BF 269/13). The BREC number for this study is BE685/18. The self-collected vulvovaginal swab samples were stored at -80^{o}C.

3.6 Extraction of DNA

The stored vaginal swabs were extracted using the PureLink ^{TM} Microbiome DNA Purification Kit (ThermoFisher Scientific, United States of America (USA) according to the manufacturer's instructions. Briefly, 300 µL of nuclease-free water (ThermoFisher Scientific, USA) was added to each thawed swab and vortexed (Vortex Genie-2, ThermoFisher Scientific, USA) at the highest speed for 2-3 minutes to homogeneously mix the microorganisms. The supernatant was removed to a clean microcentrifuge tube and was centrifuged (Masiye Labs, Alberton, South Africa) at 14 000 x g for 10 minutes. The supernatant was removed/discarded without disturbing the pellet. The pellet was re-suspended into 800 µL of lysis buffer and mixed by pipetting before being transferred into the bead tube. One hundred µL of lysis enhancer was added to further break-up the cells and incubated at 65^{o}C for 10 minutes. The samples were homogenized through bead beating for 10 minutes at maximum speed (Disruptor Genie Bead beater, USA) before centrifugation at 14 000 x g for 2 minutes. Five hundred µL of supernatant was transferred into a clean microcentrifuge tube without touching the bead pellet and debris. A volume of 900 µL of binding buffer was added and mixed by vortexing. A volume of 700 µL of the sample mixture was added in a spin

column tube and centrifuged at 14 000 x g for 1 minute; this step was repeated twice, avoiding an overflow of the sample mixture. Spin columns were added into clean collection tubes, 500 µL of the wash buffer was added into spin column tube and centrifuged at 14 000 x g for 1 minute. The flow-through was discarded, and the empty spin column tube was centrifuged at 14 000 x g for 30 seconds to completely remove the wash buffer. Spin columns were placed into clean microcentrifuge tube, 100 µL of the elution buffer was added and incubated for 1 minute. The spin column tube was centrifuged at 14 000 x g for 1 minute, before discarded. The extracted DNA was stored at -20°C.

3.7 Confirmation of *Mycoplasma genitalium* positive samples using Real-time PCR

TaqMan assay was used to confirm *M. genitalium* presence: The assay was carried out using the published method from our lab (Mitchev *et al.*, 2021).

Confirmation of *M. genitalium* positive samples was conducted using TaqMan Real-Time PCR (qPCR) assay (ThermoFisher Scientific, USA). Sexually transmitted infections panels were obtained from ThermoFisher from existing Woman's Health panels that screen 33 pathogens. *M.genitalium* was one of the 33 pathogens in this panel. A master mix was prepared by pipetting 1.25 µL TaqMan Fast Start 4x master mix (ThermoFisher Scientific, USA), 0.25 µL 6-carboxyfluorescein (FAM) labeled *M. genitalium* probe (ThermoFisher Scientific, USA), and 0.5 µL nuclease-free water into 1.5 ml microcentrifuge tube. The components were mixed, and 2 µL of the master mix was added into each well of the 384 plate (ThermoFischer Scientific, USA), and 3 µL of template DNA was added into each well to make a final total volume of 5 µL. One microliter of positive control (TaqMan™ Vaginal Microbiota Extraction Control, ThermoFisher Scientific, USA) together with 2 µL nuclease-free water, and 2 µL master mix was added to each positive control well.

The positive/standard control (TaqMan™ Vaginal Microbiota Extraction Control) was serially diluted with a 10-fold dilution, with six dilutions ranging from 10^9 to 10^3 copies. In the negative control well 3 µL of nuclease-free water was added together with 2 µL of the master mix, and the 384 well plate was sealed/covered using optical adhesive covers (ThermoFischer Scientific, USA). The components were spun down, and the assay was run on the QuantStudio™ 5 Real-Time PCR machine (ThermoFisher Scientific, USA). Amplification was performed as follows: 95°C for 30 seconds followed by 45 cycles comprising of denaturation at 95°C for 3 seconds and annealing at 60°C for 30 seconds. Detection amplification of fluorescent curve products was carried out at the single signal acquisition at end of annealing end of the annealing phase. A 10-fold serial dilution of known standard concentrations was used to generate a standard

curve, which was carried out in duplicate. Copy number calculations were based on the amplification dynamics of the positive control dilution series with a linear relationship between $\log_{10}$ gene copy numbers and cycling threshold (C_T) values. The gene copy numbers ($\log_{10}$), cycling threshold, and raw fluorescence were automatically generated by Quant Studio™ 5 Real-time PCR software system version 1.5.1 (ThermoFisher Scientific, USA). Samples with a cycling threshold (C_T) < 40 were considered positive.

3.8 Detection of *Mycoplasma genitalium* using conventional Polymerase Chain Reaction (PCR)

Conventional PCR was used for the qualitative detection of the different target genes by comparing the product sizes to a molecular weight marker using agarose gel electrophoresis. The conventional PCR process was carried out in separate rooms: master mix preparation room, amplification, and detection of samples using the agarose gel electrophoresis. The amplified products were purified using GeneJet PCR purification kit (ThermoFisher, USA) and sent for sequencing in both directions.

3.8.1 *MgPa* gene detection of *Mycoplasma genitalium*

The major immune-dominance gene (MgPa) is the major adhesion protein of *M. genitalium* microorganism, which helps attach to the different ciliated surfaces. *Mycoplasma genitalium* published primers targeting 281 bp of the major adhesion protein 140 kDa of *Mycoplasma genitalium.* Primers were synthesised by Inqaba Biotechnologies Company, MgPa-1 (Forward Primer): 5'-TGA AAC CTT AAC CCC TTG G-3', and MgPa-3 (Reverse Primer): 5'-AGG GGT TTT CCA TTT TTG C-3' (Jensen *et al.*, 1991).

A final 20 µL volume PCR reaction for each sample was prepared in the following manner: 12.39 µL of nuclease-free water, 4 µL of 5x Green Phusion buffer, 0.16 µL of 25 mM dNTP (ThermoFisher Scientific, USA), 0.25 µL of each 20 pmol primers (forward and reverse primer), 1 µL DMSO, 0.2 µL of Phusion Taq DNA polymerase, 0.25 µL of 50 mM $MgCl_2$ in a 1.5 ml microcentrifuge tube (Sigma-Aldrich, Germany). The contents were mixed, and 18 µL of the master mix was added into each 0.2 ml PCR tube (ThermoFisher Scientific, USA), and 2 µL of template DNA was added and mixed.

Amplification was conducted by loading tubes in the conventional PCR machine (SimpliAmp thermal cycler, ThermoFisher Scientific, USA). The cycling conditions were as follows: initial denaturation of one cycle at 98°C for 30 seconds, 30 cycles of denaturation at 98°C for15 seconds, annealing 64°C for 15

seconds, and extension at 72ºC for 15 seconds. Followed by a final extension of one cycle at 72ºC for 30 seconds and a final hold at 4ºC. In each amplification reaction, a negative control (nuclease-free water) and positive control were included. Positive control: A clinical sample that was previously aligned with MK4113621 strain sequence was used as a positive control. The PCR amplicons were detected using a 1.5% agarose gel electrophoresis.

3.8.2 Detection of macrolide resistance genes using conventional PCR

Published primers from previously conducted studies targeting the 147 bp variable (V) region of 23S rRNA gene were used to detect macrolide-resistant region. This region is understood to be the major region that leads to macrolide resistance (Jensen *et al.*, 2008; Le Roux *et al.*, 2018). Point mutations that are associated with macrolide resistance were also tested, targeting the 560 bp region of L22 and 670 bp region of the L4 gene (Jensen *et al.*, 2008). Inqaba Biotechnologies Company synthesized all primers (Inqaba Biotechnical Industries, Pretoria, South Africa).

Table 3.8.2.1: The primer sequences that were used to target the different regions associated with macrolide resistance

Gene	Primer sequence (5'-3')	Targeted size
23S rRNA	5'-CCATCTCTTGACTGTCTCGGCTAT-3' (F)	
	5'CCTACCTATTCTCTACATGGTGGTGTT-3' (R)	147 bp
L22 (*rpIV*)	5′-ATGGTAGGTCATAAGTTGGGTGAGTTT-3′ (F)	
	5′-AGTTCTTATTAATGCCAAACCTTAAGCC- 3′ (R)	560 bp
	5′-AAGTAATGGCTAAACTTAAAGTAATCC-3′ (F)	
L4 (*rpID*)	5′-TTTAAGAGTATGTTGGTTACATCCATAG-3′ (R)	670 bp

F- Forward primer sequence R- Reverse primer sequence

Primer sequences (forward and reverse) of genes associated with macrolide resistance mutations. The primers will target 147 bp for 23S rRNA, 560 bp for L22 and 670 bp for L4 gene proteins.

3.8.2.1 The 23S rRNA gene detection: A total volume of 20 µL was used for each conventional PCR reaction to detect 23SrRNA. Each PCR reaction consisted of 18 µL of the master mix which consisted of 12.64 µL of nuclease-free water, 4 µL of 5x Green Phusion buffer (ThermoFisher Scientific, USA), 0.25 µL of each primer (20 pmol forward and reverse primer), 0.5 µL DMSO, 0.2 µL Phusion Taq DNA polymerase, 0.25 µL of 50 mM $MgCl_2$ and 0.16 µL of 25 mM dNTP (ThermoFisher Scientific, USA) and was added to a 1.5 ml microcentrifuge tube (Sigma-Aldrich, Germany). The contents were mixed, 18 µL of the master mix was added to each 0.2 ml PCR tube (ThermoFisher Scientific, USA), and 2 µL of template DNA was added followed by mixing.

Amplification of the 147-band size of the targeted region was conducted by loading tubes in the conventional PCR machine (SimpliAmp thermal cycler, ThermoFisher Scientific, USA). The cycling conditions were as follows initial denaturation, one cycle at 98°C for 30 seconds, 30 cycles of denaturation at 98°C for 10 seconds, annealing 62°C for 10 seconds, and extension at 72°C for 10 seconds. This was followed by a final extension of one cycle at 72°C for 30 seconds and a final hold at 4°C. In each amplification reaction, a non-template negative control (nuclease-free water) and positive template control were included. All amplicons were run on a 1.5% gel.

3.8.2.2 The L4 and L22 ribosomal gene detection: A total final volume of 20 µL master mix reaction PCR was used to detect each conventional PCR reaction for the detection of L4 and L22. Each PCR reaction consisted of 18 µL of the master mix which consisted of 12.14 µL of nuclease-free water, 4 µL of 5X green Phusion buffer (ThermoFisher Scientific, USA), 0.25 µL of each primer (20 pmol forward and reverse), 1 µL DMSO, 0.2 µL of Phusion Taq DNA polymerase, 0.25 µL of 50 mM $MgCl_2$ and 0.16 µL of 25 mM dNTP (ThermoFisher Scientific, USA) and was added to a 1.5 ml microcentrifuge tube (Sigma-Aldrich, Germany).The contents were mixed, 18 µL of the master mix was added to each 0.2 ml PCR tube (ThermoFisher Scientific, USA), and 2 µL of template DNA was added followed by mixing.

Amplification of the targeted band region was conducted by loading tubes in the conventional PCR machine (SimpliAmp thermal cycler, ThermoFisher Scientific, USA). The cycling conditions were as follows initial denaturation, initial denaturation: One cycle at 98°C for 30 seconds, 30 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and final extension of one cycle at 72°C for 1 minute, followed by a final hold at 4°C. In each amplification reaction, a non-template negative control (nuclease-free water) and positive template control were included. All amplicons were run on a 1.5% gel.

3.8.3 Detection of fluoroquinolone resistance gene gyrase (*gryA)* using conventional PCR method

The deoxy ribonucleotide acid (DNA) gyraseA (*gyrA)* gene which is associated with fluoroquinolone resistance, was amplified using primers targeting 230 bp (Pond *et al.*, 2014).

Table 3.8.3.1: Primer sequences of *gyrA* gene for the amplification of the targeted region associated with fluoroquinolone resistance

Gene	Primer sequence (5'-3')	Targeted size
gyrA	5'-CCTGATGCTAGAGATGGACTTAAA-3' (F)	
	5'-AAGTTCTGCTGCAAGTTTAGATAAT-3' (R)	220 bp

F- Forward primer sequence R- Reverse primer sequence

Primer sequences that were used for the amplification of the targeted 230 base pair (bp) size of *gyrA* gene. The amplification of this region is further discussed in the following sections.

Detection of the targeted band sizes for *gyrA*: A total final volume of 20 µL master mix reaction PCR was used to detect each conventional PCR reaction for the detection of *gyrA*. Each PCR reaction consisted of 18 µL of the master mix which consisted of 12.14 µL of nuclease-free water, 4 µL of 5X green Phusion buffer (ThermoFisher Scientific, USA), 0.25 µL of each primer (20 pmol forward and reverse), 1 µL DMSO, 0.2 µL of Phusion Taq DNA polymerase, 0.25 µL of 50 mM MgCl2 and 0.16 µL of 25 mM dNTP (ThermoFisher Scientific, USA) and was added to a 1.5 ml microcentrifuge tube (Sigma-Aldrich, Germany).The contents were mixed, 18 µL of the master mix was added to each 0.2 ml PCR tube (ThermoFisher Scientific, USA), and 2 µL of template DNA was added followed by mixing.

Amplification of the targeted band region was conducted by loading tubes in the conventional PCR machine (SimpliAmp thermal cycler, ThermoFisher Scientific, USA). The cycling conditions were as follows initial denaturation, initial denaturation: One cycle at 98°C for 30 seconds, 30 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and final extension of one cycle at 72°C for 1 minute, followed by a final hold at 4°C. In each amplification reaction, a non-template negative control (nuclease-free water) and positive template control were included. All amplicons were run on a 1.5% gel.

The samples were visualized on a 1.5% agarose gel. On each gel, wells contained a molecular weight marker, a positive control, a negative control and the desired samples. Gel red (GelRed ™ Nucleic acid, Sigma-Aldrich, Germany) was added to the loading dye which allowed us to visualization the pcr products under the ultraviolet (UV) light. Gels were visualized using a UV transilluminator (SynGene, G-Box, sydr4/1153). The desired band that corresponds to the specific band size of the gene being detected was excised using a scalpel blade. The excised band size was purified using a GeneJet PCR purification spin column according to the manufacturer's instruction. The same procedure was followed for all the respective genes.

3.9 Purification of samples

The gel slice with the expected amplicon sizes was excised using a scalpel blade and transferred in a 1.5 ml microcentrifuge tube. The excised gel slice was purified using the GeneJET gel purification kit (ThermoFisher Scientific, USA) protocol according to the manufacturer's instruction. Briefly, the amplified PCR targeted size product was excised and placed into a 1.5 ml microcentrifuge tube. Two hundred microliters of binding buffer were added and incubated for 10 minutes at 55^{o}C in the water bath until the gel slice was completely dissolved. The gel slice mixture was vortexed and mixed. An aliquote of 100 µL of 100% isopropanol was added to the pcr gel mixture. Eight hundred µL of solubilized gel solution mixture was transferred to the GeneJET purification column and centrifuged at 13 000 rpm for 1 minute, and this step was repeated twice. Binding buffer volume of 100 µL was added to the GeneJET purification column, centrifuged at 13 000 rpm for 1 minute, and the flow through was discarded. Seven hundred microliters of wash buffer was added to the GeneJET column, centrifuged at 13 000 rpm for 1 minute, the flow-through was discarded, and centrifuged the empty column for another 1 minute to remove residual DNA. The GeneJET purification column was transferred into a clean 1.5 ml microcentrifuge tube, and 15 µL of elution buffer was added at the center of the purification column membrane and centrifuged for 1 minute. The purification column was discarded, and the purified DNA was sent for sequencing in both directions at KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), except for the MgPa gene.

3.10 Data analysis

All sequences obtained were blasted on National Center for Biotechnology Information (NCBI), nucleotide Basic Local Alignment Search Tool (BLASTn) software. All Fasta format sequences obtained were edited using BioEdit version 7, using Widespread Multiple Sequence Alignments (MSA) Clustal W and aligned against wild type reference strain (G-37) and with strains of known mutations: LA088 (HF572933.1), LA141 (HF572938.1), and LA202 (HF572946.1) for 23S rRNA gene.

3.10.1 Macrolide resistance gene analysis 23S rRNA, L4 and L22 of the obtained DNA sequences in comparison with the wild type sequence: Fasta sequences were aligned against *Mycoplasma genitalium* G-37 and LA141 (HF572938.1) A2058G, LA202 (HF572946.1), (Pond *et al.*, 2014) and A2059C (Tagg *et al.*, 2013). All L4 gene generated Fasta sequences were compared against the rpl4 gene which was used as a reference strain from the whole genome of G-37 (L43967.2). The L22 gene Fasta sequences generated were compared against the G-37 whole genome (L43967.2) gene rpl22.

3.10.2 Fluoroquinolone gene *gyrA* analysis for the detection of mutations when compared with the wild type: Fasta sequences obtained from KRISP were compared against deoxyribonucleotide (DNA) gyrase subunitA (*M. genitalium* G-37, L43967.2).

CHAPTER 4

RESULTS

4.1 Study ethical clearance: The protocol for the study was approved by the University of KwaZulu-Natal (UKZN) Biomedical Research Ethics Committee (BREC) reference no. BE685/18 (Appendix D).

4.2 Characteristics of the cohort according to the categorical risk factors: sociodemographic, behavioral, and sexual behavior detected for *Mycoplasma genitalium* resistance targets

A total of 100 female vaginal swab samples were confirmed to be positive for *M. genitalium*. Figure 4.1 outlines how the 100 samples were obtained (Centre for the AIDS Programme of Research in South Africa (CAPRISA) HIV Incidence Provincial Surveillance System (HIPPS) and summarizes the presence and absent of gene mutations obtained using sequencing method. The interquartile range age of the cohort was 15 to 25 years, with a standard deviation of 2.40 and a standard error of 0.24, as shown in table 4.1. In the population, human immunodeficiency virus (HIV) positive individuals were 58%, and 42% were HIV negative. Ninety-two percent of the participants did not consume alcohol, 90% were single, 52% had incomplete secondary, and four percent with tertiary education. In the cohort, other STIs were detected this includes: herpes simplex virus (HSV) serology in 64% individuals, 26% for *Chlamydia trachomatis* (*C. trachomatis*), 14% for *Trichomonas vaginalis* (*T. vaginalis)*, and nine percent for *Neisseria gonorrheae (N. gonorrheae)*. There is less condom usage in the population during first sex, with 22% individuals observed, compared to 48 individuals who used condoms in the last 12 months, as shown in table 4.1.

Cohort description

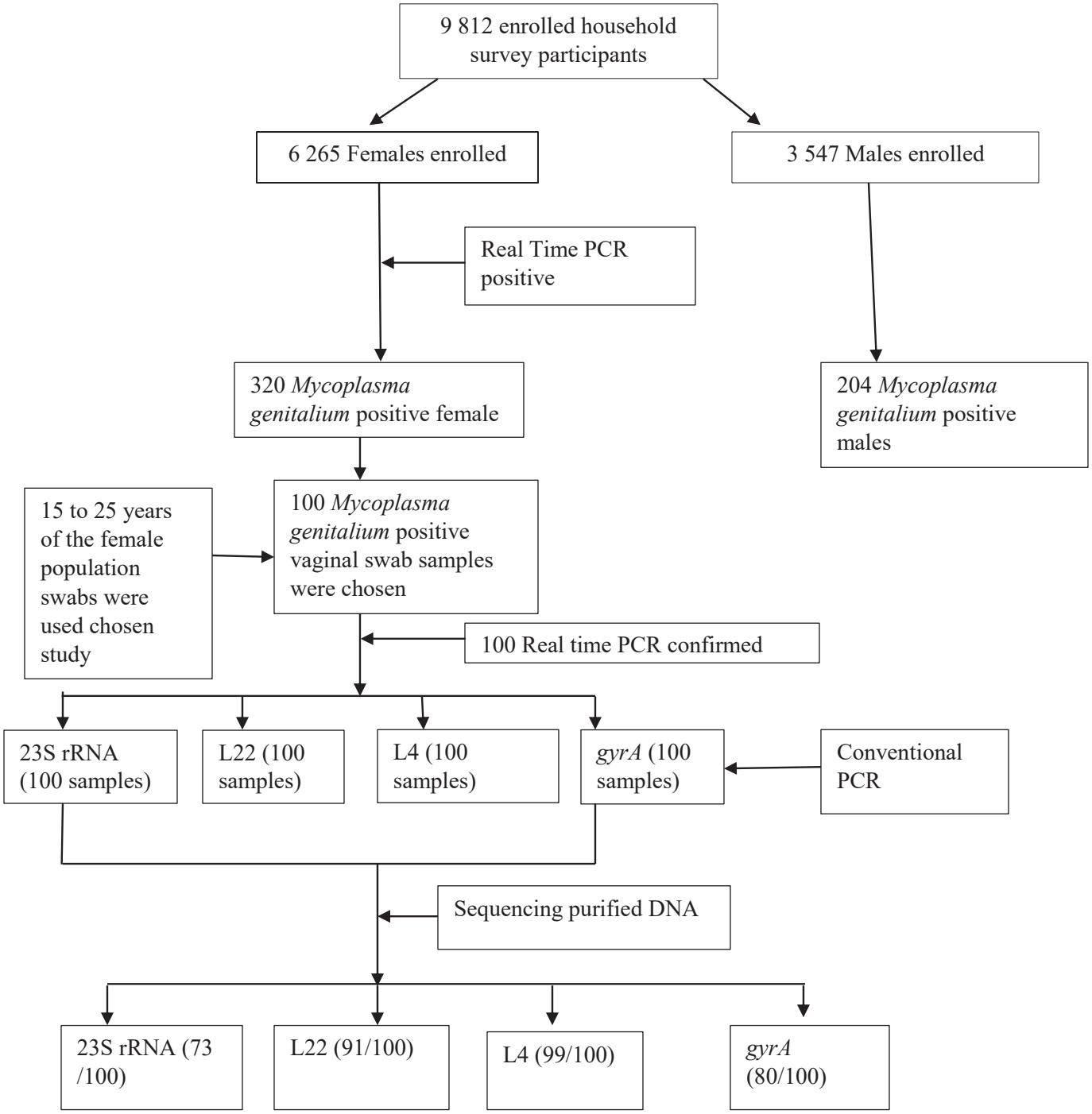


Figure 4.1: **Cohort description for testing macrolide resistance and chosen vaginal swab samples description used for testing resistance of macrolides and fluoroquinolone regiments**

Seventy-three samples for 23S rRNA, 91 for L22, 99 for L4 and 80 for *gryA* were amplified. In the samples that failed to amplify for sequencing that is for 23s rRNA, L4, L22 and *gryA*. It could be speculated that the samples failed because the concentration was not high enough to detect these genes. Amplification was attempted on these samples atleast more than three time.

Table 4.1: The cohort's characteristics using demographic: behavioral, sociodemographic, and sexual behavior of the female population

Population demographic	Total [(n =100 (%)]
Interquartile range (IQR) age and range	15 to 25 years
STD	2.40
STD Error	0.24
HIV	
Positive	42
Negative	58
Education	
No schooling	2
Primary /Incomplete secondary	52
Completed secondary	42
Tertiary	4
Marital status	
Single	90
Single/Have been living together (husband and wife)	2
Living together (husband and wife)	5
Legally married	3
Alcohol consumption	
Yes	8
Diagnosed by a doctor or nurse with STI	
Yes	4
Other STIs	
Yes *N. gonorrhoeae*	9
Yes *C. trachomatis*	26
Yes *T. vaginalis*	14
Yes, herpes simplex virus 2 *(*HSV)	64
Condom usage first sex	
No	65
Yes	22
Don’t remember	3

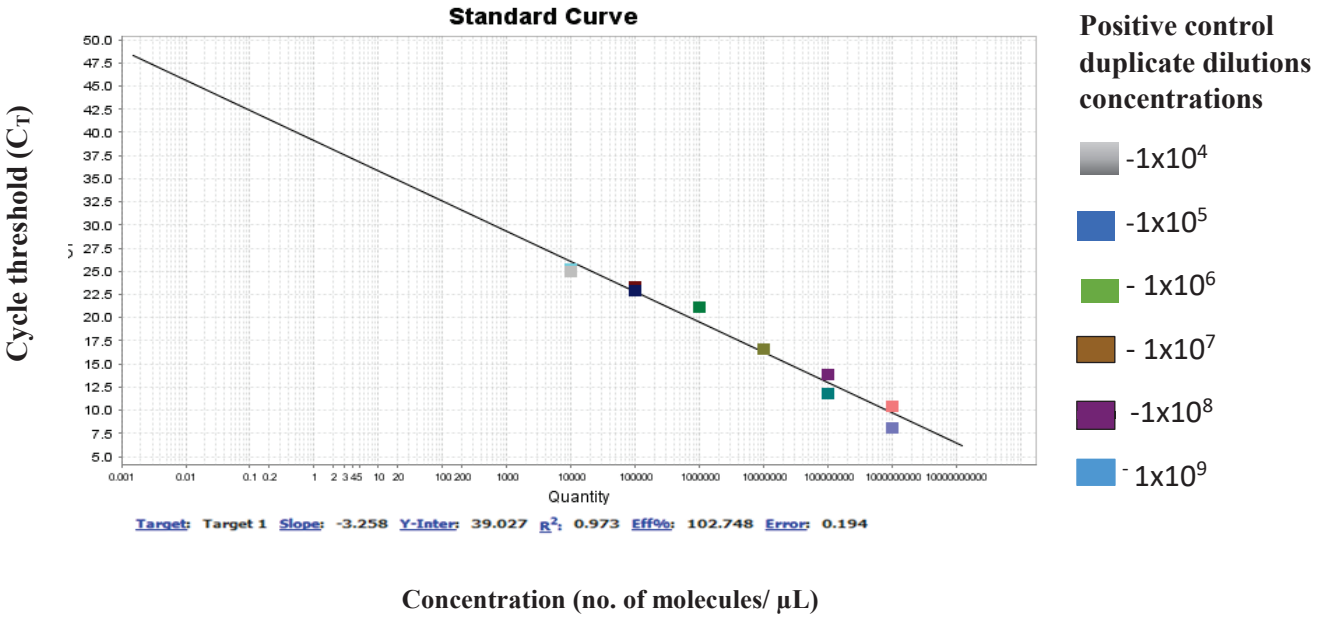
Population demographic	Total [(n =100 (%)]
Condom usage last 12 months	
No	15
Yes	48
Don't remember	37
STI Treatment	
Yes	4

Table 4.1 Description of the cohort's demographic characteristics

Description of the patient's demographic characteristics in percentages. These includes behavioral, sociodemographic, and sexual behavior of the female population

4.3 Detection of *Mycoplasma genitalium* using real-time PCR (qPCR)

Duplicated 10-fold dilutions of standard (TaqMan™ Vaginal Microbiota Extraction Control; cat no. A32039) were used to generate the standard curve (figure 4.2). Figure 4.2 shows a linear regression between the controls that were used in the standard curve. The standard curve's efficiency was 102%, calculated from the slope of the standard curve, -3,258, and the R^2 of the curve was 0.973. The acceptable efficiency for a standard curve ranges from 80 to 120%.



Concentration (no. of molecules/ µL)

Figure 4.2: The calibration curve shows the CPs (cycle number) of each standard control plotted against the logarithmic concentration to produce a standard curve. The standards were run in duplicates together with the sample.

Amplification plot of samples and standards: Few samples were amplified at the beginning of the cycle, and most samples were amplified between 20-35 cycles. Samples that amplified much early had higher *M. genitalium* concentrations these samples were easier to work with for downstream processes. Cycle threshold (C_T) values < 40 cycles were considered positive, as shown in figure 4.

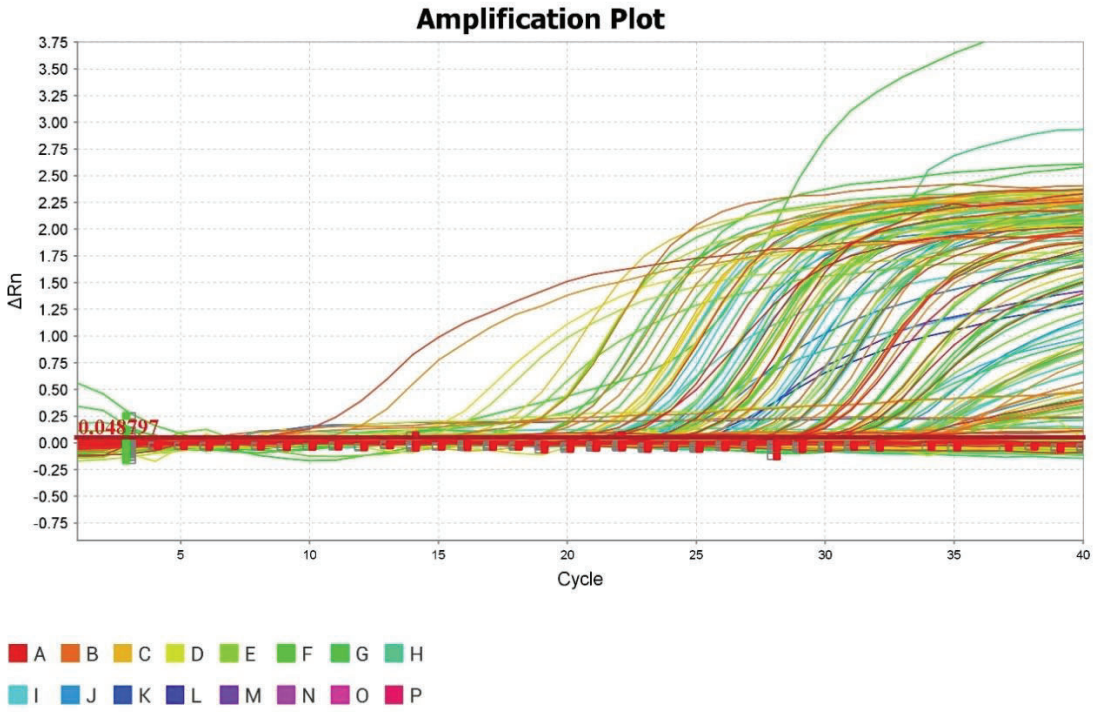


Figure 4.3: Representative results of different *Mycoplasma genitalium* positive samples using real-time PCR (q-PCR). Ct < 40 cycles were considered positive

4.4 Conventional PCR for *Mycoplasma genitalium* gene detection

Specific band sizes for each targeted gene were detected using conventional PCR.

Multiple sequence alignment of each targeted gene using DNA sequencing method

Representative figures showing sequence alignments of ~ 20 samples with or without mutations for all the genes tested.

4.4.1 The major immune-dominace (*MgPa*) gene detection

Figure 4.4 shows 281 bp band size detected for the *MgPa* gene size for *Mycoplasma genitalium*.

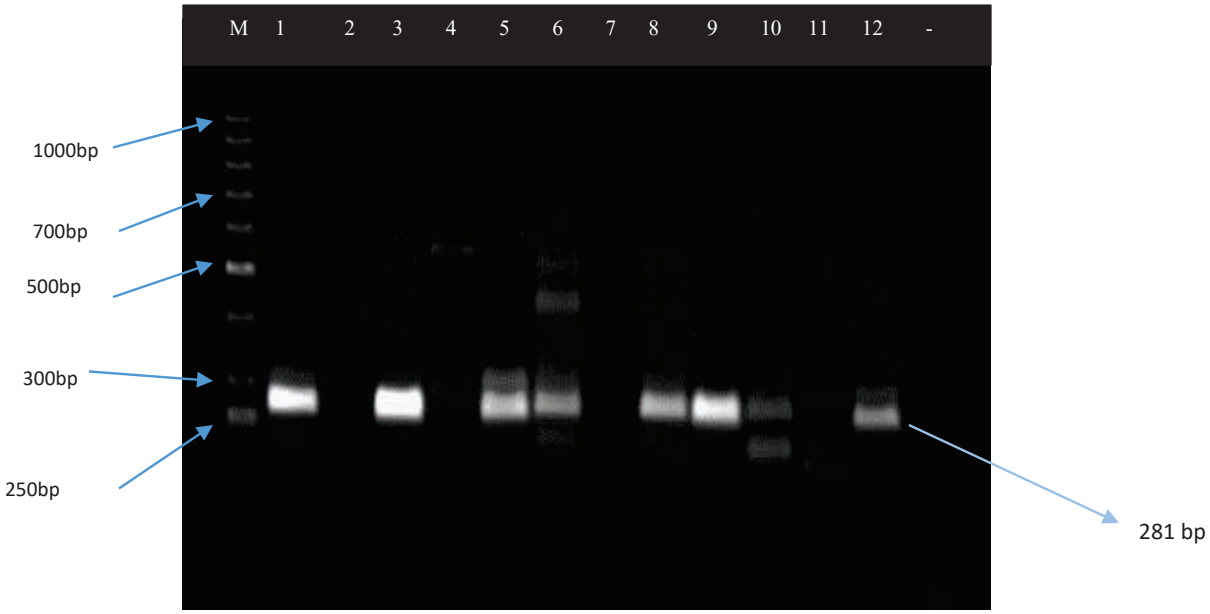


Figure 4.4: Agarose gel electrophoresis image of MgPa PCR amplicons from conventional PCR. M: 50 bp DNA ladder, **Lane 1-10:** Positive *M. genitalium* samples, **Lane 11:** positive control and **Lane 12:** Negative control.

4.4.2 Macrolide resistance

23S rRNA gene: A 147 bp product was amplified for the detection of mutations in 23S rRNA gene. The mutations in this region, V region is associated with macrolide azithromycin resistance. Figure 6. In the 73 samples that were successfully sequenced, five samples harboured mutations. Two samples harboured mutations at the same position: A2072G (2059 *E. coli* numbering), refer to figure 4.7. Other mutations were detected in the following positions: A2071T (2058, *E. coli* numbering), A2072C (2059, *E. coli* numbering), A2072T (2059, *E. coli* numbering), refer to figure 7. Mutation positions are given according to the *Mycoplasma genitalium* numbering.

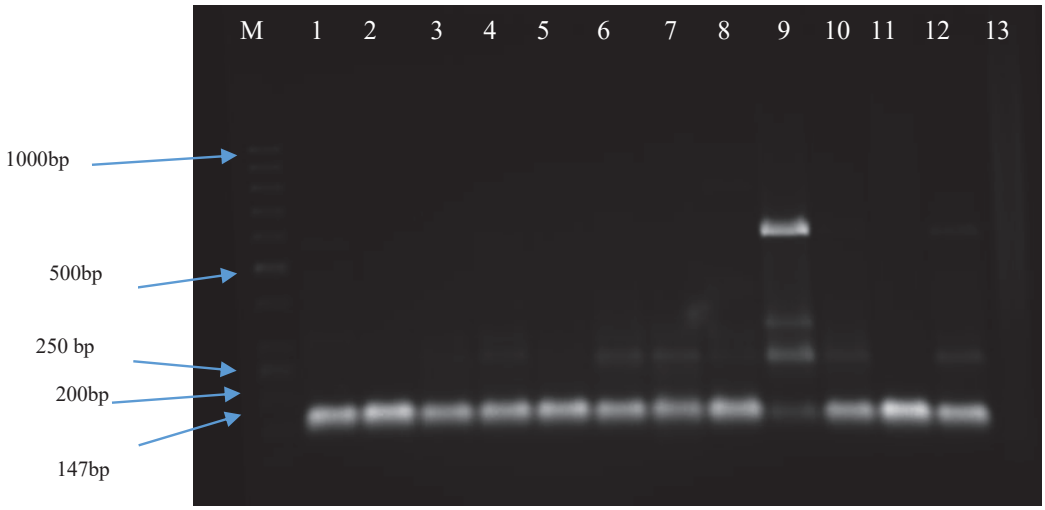


Figure 4.5: Representation of the 147 bp product of 23S rRNA gene from agarose gel electrophoresis. **M:** 50 bp DNA ladder, **Lane 1-11:** clinical positive Mg samples, **Lane 12:** Positive control and **Lane13**: Negative control

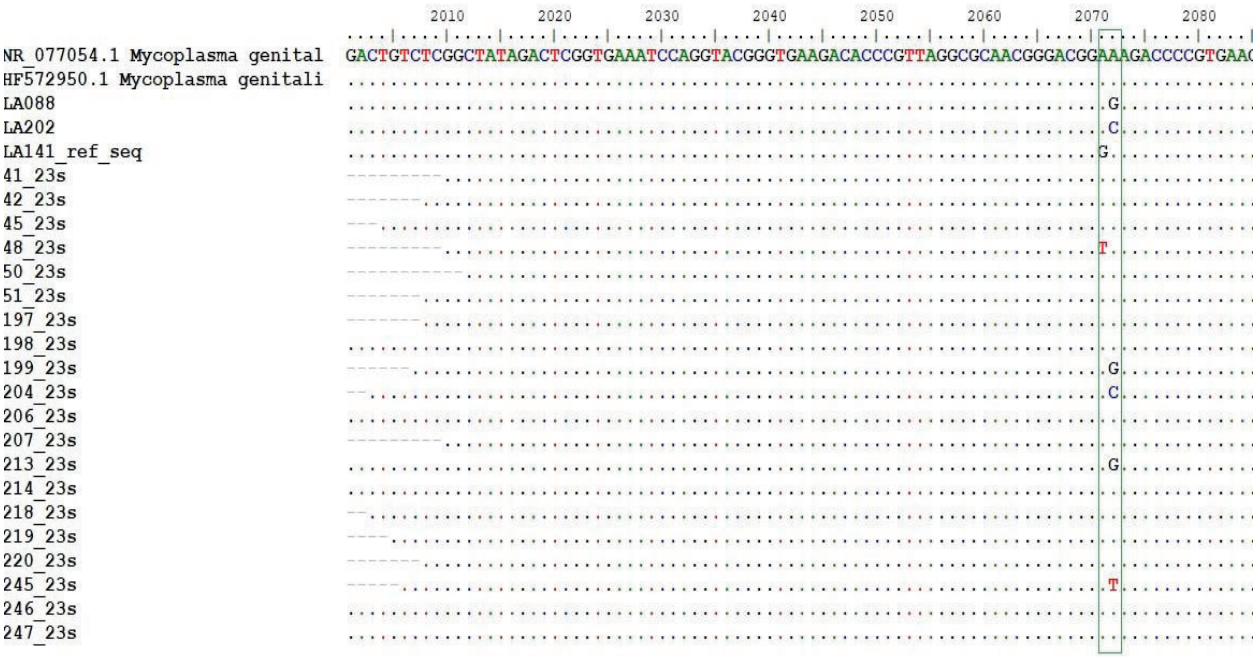


Figure 4.6: Multiple sequence alignments of 23S rRNA gene in comparison with wild type reference strain *M. genitalium* G-37 and with sequences of known mutations

Table 4.2: Detection of mutations associated with macrolide resistance in 23S rRNA

Sample no.	Mutation	Amino acid change
48	A2071T	Start690stop (K690stop)
204	A2072C	K690T
213	A2072G	K690R
199	A2072G	K690R
245	A2072T	K690I

Table 4.2 The detected mutation in 23S rRNA gene with the amino acid changes

The mutations detected in the five samples led to a change in the amino acids change. Lysine amino acid was mostly mutated and was changed to other amino acids.

4.3.3 L4 *(rpID)* gene mutation detection

Six hundred seventy base-pair PCR products were detected for the presence of the L4 *(rpIV)* gene, Figure 4.8. Four samples harboured mutations from the 99 PCR amplicons that were successfully sequenced. None led to amino acid changes, as these mutations were synonymous.

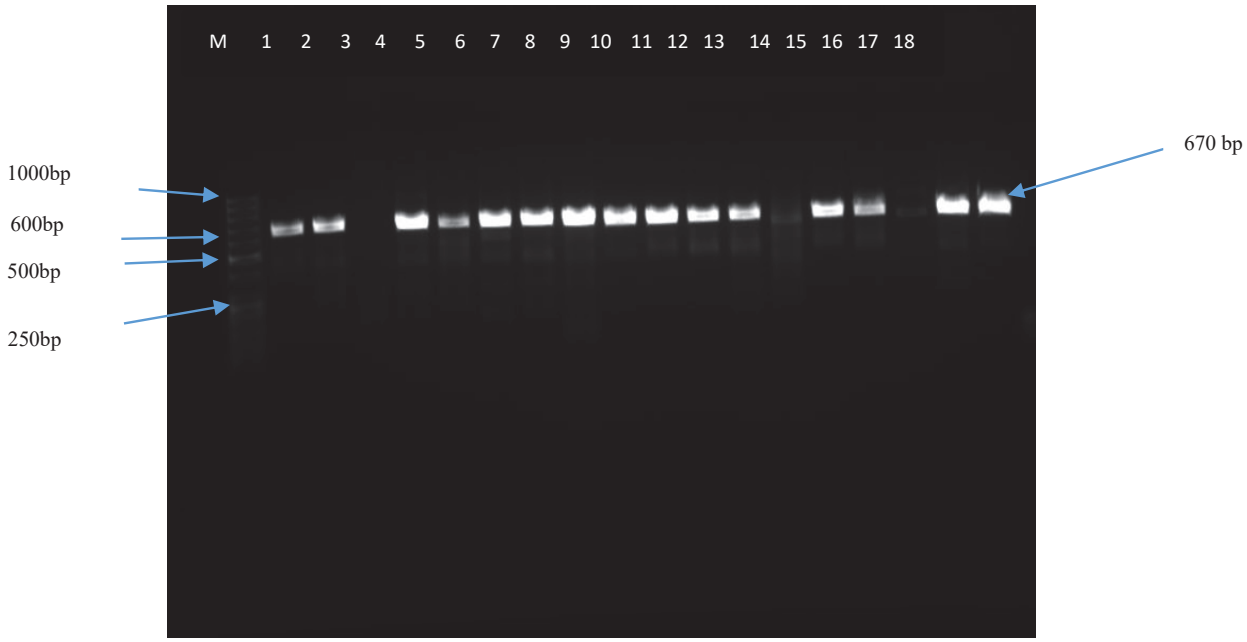


Figure 4.7: Amplicon sizes of the amplified L4 PCR products. M: 50 bp DNA ladder, **Lane 1, 2, and 4-17:** positive *M. genitalium* samples, **Lane 18:** Positive control and **Lane 3**: Negative control

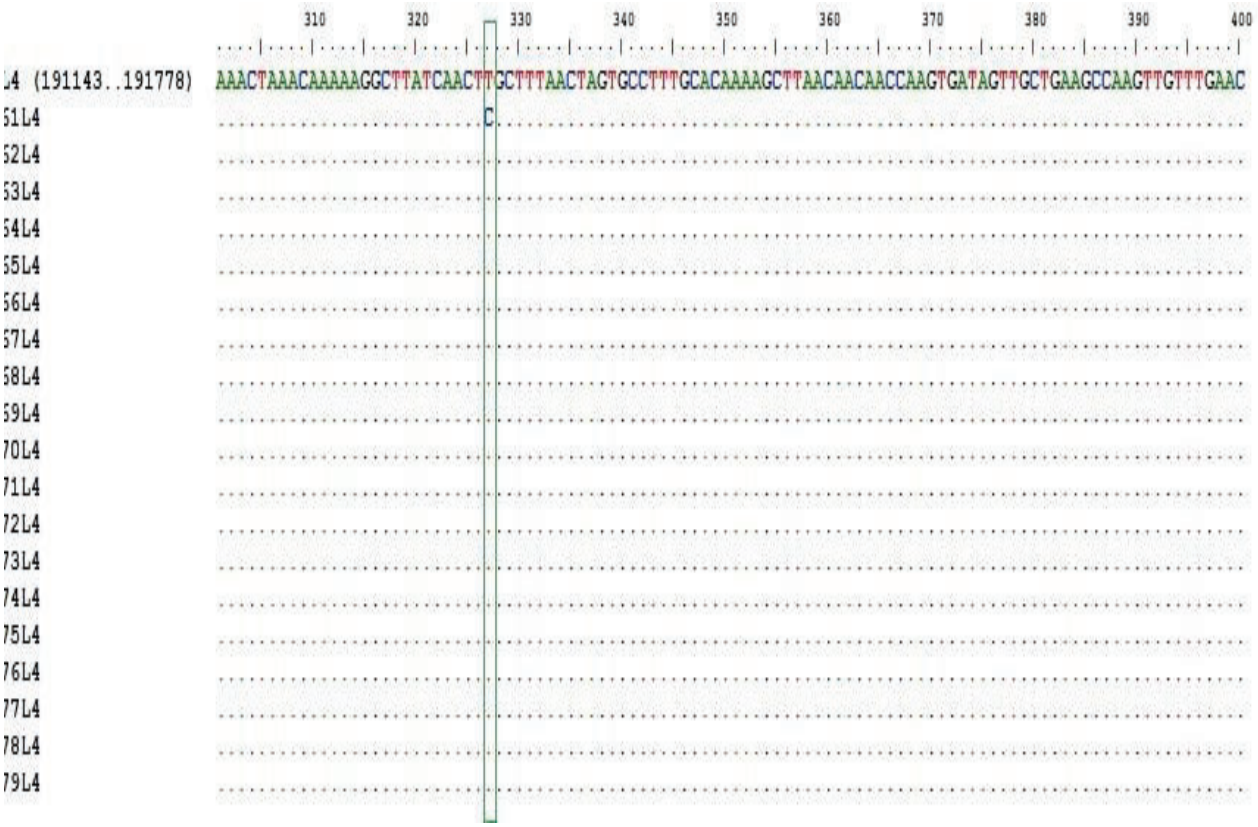


Figure 4.8A: Nucleotide base changes of the PCR products for L4 gene protein

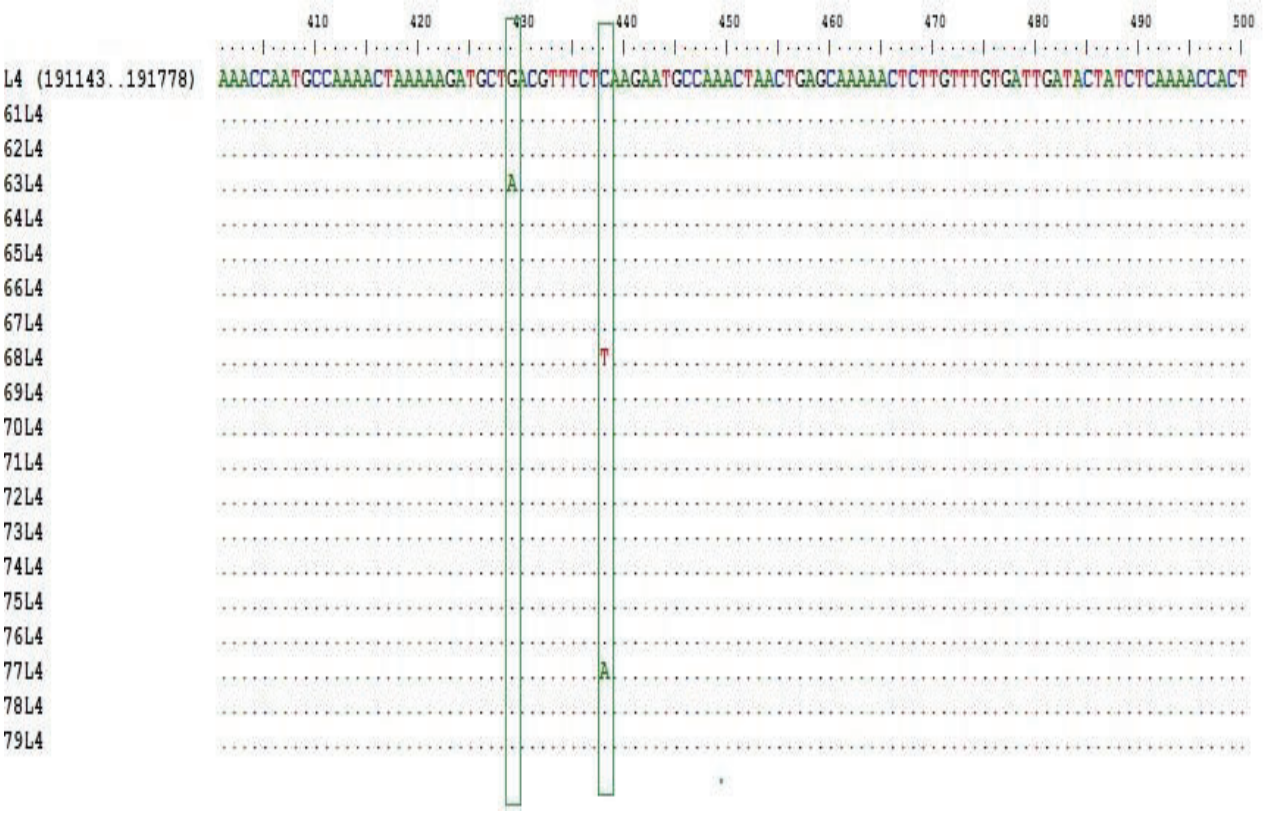


Figure 4.8B: Representation of the different nucleotide base changes of the L4 gene protein

Table 4.3: Mutations detected in L4 protein gene

Sample no.	Mutation	Amino acid change
61	T327C	L109L
63	G429A	L143L
68	C438T	L146L
77	C438A	L146L

Table 4.3: The detected mutations for L4 gene protein led to silent mutations as none led to an amino acid change. These mutations occurred at different positions in the targeted gene. This includes T327C, G429A, C438T and C438A (refer table 4.3 above).

4.3.4 L22 *(rpI22)* gene

Figure 4.10 shows a representative image of PCR products run on an agarose gel. The desired fragment size was 560 bp. Mutations obtained from the 99 that were successfully sequenced after multiple sequence alignment (MSA) showed changes in the nucleotide sequences, figure 4.11A and B. Samples that did not amplify had a high CT value above 33 threshold cycle. The recommended concentration (5-10 ng) for sample was sent for sequencing. This resulted in successful chromatograms for sequencing data. Three samples had mutations from a total of ninety-one after sequencing. Mutations that were observed all had silent mutations.

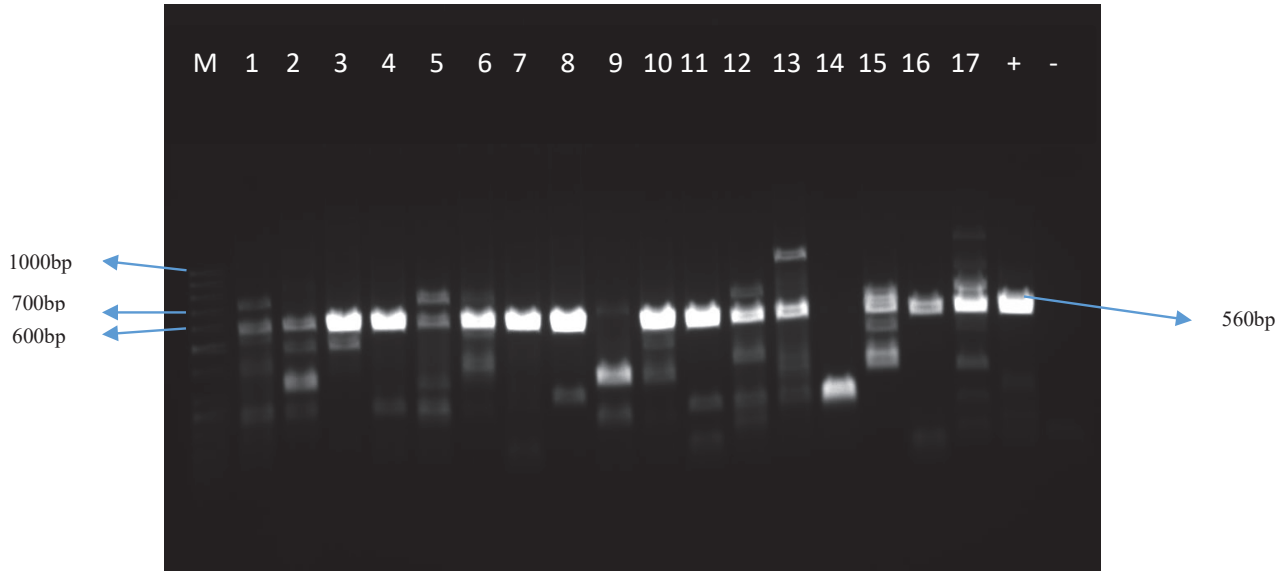


Figure 4.9: Representative Agarose gel electrophoresis image of ribosomal gene protein L22. **M**: 50 bp molecular weight marker, **Lane 1-17-** Clinical samples, **Lane (+)** - Positive control and **Lane (-)** Negative control

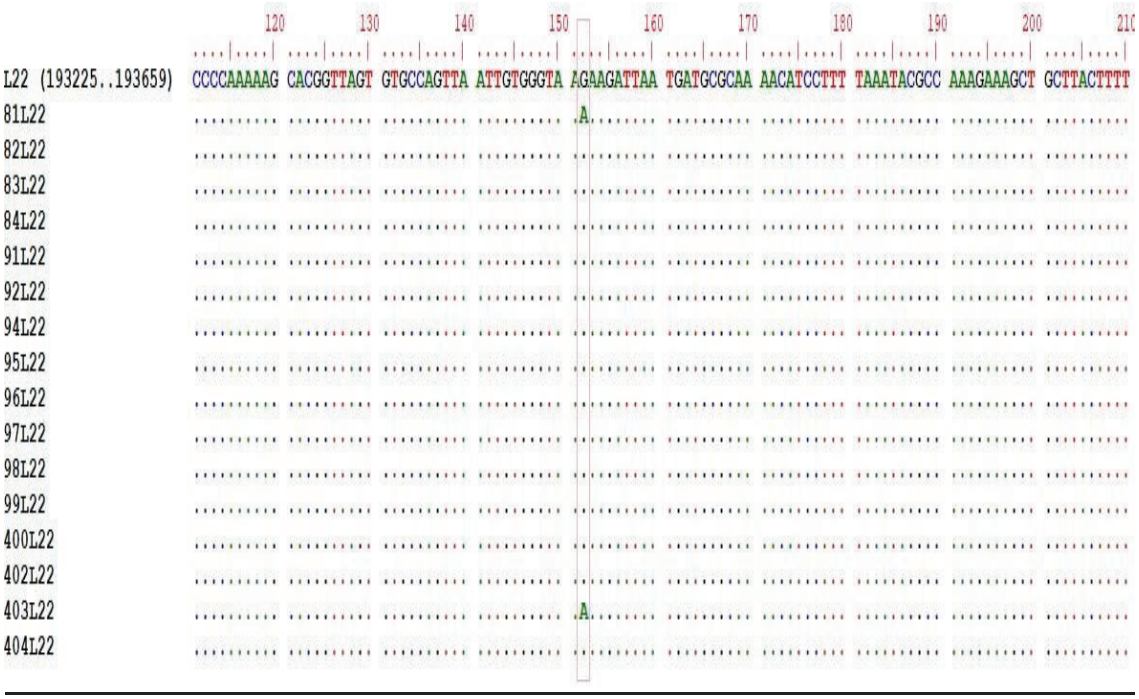


Figure 4.10A: Representation of mutations detected from the sequenced PCR products of L22 protein gene

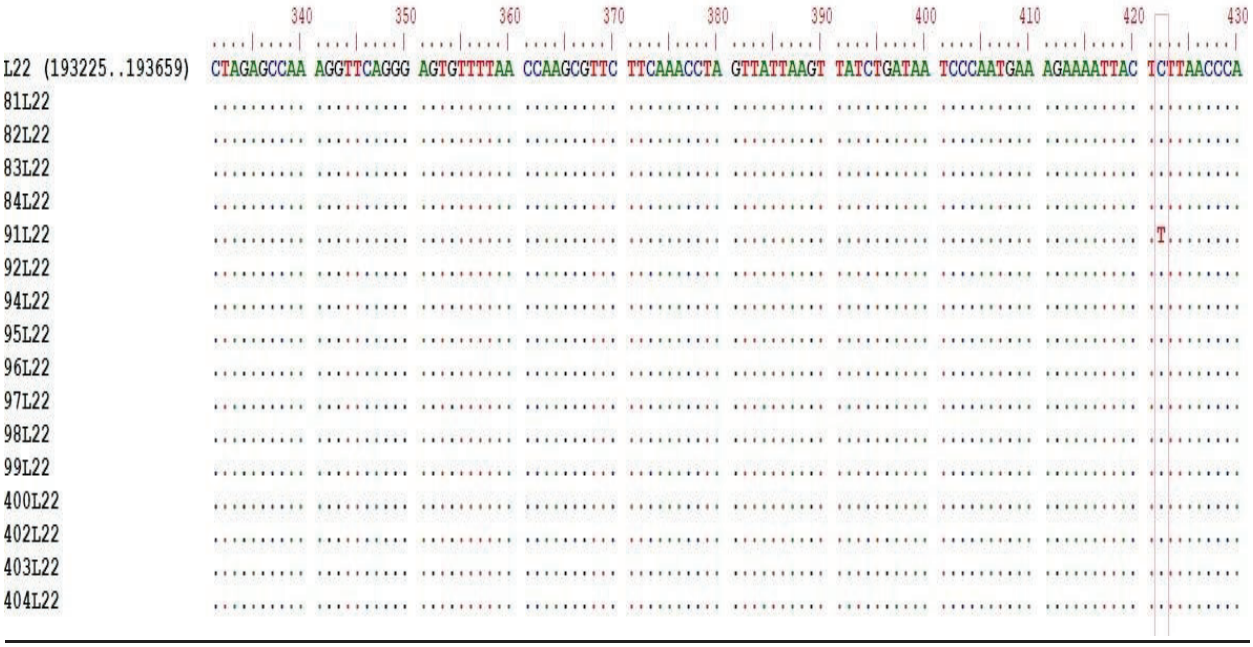


Figure 4.10B: **Nucleotide base changes for each sample in L22 gene protein**

Table 4.4: Samples showing the mutations in L22 protein gene with the amino acid changes

Sample no.	Mutation	Amino acid change
81	G81A	K27K
403	G81A	K27K
91	C351T	L117L

Table 4.4: Detected mutations led to the same amino acid change (lysine and leucine change) resulting in silent mutations. These mutations were detected at different positions in the gene (27 and 117).

4.4 Fluoroquinolone conventional PCR detection.

Figure 4.12 shows a representative plot of PCR products run on an agarose gel**.** The *gyrA* gene amplification of the targeted band sizes was detected in 100 samples. The PCR amplicons detected were sequenced. Sequences obtained were compared against DNA gyrase subunit A (*Mycoplasma genitalium* G37, accession no. L43967.2). Six samples harboured mutations, and of these, three led to amino acid changes.

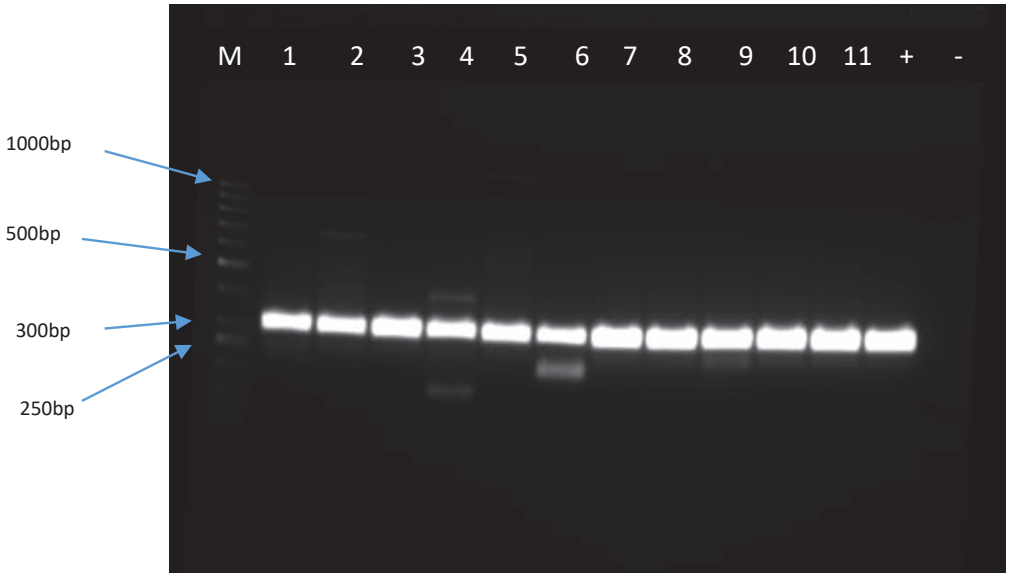


Figure 4.11: Representative Agarose gel electrophoresis of *gyrA* conventional PCR amplicons. M: 50 bp DNA ladder, **Lane 1-11**: Positive *M. genitalium* samples, **+ve:** Positive control and **-ve**: Negative control

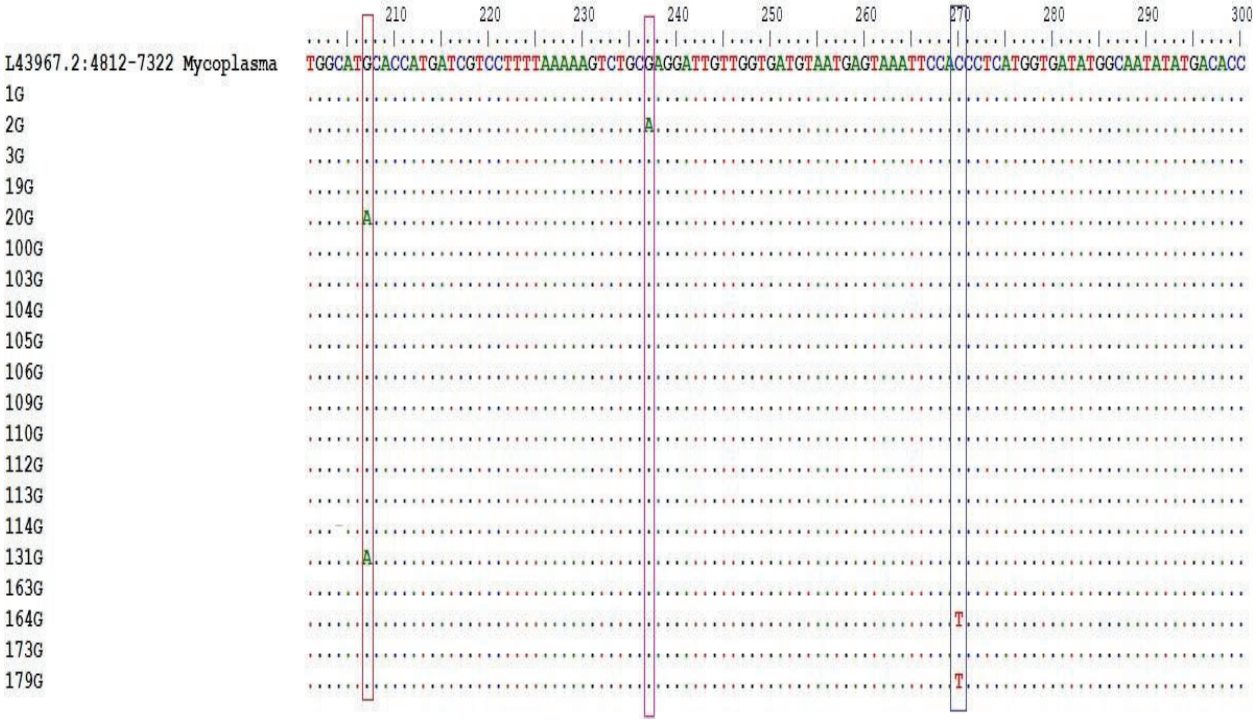


Figure 4.12A: Nucleotide base changes of *gyrA* gene in comparison with the reference strain

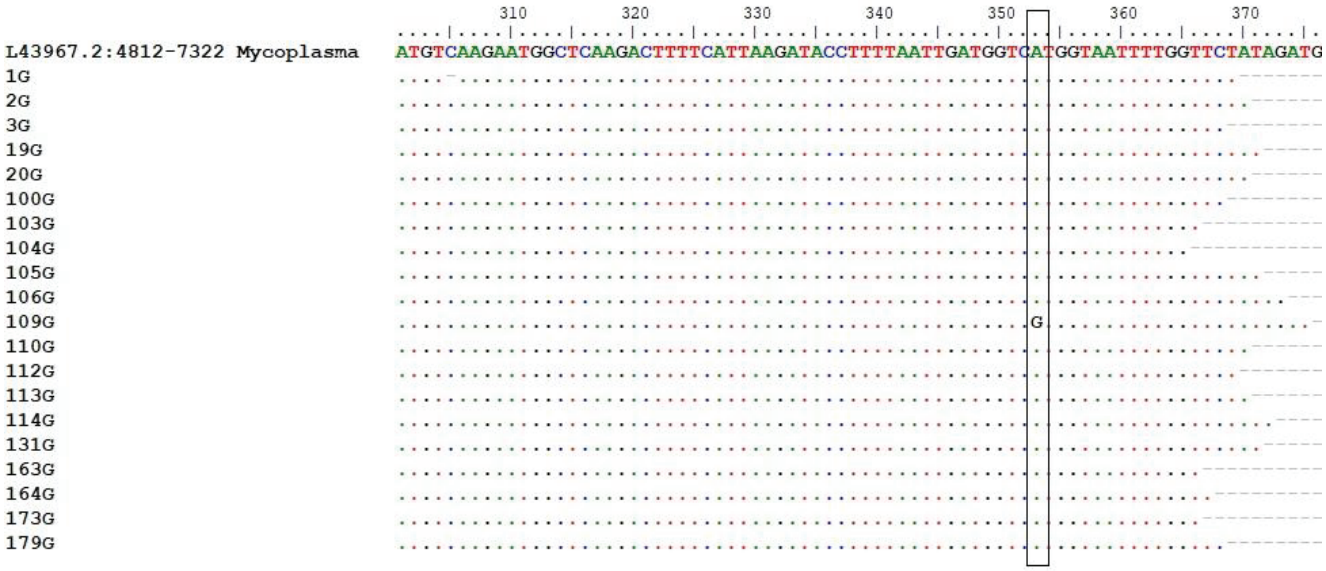


Figure 4.12B: **Nucleic acid changes of *gyrA* gene in comparison with the reference strain**

Table 4.5: Representation of the different mutations detected *gyrA* gene

Sample no.	Mutations	Amino acid change
2	G237A	A79A
20	G207A	M69I
109	A353G	H117R
131	G207A	M69I
164	C270T	H90H
179	C270T	H90H

Table 4.5: Represents mutations detected in *gyrA* gene that and amino acid changes

Three mutations detected were silent mutations and some led to amino acid changes in the following positions G207A resulting in methionine to isoleucine. The A353G mutation led to histamine to arginine in the gene. Silent mutations detected were at positions C270T (H90H) and G237A A79A).

Table 4.6: The positions of point mutations and amino acid changes in the targeted different genes

Gene	Mutation (nucleotide change)	Amino acid change	Sample No. (n=100)	Frequency in population Sequenced (%)	References
23S rRNA	A2071T (2058)	Start690Stop	8		(Twin *et al.*, 2012, Tagg *et al.*, 2013)
	*A2072G (2059)	K690R	38 and 52		(Jensen *et al.*, 2008, Tagg *et al.*, 2013)
	A2072C	K690T	43		(Chrisment *et al.*, 2012)
	A2072T	K690I	84		(Chrisment *et al.*, 2012, Pond *et al.*, 2014, Le Roy *et al.*, 2016)
Total no. of mutations	**5**			7% (5/73)	
L4	T327C	L109L	1		(Read *et al.*, 2018)
	G429A	L143L	3		(Read *et al.*, 2018)
	C438T	L146L	8		(Read *et al.*, 2018)
	C438A	L146L	17		(Read *et al.*, 2018)
Total no. of mutations	**4**			4% (4/99)	
L22	*G81A	K27K	1 and 80		(Read *et al.*, 2018)
	C351T	L117L	11		(Read *et al.*, 2018)
Total no. of mutations	**3**			3% (3/91)	
gyrA	*G207A	M69I	50 and 51		(Muller *et al.*, 2019)
	G237A	A79A	2		N/A
	*C270T	H90H	84 and 99		(Tagg *et al.*, 2013)
	A353G	H118R	29		(Muller *et al.*, 2019)
Total no. of mutations	**6**			8% (6/80)	

Numbering is given according to *M. genitalium* G-37 genome accession number L43967.2

*Mutations that appeared twice. Parentheses show *Escherichia coli* numbering. Frequencies are from the total number of mutations per gene.

Table 4.6 Shows the different mutations detected for each targeted gene tested

There was no amino acid change in the ribosomal gene proteins L22 and L4. In the mutations detected for fluoroquinolone, *gyrA* gene three led to an amino acid change of M69I and H118R, while three had no amino acid change.

Table 4.7: Prevalence of mutations detected in macrolides and fluoroquinolone resistance associated genes

	Gene	Mutations prevalence
Macrolides	23S rRNA	7% (5/73) x100
	L4	4% (4/99) x100
	L22	3% (3/91) x100
Fluoroquinolone	*gyrA*	8% (6/80) x100

Table 4.6: Representation of the different mutation prevalence detected in each gene

The total number of each gene was taken from table 4 above. The prevalence of macrolide mutations are as follows: 23S rRNA was 7%, L4 was 4%, and L22 was 3%. Fluoroquinolone mutational prevalence for *gyrA* gene was 8%.

CHAPTER 5

DISCUSSION

Mycoplasma genitalium has been shown to be the causative agent of various genital tract infections in both men and women. Therefore, controlling infections that are caused by this pathogen is a matter of concern globally due to the emergence of resistant strains. *Mycoplasma genitalium* is mostly treated using the syndromic approach by most sexually transmitted infections (STI) management treatment guidelines, especially in developing countries, where infections are treated based on signs and symptoms, without laboratory diagnosis. However, in previous and current studies, reports of an increase in treatment failure to both the first and second-line treatment regimens are being detected, and the numbers vary across the globe (Murray *et al.*, 2017; Read *et al.*, 2019a). This was part of a larger study Centre for the AIDS Programme of Research in South Africa (CAPRISA) HIV Incidence Provincial Surveillance System (HIPPS) and the samples were chosen based on similar ages. The age range was 15 to 25 years and 100 samples were chosen for the study. All 100 samples were confirmed genotypically to be *M. genitalium* positive using TaqMan real time PCR. Amplification on all genes were attempted for 23S rRNA, L22, L4 and *gryA.* Seventy-three samples for 23S rRNA, 91 for L22, 99 for L4 and 80 for *gryA* amplified. Sanger sequencing was performed on amplified PCR products.

In South Africa, there is limited *M. genitalium* resistance prevalence data. The presence of high levels of *M. genitalium* resistance in the different population groups (women, pregnant women, homosexual men (MSM), and heterosexual men) can increase the transmission of the resistant strains in the population (Murray *et al.*, 2017; Read *et al.*, 2019b). A study conducted by Trevis *et al* 2018 showed that the prevalence of resistance differs in different geographical areas (Braam *et al.*, 2017; Trevis *et al.*, 2018). In this study, five samples (5/73) had mutations in the 23S rRNA gene which is associated with macrolide resistance. Mutations detected were A2071T (start690stop), A2072C (K690T), A2072T (K690I), and two harbored A2072G (K690R). These positions are known to be the hot sport for macrolide resistance and are associated with macrolide 1 g azithromycin treatment failure (Ito *et al.*, 2011). It could be speculated that a change in the amino acid can result in a phenotypic change, and are linked with azithromycin resistance.

The A2072G (*M. genitalium* numbering) mutations are associated with high levels of resistance which was previously reported by Jensen *et al*, 2008 and Le Roux *et al*, 2018 (Jensen *et al.*, 2008; Le Roux *et al.*, 2018). This mutation has mostly been observed in Australia, and some parts of the world (Tagg *et al.*, 2013; Le Roux *et al.*, 2018). In this study we found adenine to thymine (A to T) nucleotide change while in a study carried in Limpopo, South Africa a change of adenine to guanine (A to G) was observed at the same position 2071 (Hay *et al.*, 2015).

Other studies from the Netherlands also showed similar mutations in their population at A2071T *M. genitalium* numbering, (Nijhuis *et al.*, 2015; Braam *et al.*, 2017). Braam and colleagues associated transition at this position with a clonal spread as this mutation and is considered to be rare in other countries as it was only seen to spread in different parts of the Netherlands (Braam *et al.*, 2017). Braam and colleagues suggested that this is not a single outbreak (Braam *et al.*, 2017). The A2058T mutation position is considered the third most frequently detected mutation for macrolide resistance (Kristiansen *et al.*, 2016) . One sample had a mutation at position A2072C, *M. genitalium* numbering which is similar to other studies (Chrisment *et al.*, 2012; Braam *et al.*, 2018, Ke *et al.*, 2020). Similar results were obtained in Australia, Britain, and China at the same position A2072C, *M. genitalium* numbering (Pond *et al.*, 2014; Ke *et al.*, 2020; Pitt *et al.*, 2020).

Diner and Hayes in 2009, showed point mutations in the ribosomal proteins L4 and L22 to lead to missense mutations, deletions, and insertions (Diner and Hayes, 2009). Results obtained in this study did not lead to amino acid changes (see table 4.6), however, previous studies conducted in Japan demonstrated, missense mutations on L4 and L22 in a group of the participants with treatment failure (Ito *et al.*, 2011; Shimada *et al.*, 2011). Synonymous mutations detected in our study are similar to results obtained by Read *et al* (Read *et al.*, 2018). In this study, the silent mutations that were found were mostly leucine amino acids.

Mutations associated with macrolide resistance in the different studies brings about concern of the effectiveness of the current drug regimens that are used in treating and managing STIs in South Africa. A single dose of 1 g azithromycin is the recommended regimen to be used in treating sexually transmitted infections (Department of Health, 2015). This was incorporated into syndromic STI management guidelines of South Africa in 2015. This was after the poor eradication of STIs in the drugs that were previously used due to the emerged development of resistance (Mahlangu *et al.*, 2019). Studies that evaluated the syndromic management approach showed that it is becoming less effective in treating infections as it relies upon symptoms that are being presented which can also increase resistance against *M. genitalium* infections (Horner *et al.*, 2014).

Results obtained in this study and previously conducted studies in South Africa are indicating the establishment of diverse resistant-associated mutations of *M. genitalium* to either macrolides or fluoroquinolones regiments although in low prevalence (Hay *et al.*, 2015; Le Roux *et al.*, 2018; Maduna *et al.*, 2019; Laumen *et al.*, 2020). This study's prevalence of macrolide-resistant was 7% for 23S rRNA, 4% for L4, 3% for L22. Fluoroquinolone, *gryA* gene prevalence was 8%. Hayes and colleagues showed

similar results in a study conducted in Limpopo (Hay *et al.*, 2015). In 2018, 12.5% (1/8) of 23S rRNA mutation was observed in a female population attending termination of pregnancy (TOP), Pretoria, South Africa (Le Roux *et al.*, 2018). Macrolide (23S rRNA) mutations observed in this study showed different mutations compared to other studies, demonstrating different mutations circulating in our population group. In comparison, with studies that have been conducted in South Africa observed only a single change of nucleotide base (adenine to guanine) at either position 2058 or 2059 (Hay *et al.*, 2015; Le Roux *et al.*, 2018). This could be caused by the sexual behavior of the selected group (young women), presence of other STIs in the population which could have led the individuals to be exposed to antibiotics. (Wand *et al.*, 2020). In this study there was no association with the clinical and behavioral data.

Razin and colleagues showed that mycoplasmas have a high mutational rate, which can generate variable mutants at an increased rate (Razin *et al.*, 1998; Twin *et al.*, 2012). Suggesting, prolonged usage of azithromycin may increase the possibility of spreading *M. genitalium* resistant infections in primary health care facilities (PHC) of South Africa in the near future (Laumen *et al.*, 2020).

In 2019, in a case report study conducted in Pretoria, South Africa, showed *M. genitalium* to be multi-drug resistant after a man failed 1 g azithromycin and 250 mg ceftriaxone with an extended treatment of 1 g ceftriaxone and 2 g azithromycin (Maduna *et al.*, 2019). Molecular methods were used for testing, and A2071G, 23S rRNA mutation combined with C234T (Pro-62→Ser) *parC* mutations were detected (Maduna *et al.*, 2019). This suggested that *M. genitalium* is more likely to escape the syndromic treatment approach as the test of cure is not recommended after first-line treatment unless infection symptoms persist. Testing STI infections after first-line treatment failure can be adopted to decrease generating more resistant strains (Maduna *et al.*, 2019). The new Australian and UK guidelines can be adopted, which treats for seven days with 100 mg doxycycline and azithromycin afterwards to prevent selective pressure from occurring so adequate treatment can be provided in primary health care (Read *et al.*, 2019a; Soni *et al.*, 2019; Sweeney *et al.*, 2019; De Baetselier *et al.*, 2020).

Fluoroquinolone, a moxifloxacin antibiotic, is a second-line drug that is usually recommended to be used by most STI guidelines across the globe in treating *M. genitalium* infections that have macrolide (azithromycin) failure. These antibiotics work through the inhibition of DNA synthesis in the *parC* gene (topoisomerase IV region) and *gyrA* (DNA gyrase A subunit) (Bebear *et al.*, 1999; Ke *et al.*, 2020).

Mutations detected at positions G207A and C207T were detected in two participants. The C207T alteration mutations were previously reported in two retrospective studies conducted in Sydney, Australia, and in Sendai, Japan, in a single sample where the prevalence of macrolide and fluoroquinolone was investigated in clinical specimens (Tagg *et al.*, 2013; Kikuchi *et al.*, 2014). Two samples harboured the G207A mutation, which led to amino acid change (M69I), however, this mutation has not been linked with clinical significance of fluoroquinolones resistance (Muller *et al.*, 2019; De Baetselier *et al.*, 2020). These mutations have been reported by Muller et al, 2019 in South Africa.

An alteration at position A353G (H118R) has previously been reported in South Africa, although it was not linked with fluoroquinolone resistance (Muller *et al.*, 2019). In a recent study from Belgium De Baetselier and colleagues associated *gyrA* gene mutations with low fluoroquinolone resistance (De Baetselier *et al.*, 2020). In the three *gyrA* gene mutations detected none led to an amino acid change (refer table 4.6 above). The most common position G285C (methionine – isoleucine) which is associated with triggering fluoroquinolone resistance was not detected in our study (Tagg *et al.*, 2013).

CONCLUSION

In conclusion, there is low macrolide and fluoroquinolone mutations prevalence that are circulating in KwaZulu-Natal (KZN) province from the tested female population in 2014 to 2015. Macrolide and fluoroquinolone mutations detected are similar to the other studies previously conducted in South Africa and in other part of the world. Although a resistance to macrolides and fluoroquinolones is low it is worrisome, for the future effectiveness of the current primary drug of choice azithromycin. There is a need for surveillance of mutation targets to more current time point population in KZN.

STUDY LIMITATIONS

The study samples were collected in one city (Pietermaritzburg), although in a rural and semi-urban area. These results limit usage in the general population as a smaller sample number was investigated for macrolide and fluoroquinolone resistance. This was because of financial constraints and a larger study need to be performed. The samples used were self-collected vulvovaginal swabs, which could have resulted in a low DNA amount. These samples yielded high cycling threshold (CT) values on the TaqMan PCR. Sequencing for the targeted genes failed in samples with low concentration of DNA Sample were stored for the time of collection which may have led to low DNA concentrations due to long term storage. The behavioral, sociodemographic, and clinical data of the study was entirely reliant on the information given by the participant, which could have created some biasness. There was no

association with the behavioral characteristics. New PCR technologies should be adopted for faster screening of mutations such as the MG-Flex assay and SpeeDx ResistancePlus MG test, which test and detect the most common mutations for macrolides resistance (A2058T, A2058C, A2058T, A2059C, A2058G, and A2059G). This was not performed in the study as we used Sanger sequencing.

CHAPTER 6

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APPENDIX

APPENDICES

APPENDIX A: Experimental reagents and procedures

1. TBE buffer used for the preparation of all gel electrophoresis used

A concentration of 1X buffer was used for the preparation of all gel electrophoresis used for the detection of amplicons.

2. TBE buffer preparation

108 g Tris, 55 g Boric acid, and 9.3 g EDTA were dissolved in 800 ml of distilled water, and the volume was adjusted to 1000 ml. The buffer was diluted to 1X by adding 100 ml of the 10X TBE buffer into 900 ml of distilled water.

3. Agarose gel electrophoresis preparation

All amplified amplicons were analysed on 1.5% agarose gel in the following manner. The open ends of the casting tray were sealed with masking tape, and a comb was inserted towards the end of the casting tray. A 1.5% agarose gel was prepared by dissolving three agarose tablets 0.5 g (ThermoFisher Scientific, USA) in a conical flask with 100 ml of the 1x TBE buffer. The mixture was dissolved by heating and letting cool, poured into the casting tray, and allowed to solidify (approximately 25-30 minutes). The comb was removed, and the casting tray containing the gel was placed in an electrophoresis tank. Approximately 800 ml of 1x TBE buffer was added into the tank, such that the gel is completely covered.

4. Agarose gel electrophoresis for the detection of all the targeted sizes for each gene (MgPa, 23S rRNA, L4, L22, and *gryA*)

On one end of the gel, a 50 bp gene ruler marker (ThermoFisher Scientific, USA) was added to the well and was used for band size estimation. This was carried out by mixing 2 µL of 6X DNA loading dye (ThermoFisher Scientific, USA) containing 20 µL of gel red added (GelRed TM Nucleic acid, Sigma-Aldrich, Germany) with 1 µL of the molecular weight marker.

The prepared 1.5% agarose gel (same as above) was loaded with 20 µL of each sample which was mixed with 4 µL of the loading dye on a parafilm (ThermoFisher Scientific, USA). The mixture was loaded/ added to each well. The gel was run for approximately 45- 60 minutes at 100 volts. The gel was transferred to the gel dock for visualization (SynGene, G-Box, sydr4/1153) and photographed under UV light. The results were viewed on a computer. The desired band sizes were excised on the UV transilluminator machine (SynGene, sygv/4604g) and transferred into a 1.5 ml microcentrifuge tube (Sigma-Aldrich, Germany) for purification. The same procedure was followed for all genes.

APPENDIX B (Multiple sequence alignments for each gene)

1A. Macrolide 23S rRNA gene sequence alignments

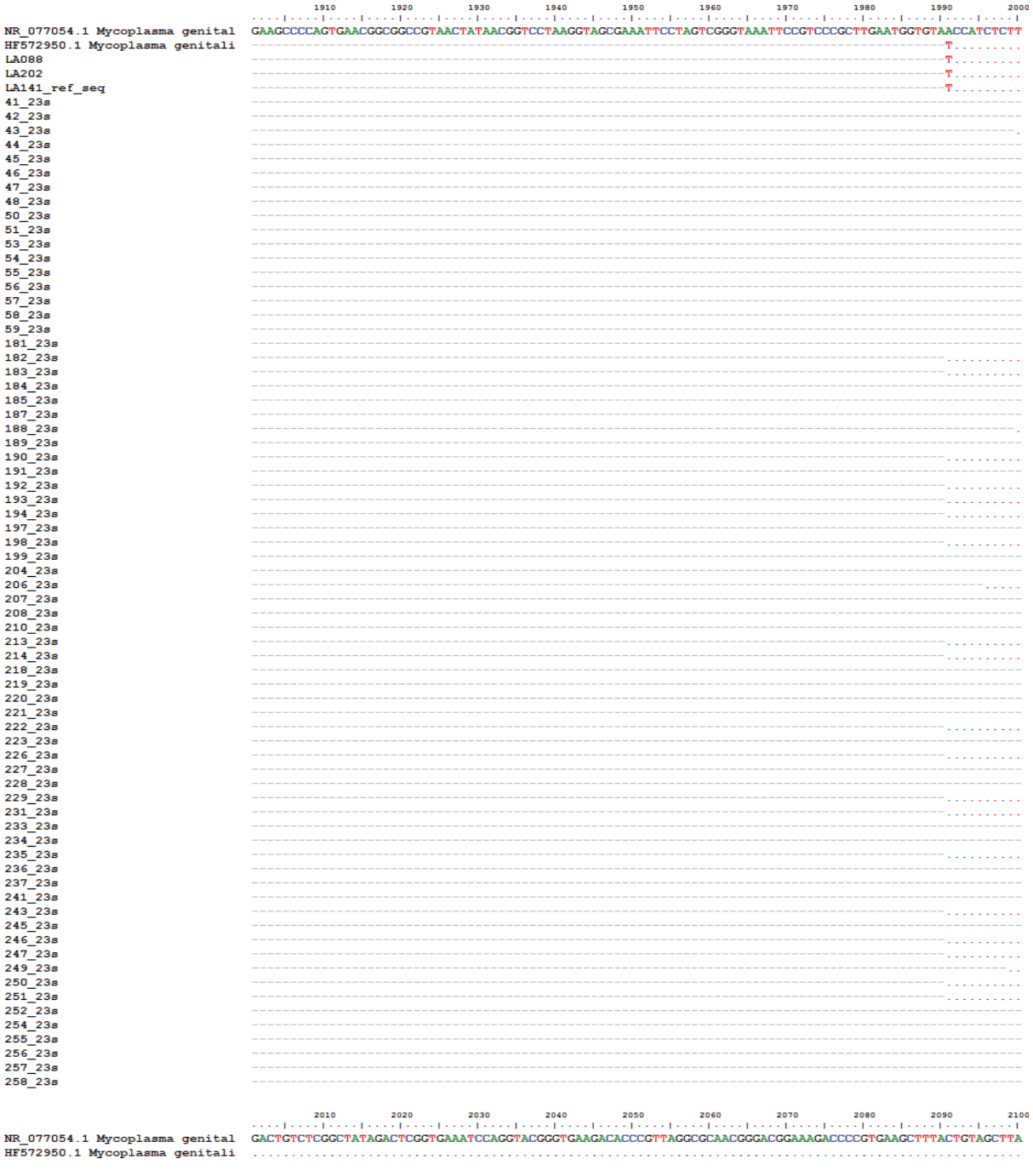

Figure 1A: Representation of 23S rRNA sequences in comparison with the wild type reference and sequences of known mutations

1B. Macrolide 23S rRNA gene sequence alignments

Figure 1B: Sequences of 23S rRNA showing the presence of mutations in the different samples

1C. Macrolide 23S rRNA gene sequence alignments

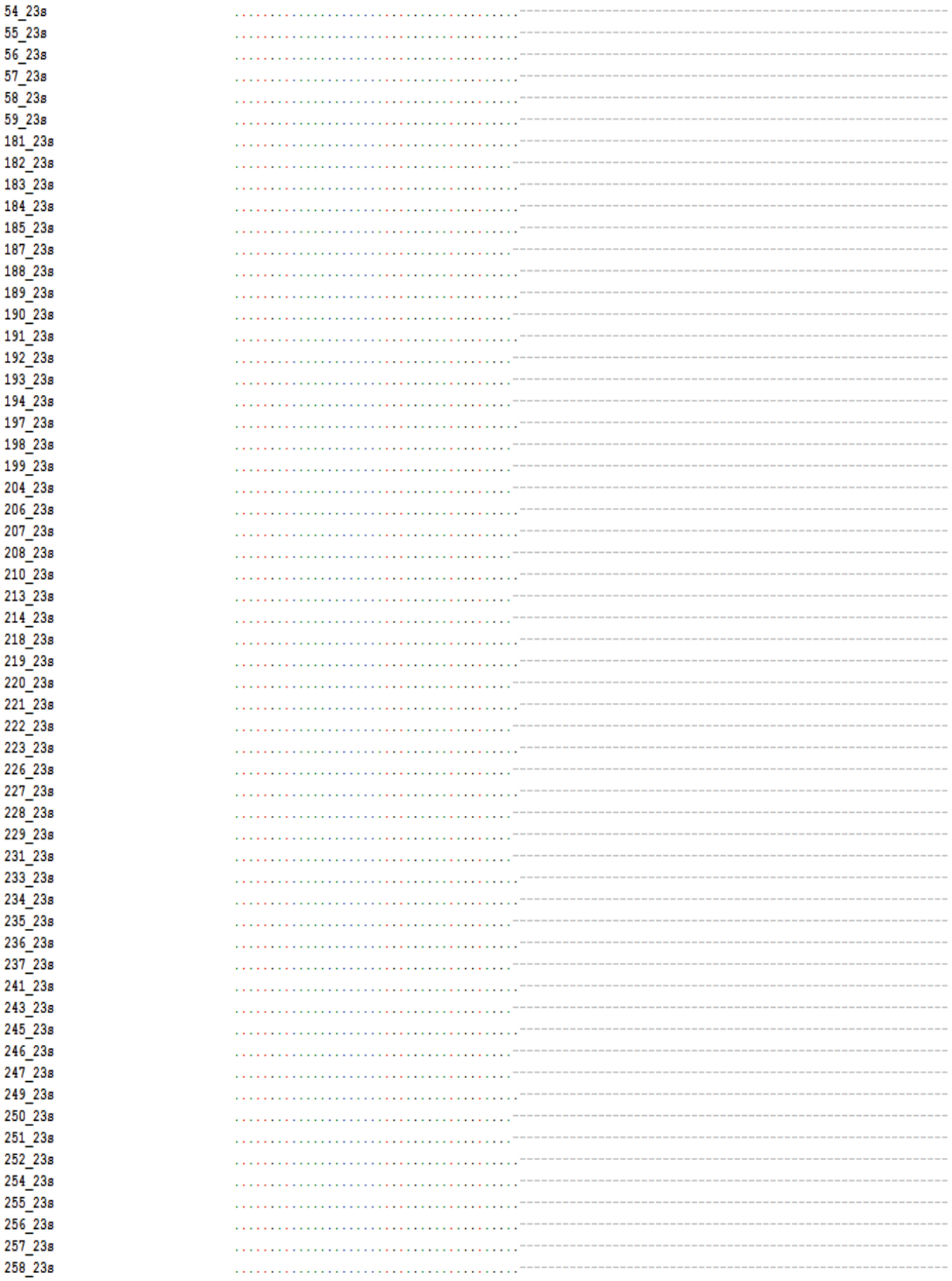

Figure 1C: Sequence alignments of 23S rRNA gene after DNA sequencing

2A. Multiple sequence alignments of L4 gene

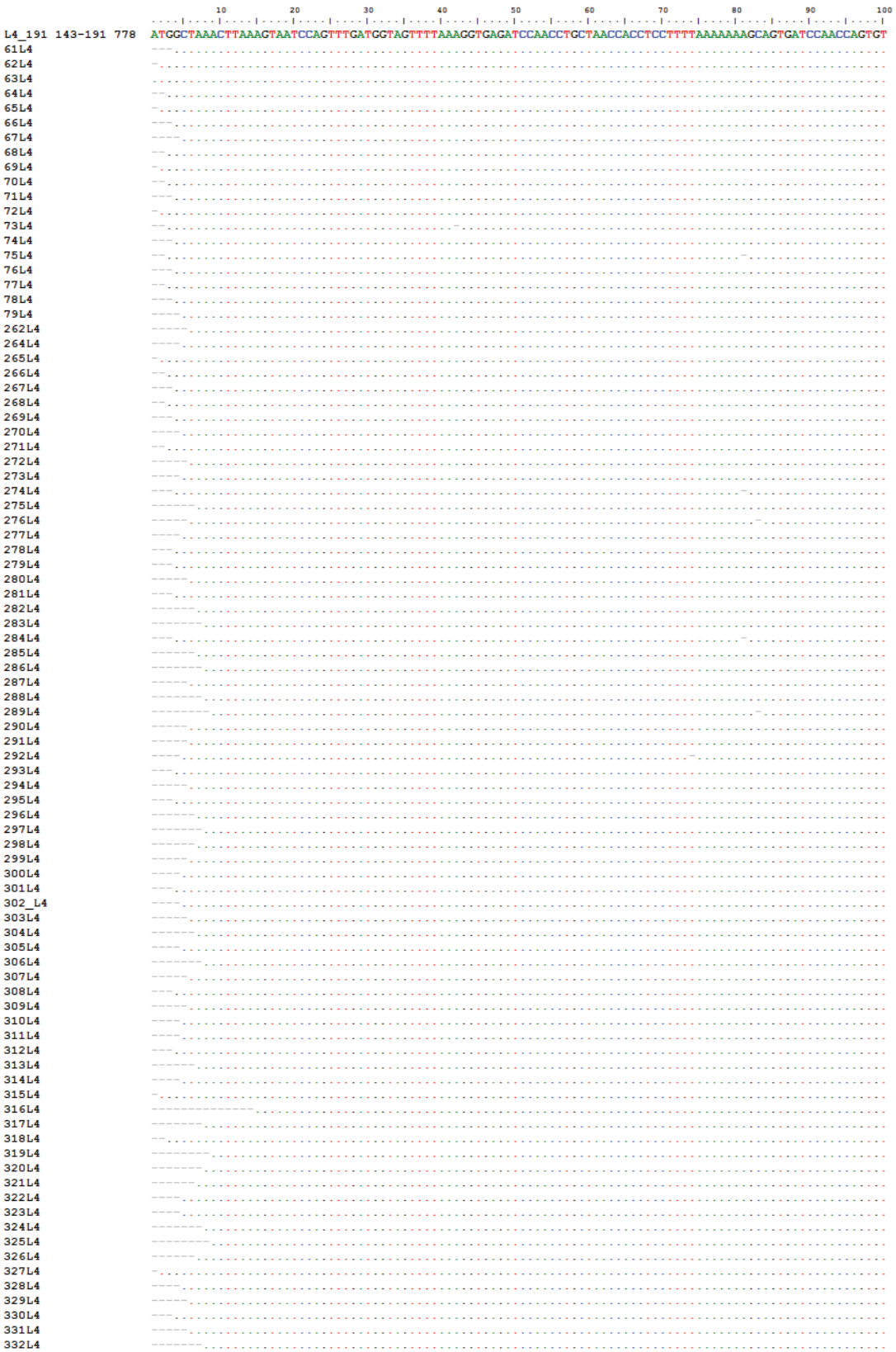

Figure 2A: Multiple sequence alignments of L4 gene protein after sequencing

2B. Multiple sequence alignments of L4 gene

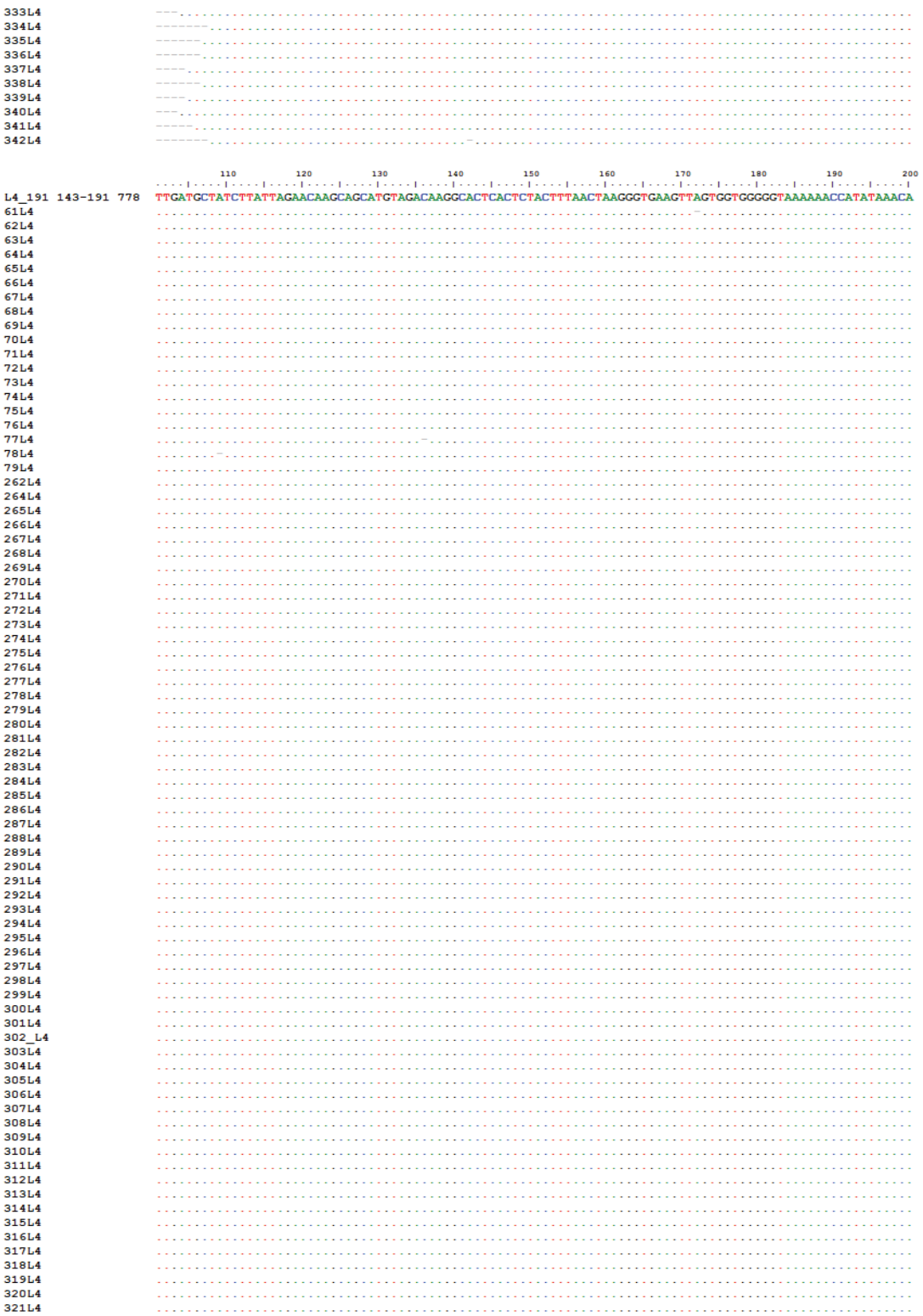

Figure 2B: Shows the alignments of L4 gene after sequencing of the PCR amplicons

2C. Multiple sequence alignments of L4 gene

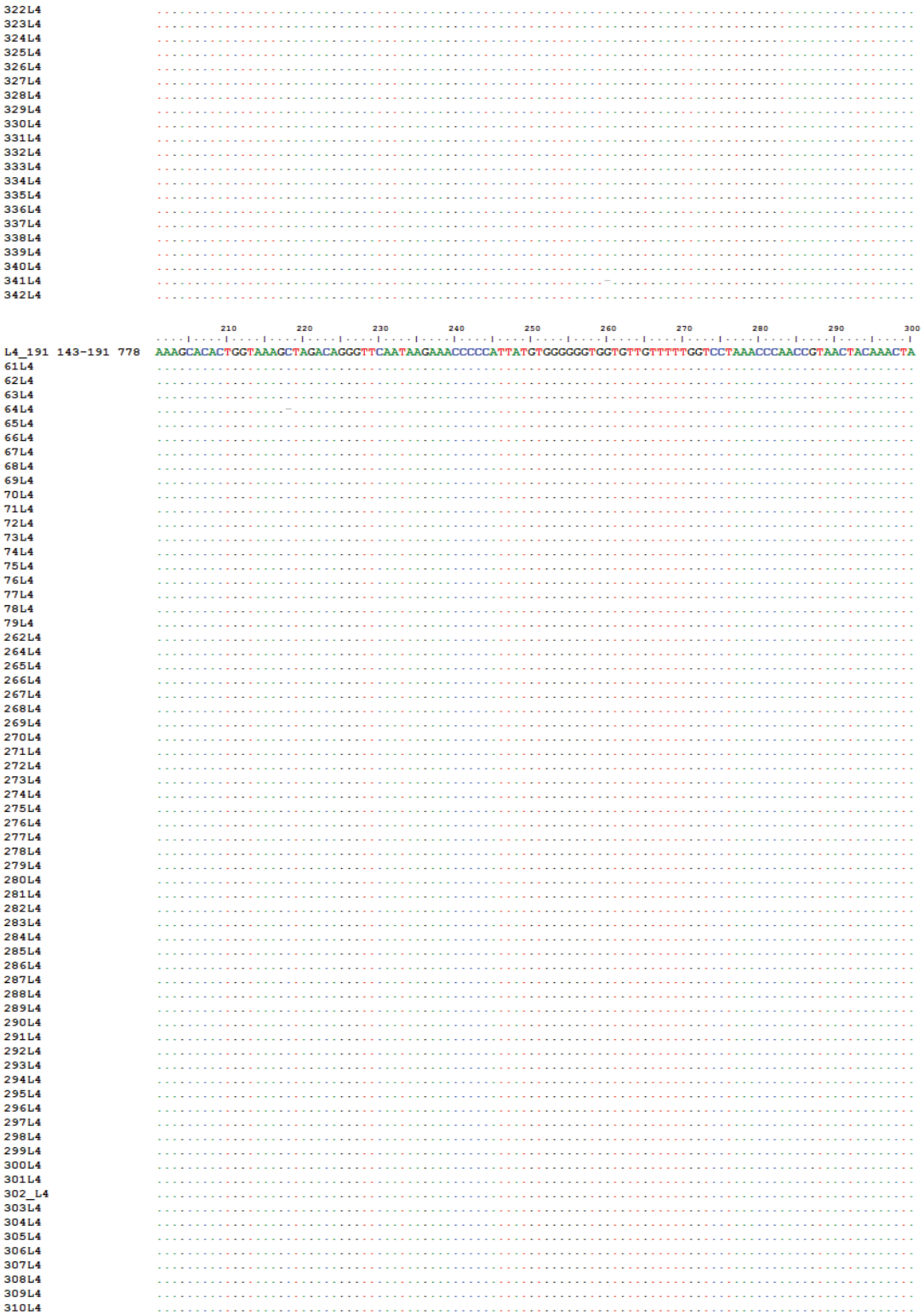

Figure 2C: L4 protein gene multiple sequence alignments after DNA sequencing

2D. Multiple sequence alignments of L4 gene

Figure 2D: L4 protein gene multiple sequence alignments in comparison with the wild type

2E. Multiple sequence alignments of L4 gene

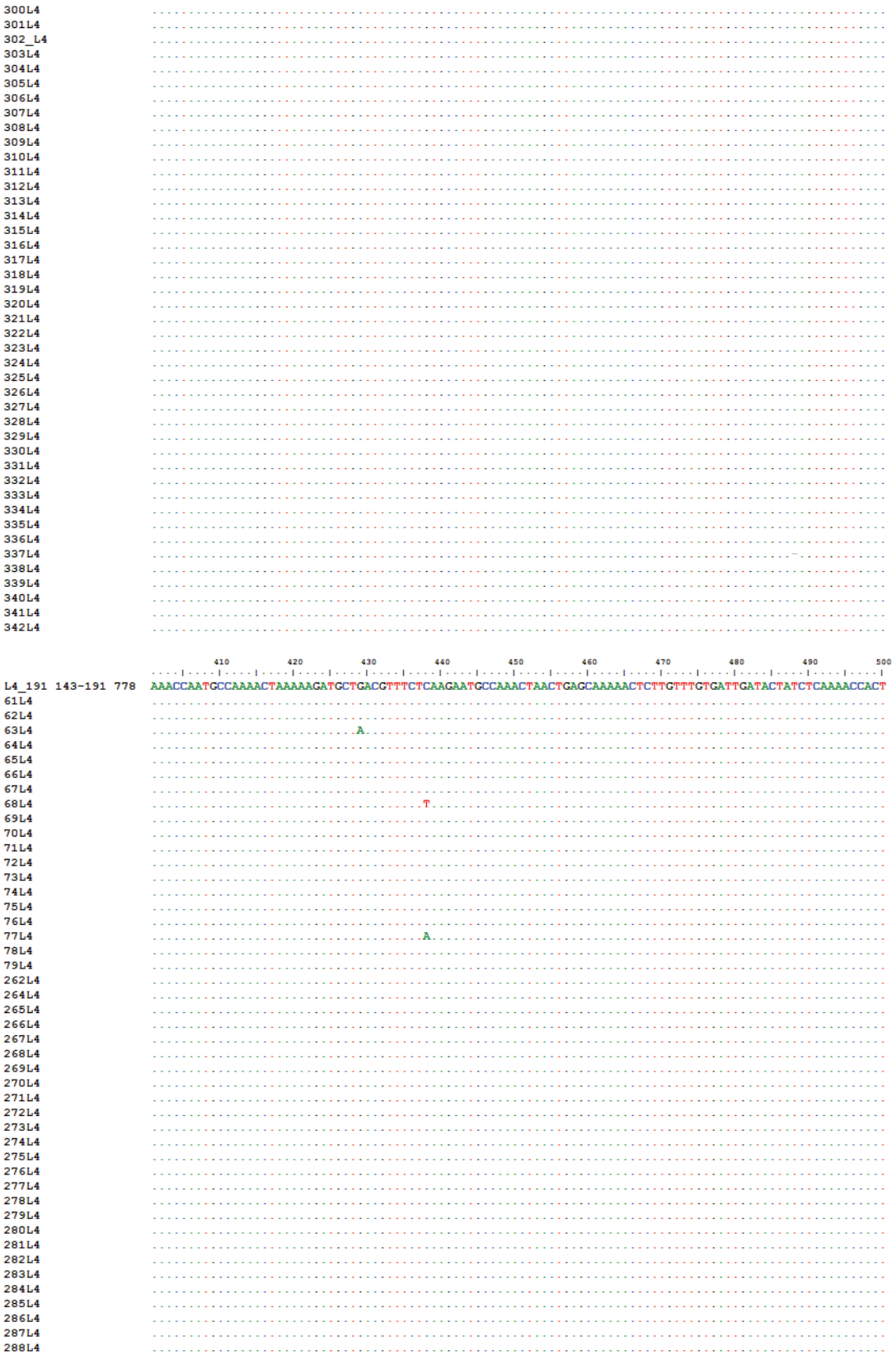

Figure 2E: L4 protein gene sequences with the detected mutations

2F. L4 protein gene sequences with the detected mutations

Figure 2F: L4 protein gene multiple sequences compared with the wild type reference strain

2G. L4 protein gene sequences with the detected mutations

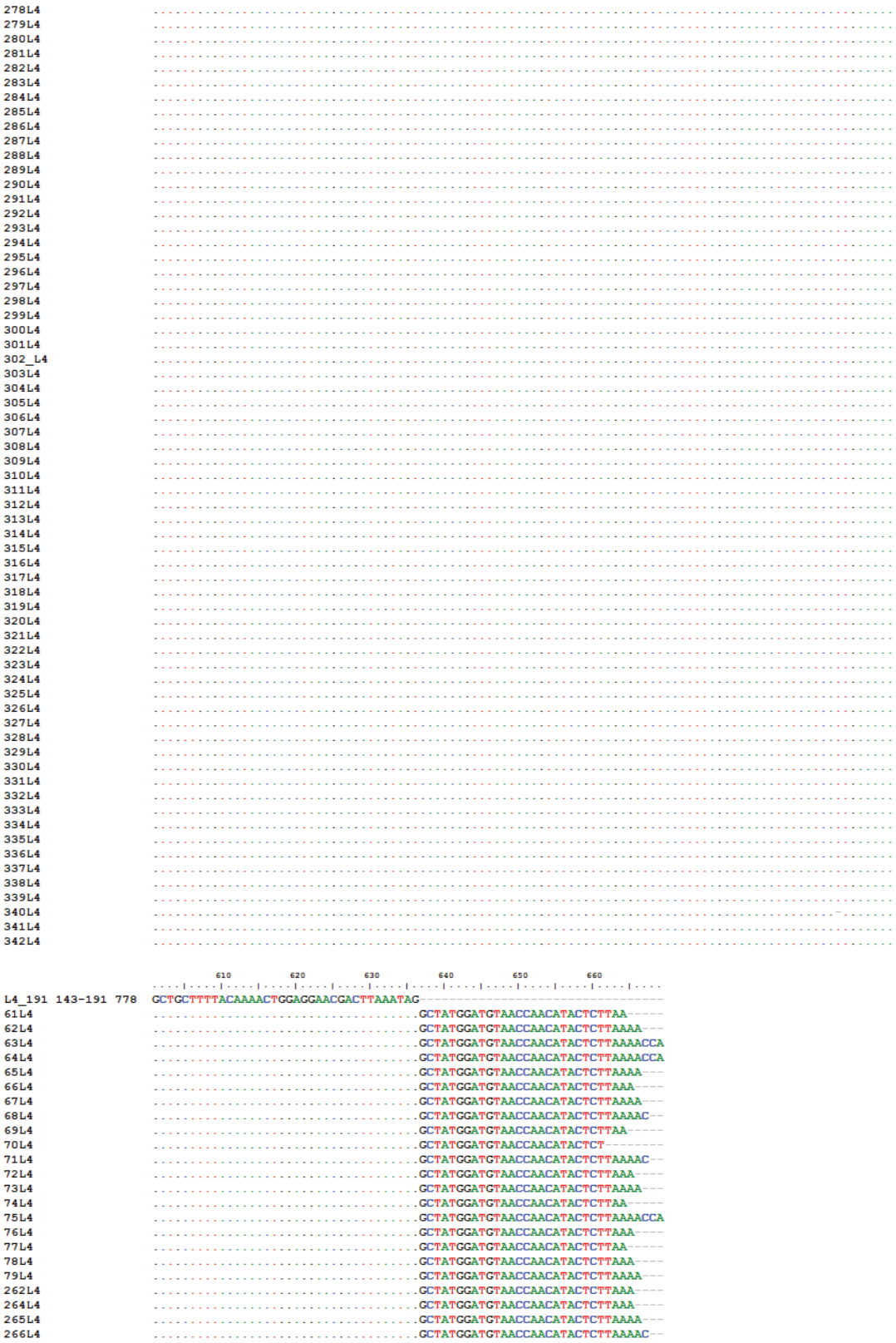

Figure 2G: L4 protein gene in comparison with the reference strain

Figure 2H. L4 protein gene sequences with the detected mutations

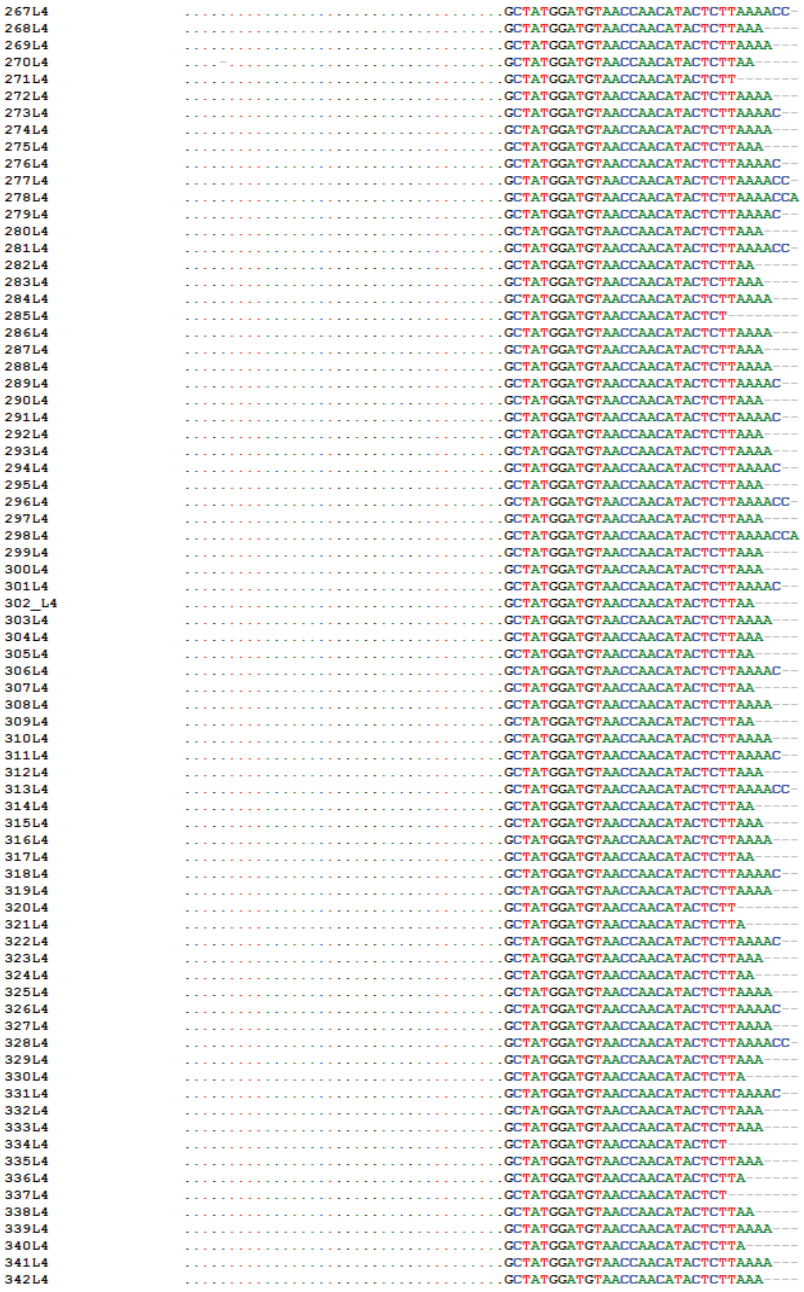

Figure 2H: L4 protein gene multiple sequence alignments in comparison with the reference strain

3. Multiple Sequence Alignments: *gryA* gene

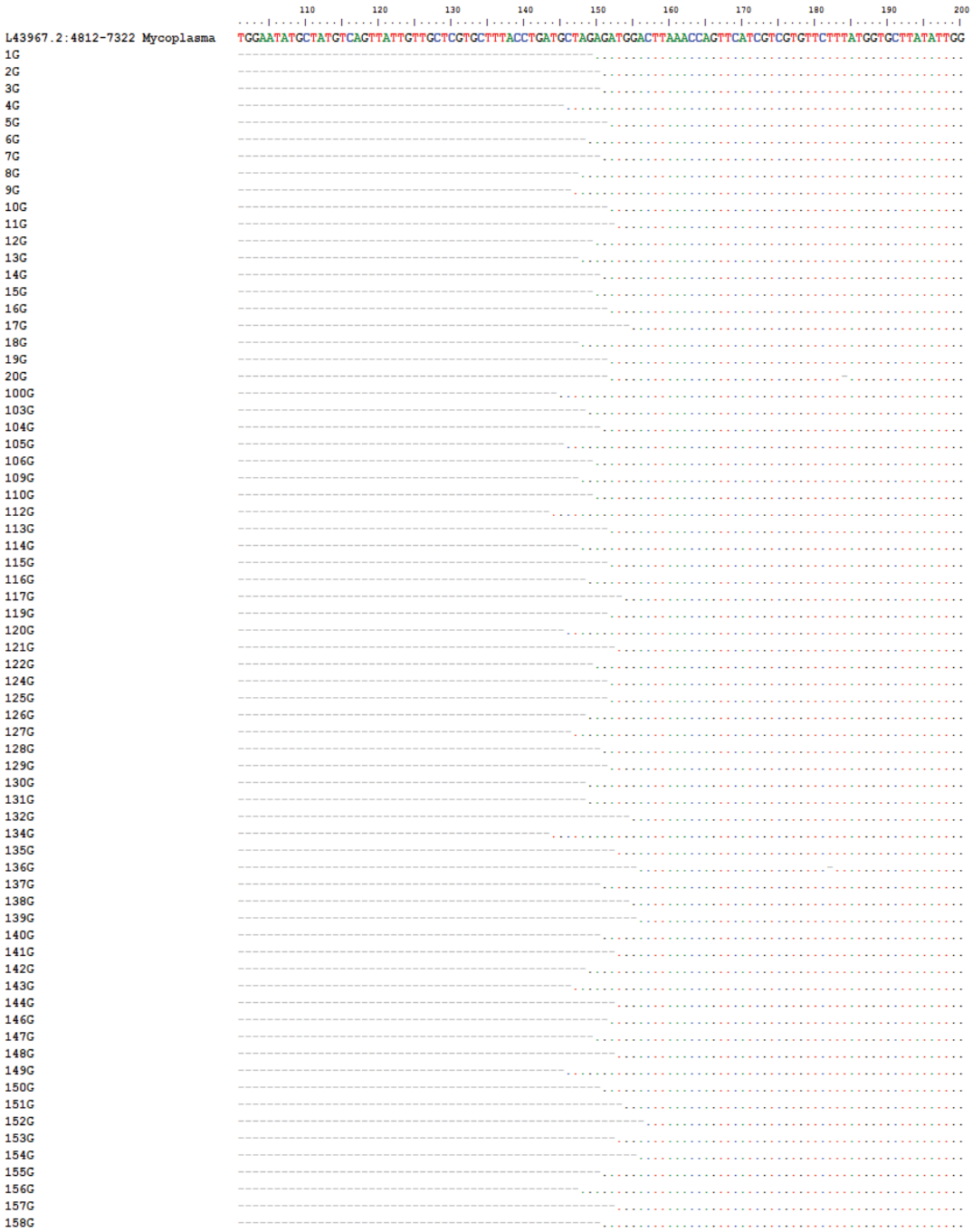

Figure 3A: The *gyrA* gene multiple sequence alignments for the identification of mutations

3B. Multiple Sequence Alignments: *gryA* gene

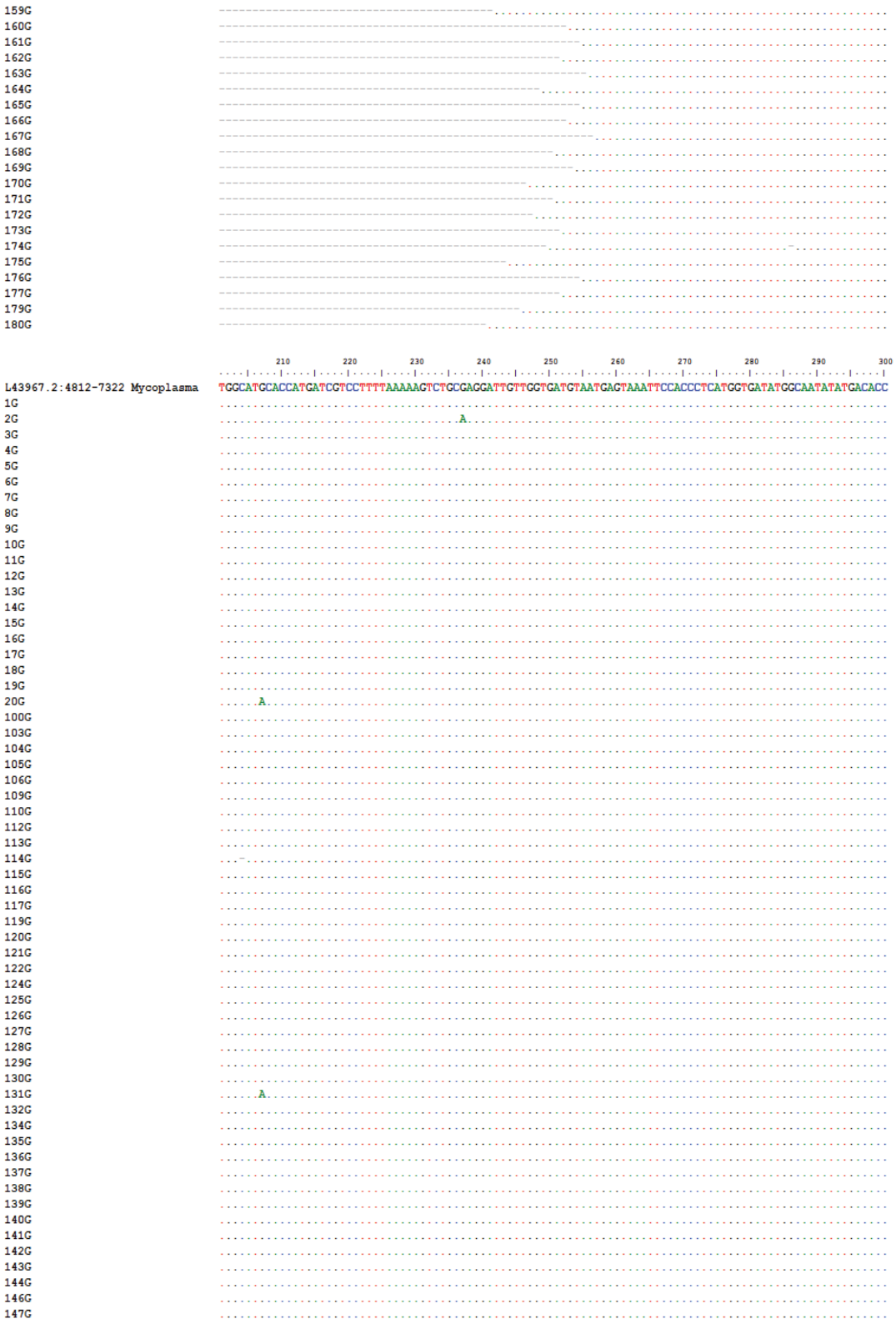

Figure 3B: Multiple sequence alignments of *gryA* gene in comparison with the wild type refence strain

3C. Multiple Sequence Alignments: *gryA* gene

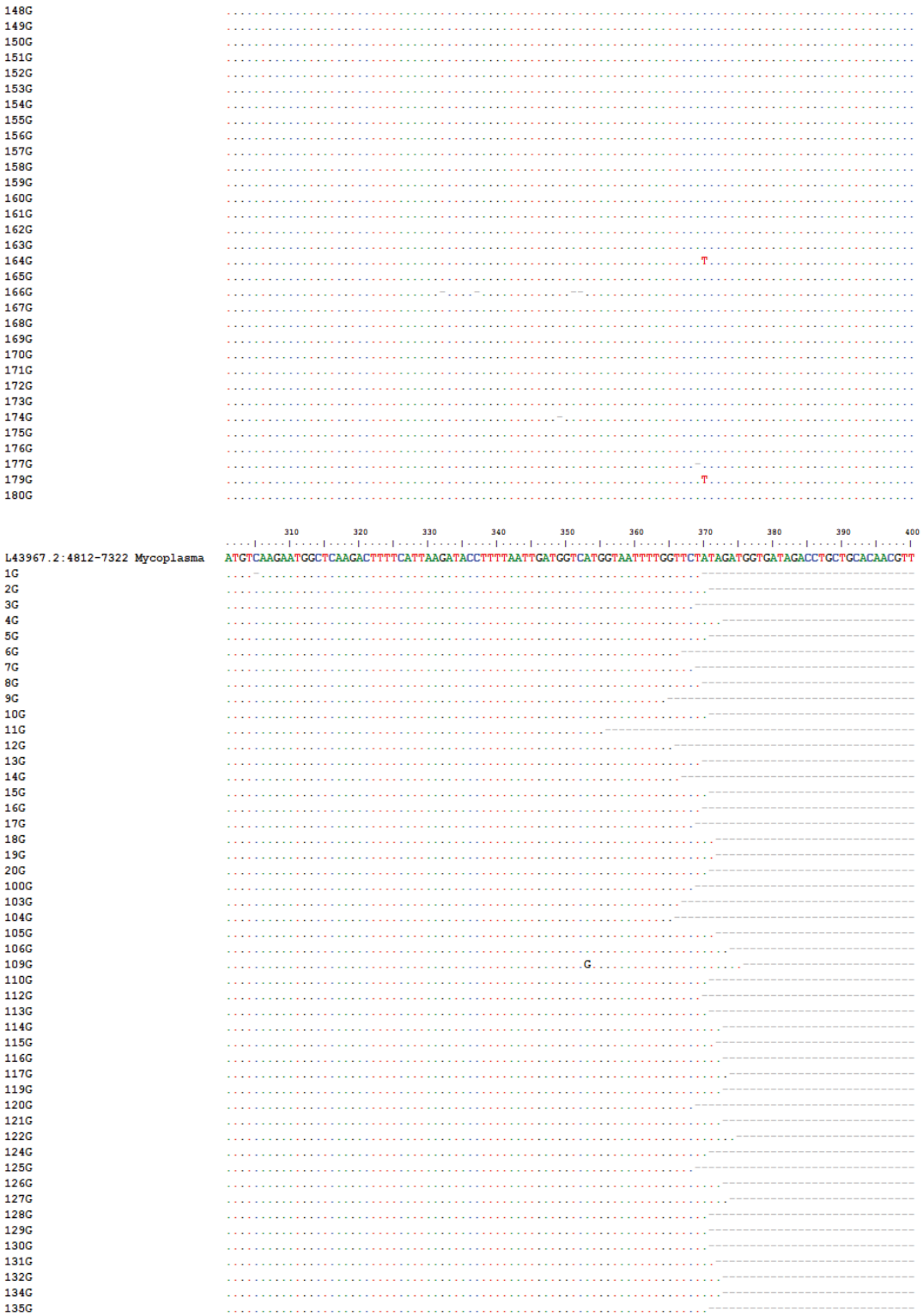

Figure 3C: The *gryA* gene multiple sequence alignments showing mutations at different positions

3D: Multiple Sequence Alignments: *gryA* gene

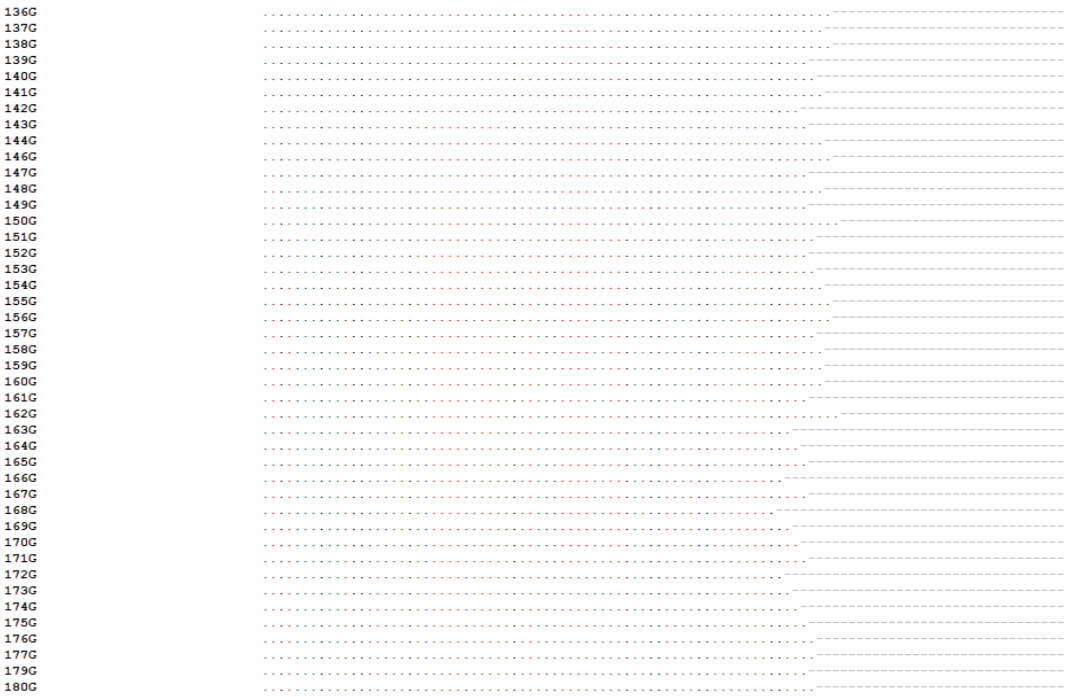

Figure 3D: Representation of mutations detected in *gryA* gene when compared with the wild type reference strain

4. Sequences alignments of L22 gene alignments

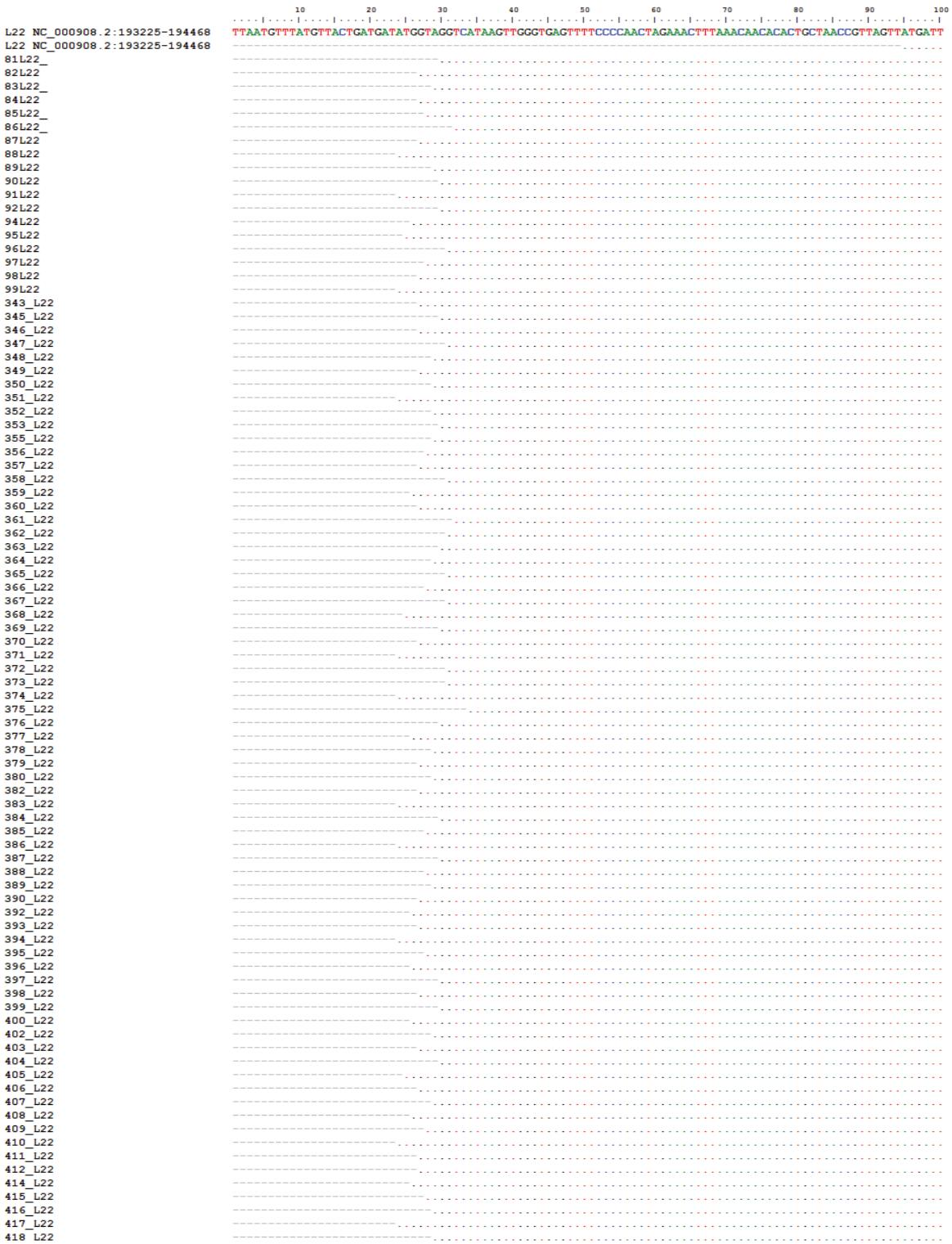

Figure 4A: Multiple sequence alignments of L22 gene from the successful sequences

4B. Sequences alignments of L22 gene alignments

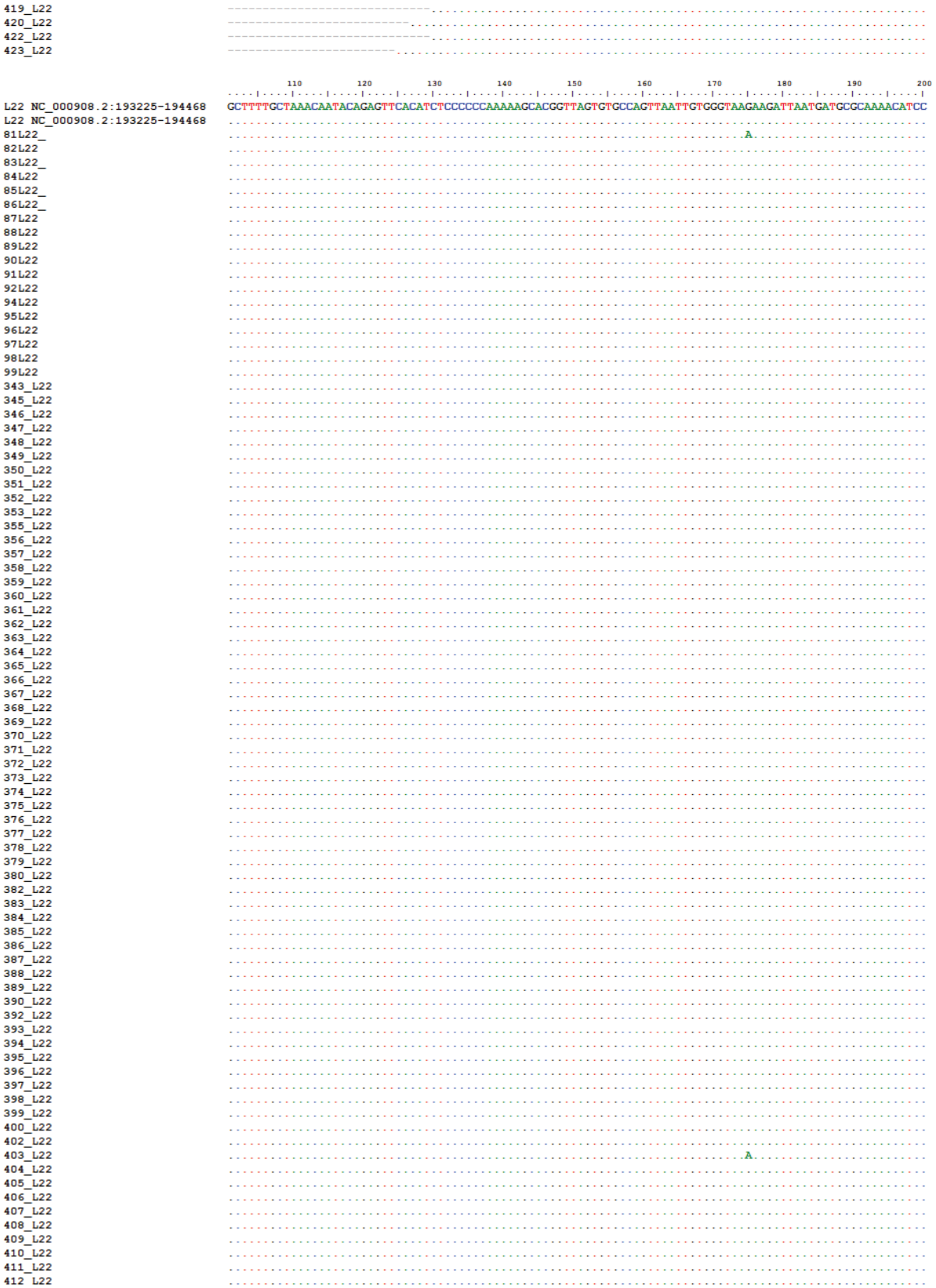

Figure 4B: Representation of the mutations detected for L22 protein gene

4C. Sequences alignments of L22 gene

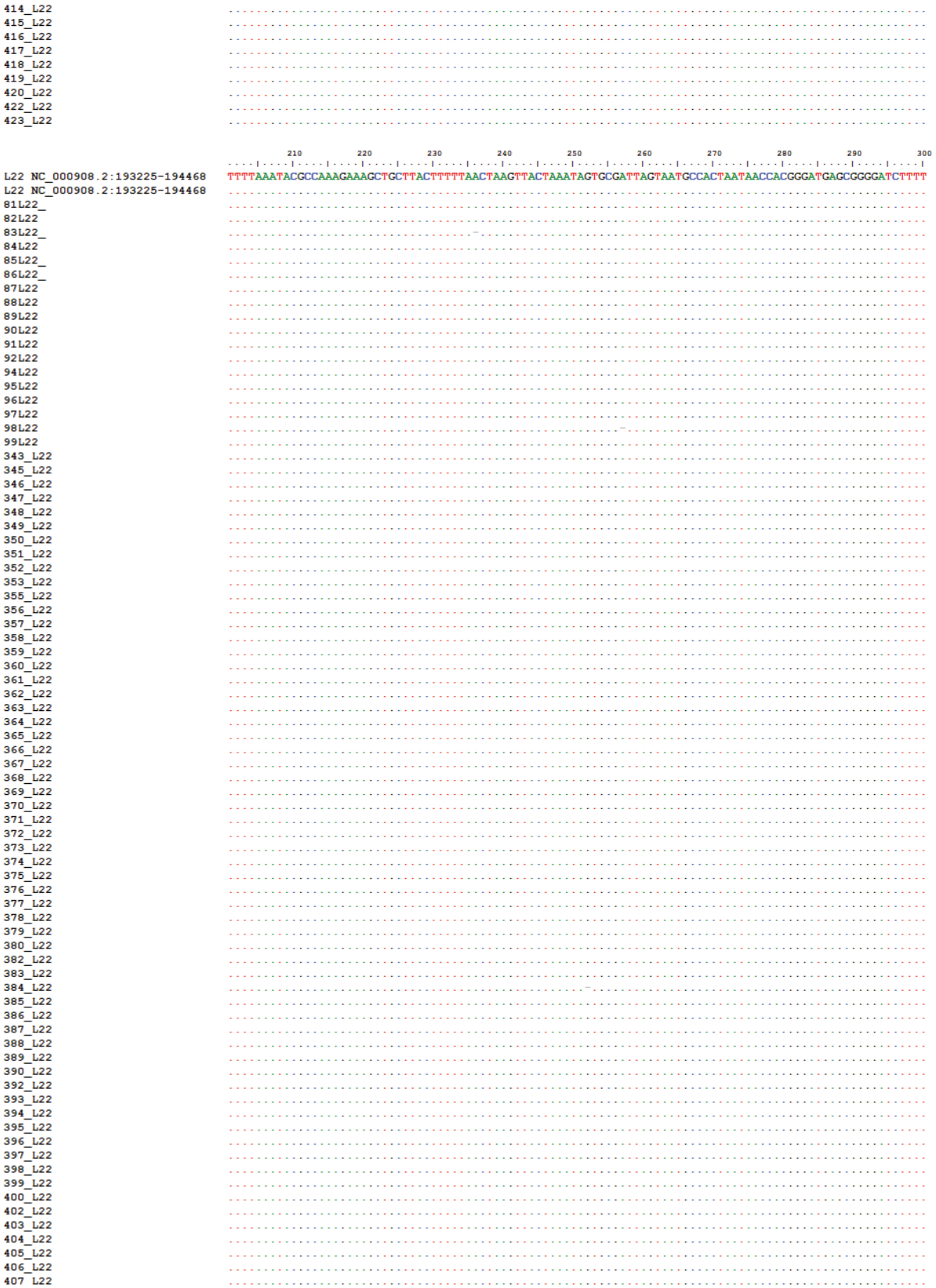

Figure 4C: Multiple sequence alignments of L22 protein gene when compared with the reference strain

4D. Sequences alignments of L22 gene alignments

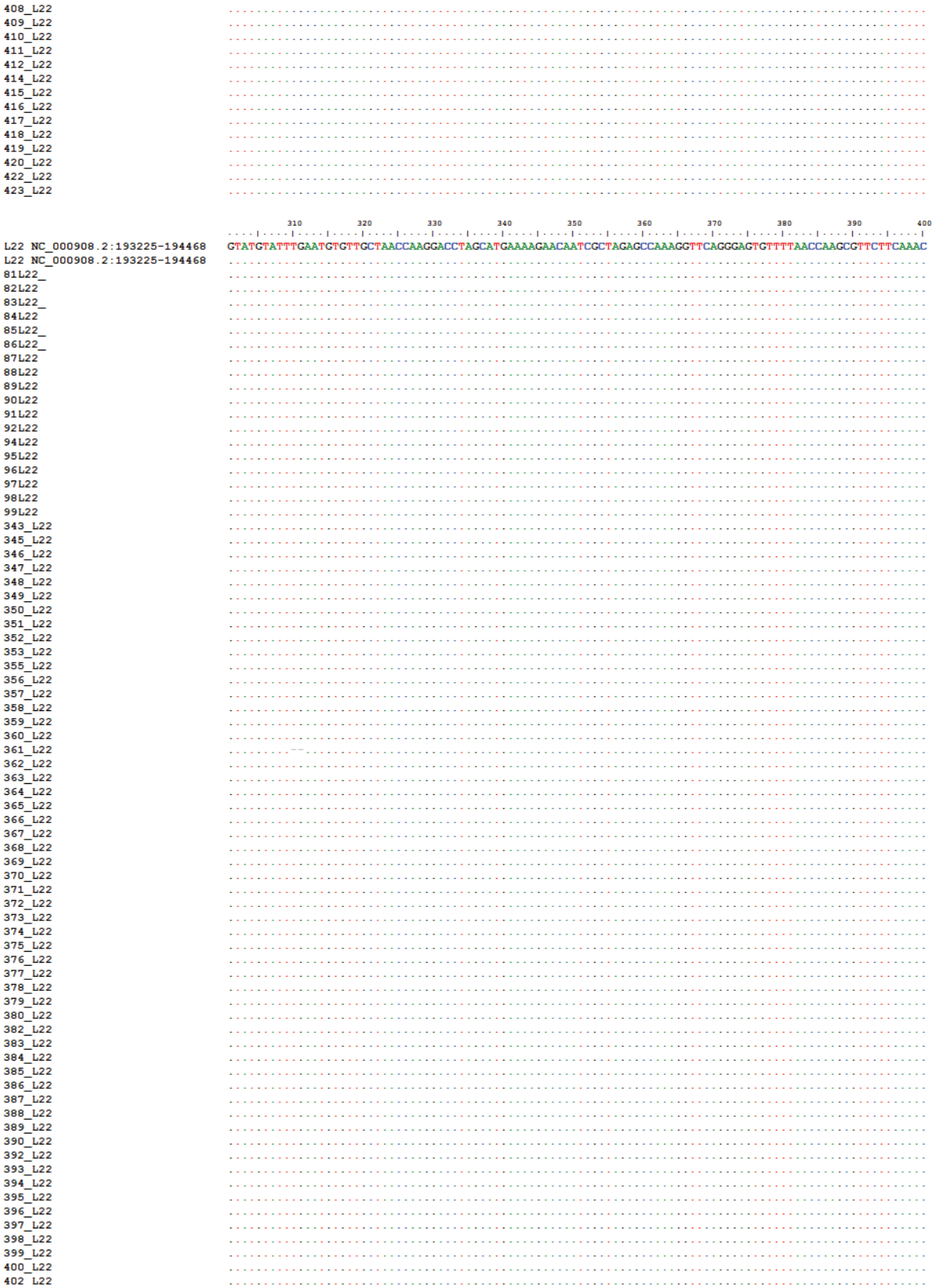

Figure 4D: Multiple sequence alignments of L22 protein gene compared with protein gene

4E. Sequences alignments of L22 gene alignments

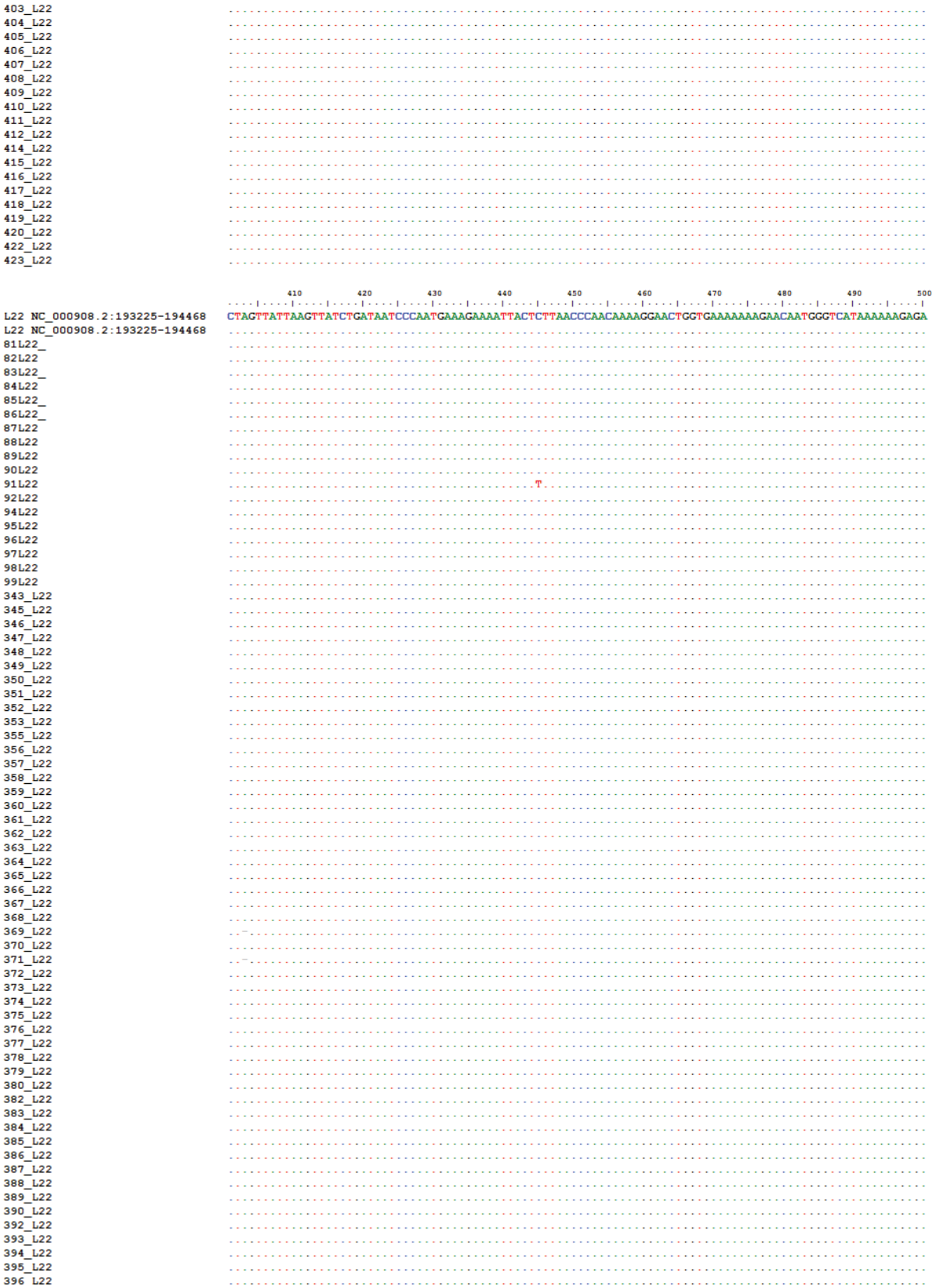

Figure 4E: L22 gene sequences compared with the wild type reference strain

4F: Sequences alignments of L22 gene alignments

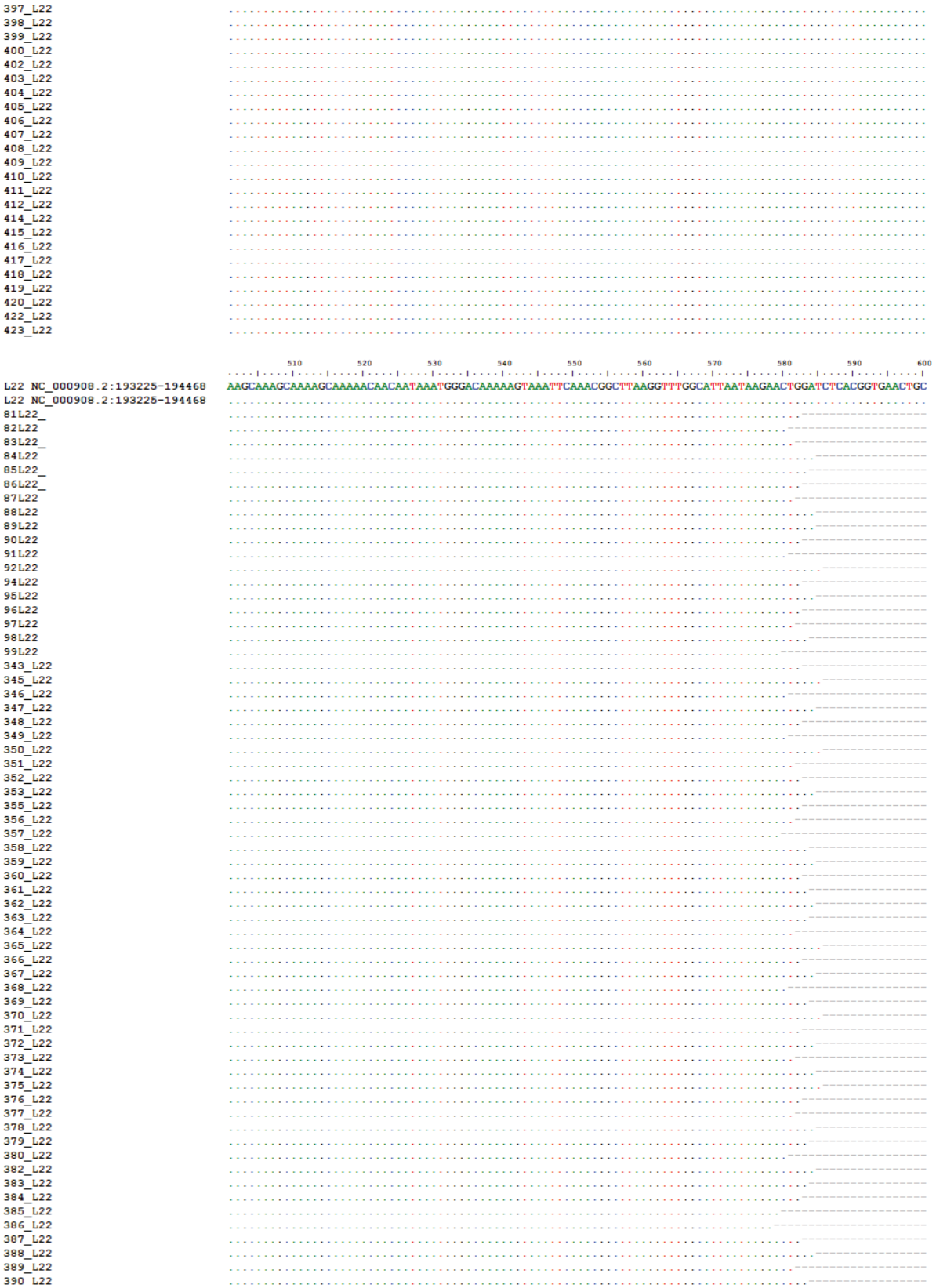

Figure 4F: Multiple sequences of L22 protein gene after successfully sequenced

4G. Sequences alignments of L22 gene alignments

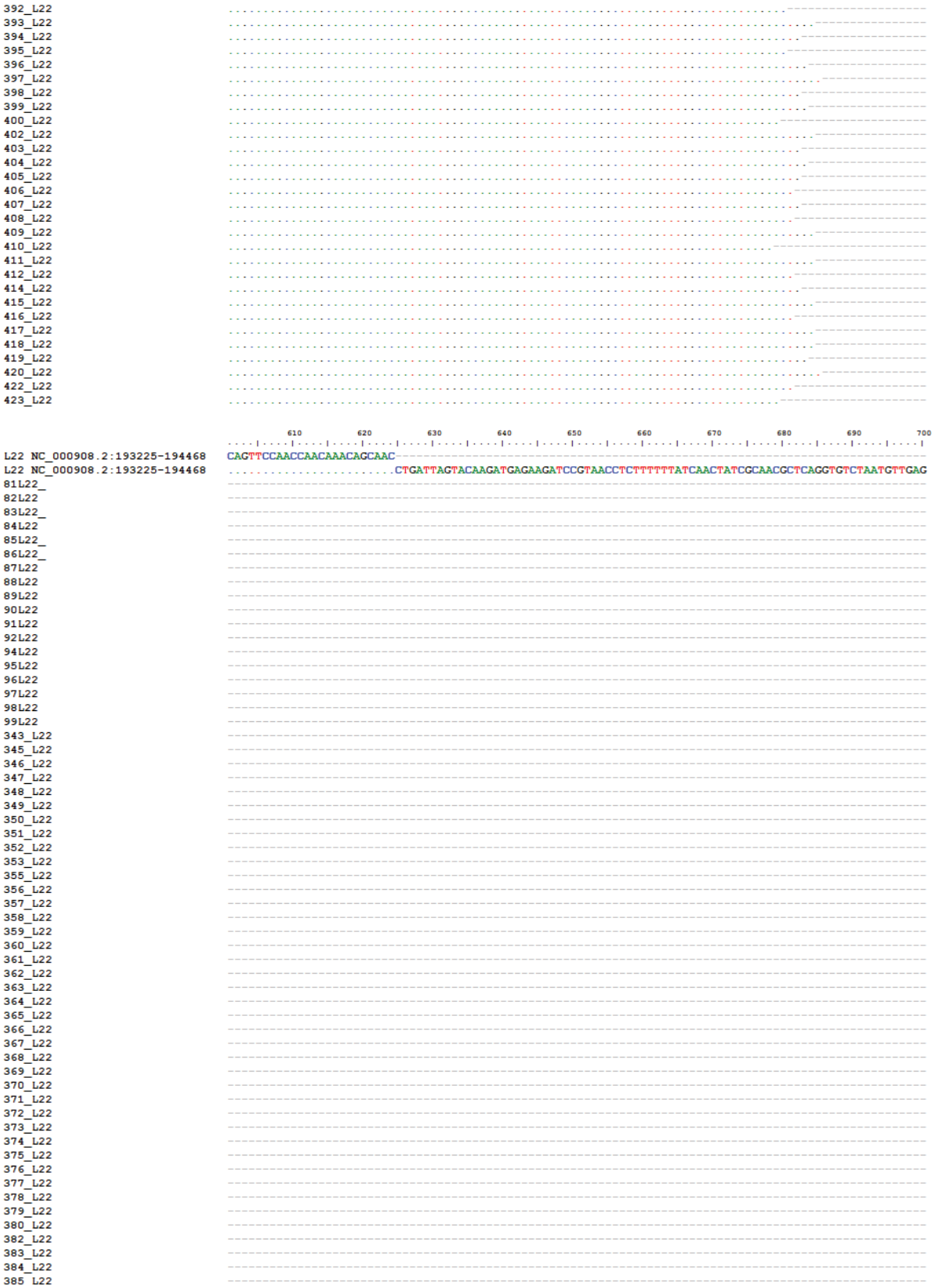

Figure 4G: Representation of all the successful sequences after DNA sequencing

Mutations detected a change of KtoK and LtoL amino acid base changes. These mutations did not lead to an amino acid change and were all silent mutations.

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APPENDIX C

Table 1A: One hundred *M. genitalium* positive female population sample list collected from CAPRISA HIPPS cross sectional study

Sample G20 for *gryA* is sample G130 on the sample list.

Sample No.	Collection Date	*gyrA*	23S rRNA	L4	L22
1	08/12/2014	G1	23S 41	L4 61	L22 81
2	09/30/2014	G2	23S 42	L4 62	L22 82
3	01/16/2015	G 3	23S 43	L4 63	L22 83
4	05/05/2015	G 4	23S 44	L4 64	L22 84
5	06/22/2015	G5	23S 45	L4 65	L22 85
6	10/10/2014	G6	23S 46	L4 66	L22 86
7	09/15/2014	G 7	23S 47	L4 67	L22 87
8	02/27/2015	G 8	23S 48	L4 68	L22 88
9	10/06/2014	G 9	23S 49	L4 69	L22 89
10	04/29/2015	G 10	23S 50	L4 70	L22 90
11	10/30/2014	G 11	23S 51	L4 71	L22 91
12	03/27/2015	G 12	23S 52	L4 72	L22 92
13	01/20/2015	G 13	23S 53	L4 73	L22 93
14	04/20/2015	G 14	23S 54	L4 74	L22 94
15	03/26/2015	G 15	23S 55	L4 75	L22 95
16	05/11/2015	G 16	23S 56	L4 76	L22 96
17	03/25/2015	G 17	23S 57	L4 77	L22 97
18	01/16/2015	G 18	23S 58	L4 78	L22 98
19	09/22/2014	G 19	23S 59	L4 79	L22 99
20	02/27/2015	G 100	23S 181	L4 262	L22 343
21	06/03/2015	G 101	23S 182	L4 263	L22 344
22	05/06/2015	G 102	23S 183	L4 264	L22 345
23	07/10/2014	G 103	23S 184	L4 265	L22 346
24	08/29/2014	G 104	23S 185	L4 266	L22 347
25	11/26/2014	G 105	23S 186	L4 267	L22 348
26	09/25/2014	G 106	23S 187	L4 268	L22 349
27	10/03/2014	G 107	23S 188	L4 269	L22 350
28	09/22/2014	G 108	23S 189	L4 270	L22 351
29	11/26/2014	G 109	23S 190	L4 271	L22 352
30	01/09/2015	G 110	23S 191	L4 272	L22 353
31	12/10/2014	G 111	23S 192	L4 273	L22 354
32	01/28/2015	G 112	23S 193	L4 274	L22 355
33	04/30/2015	G 113	23S 194	L4 275	L22 356
34	06/03/2015	G 114	23S 195	L4 276	L22 357

Table 1B: One hundred *M. genitalium* positive female population sample list collected from CAPRISA HIPPS cross sectional study

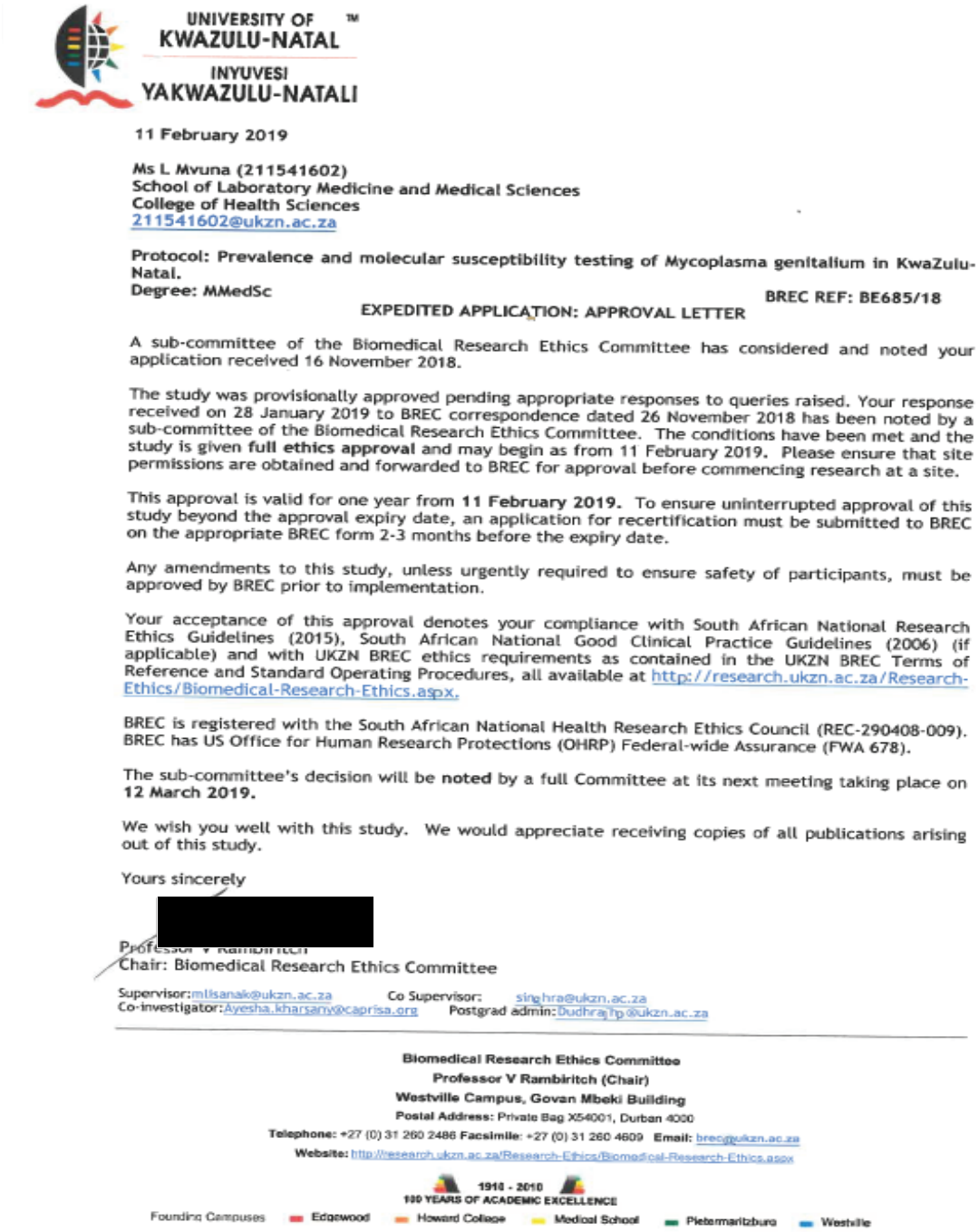
Sample No.	Collection Date	*gyrA*	23S rRNA	L4	L22
35	06/15/2015	G 115	23S 196	L4 277	L22 358
36	08/21/2014	G 116	23S 197	L4 278	L22 359
37	08/06/2014	G 117	23S 198	L4 279	L22 360
38	10/01/2014	G 118	23S 199	L4 280	L22 361
39	10/15/2014	G 119	23S 200	L4 281	L22 362
40	10/06/2014	G 120	23S 201	L4 282	L22 363
41	11/05/2014	G 121	23S 202	L4 283	L22 364
42	04/13/2015	G 122	23S 203	L4284	L22 365
43	04/17/2015	G 123	23S 204	L4 285	L22 366
44	08/01/2014	G 124	23S 205	L4 286	L22 367
45	08/22/2014	G 125	23S 206	L4 287	L22 368
46	08/20/2014	G 126	23S 207	L4 288	L22 369
47	09/11/2014	G 127	23S 208	L4 289	L22 370
48	09/17/2014	G 128	23S 209	L4 290	L22 371
49	11/11/2014	G 129	23S 210	L4 291	L22 372
50	10/28/2014	G 20	23S 211	L4 292	L22 373
51	12/02/2014	G 131	23S 212	L4 293	L22 374
52	02/18/2015	G 132	23S 213	L4 294	L22 375
53	02/27/2015	G 133	23S 214	L4 295	L22 376
54	03/27/2015	G 134	23S 215	L4 296	L22 377
55	05/27/2015	G 135	23S 216	L4 297	L22 378
56	05/15/2015	G 136	23S 217	L4 298	L22 379
57	08/20/2014	G 137	23S 218	L4 299	L22 380
58	08/27/2014	G 138	23S 219	L4 300	L22 381
59	10/27/2014	G 139	23S 220	L4 301	L22 382
60	10/02/2014	G 140	23S 221	L4 302	L22 383
61	10/29/2014	G 141	23S 222	L4 303	L22 384
62	12/10/2014	G 142	23S 223	L4 304	L22 385
63	05/20/2015	G 143	23S 224	L4 305	L22 386
64	02/24/2015	G 144	23S 225	L4 306	L22 387
65	04/17/2015	G 145	23S 226	L4 307	L22 388
66	06/10/2015	G 146	23S 227	L4 308	L22 389
67	08/01/2014	G 147	23S 228	L4 309	L22 390
68	9/30/2014	G 148	23S 229	L4 310	L22 391
69	05/05/2015	G 149	23S 230	L4 311	L22 392
70	12/03/2014	G 150	23S 231	L4 312	L22 393
71	08/06/2014	G 151	23S 232	L4 313	L22 394
72	08/20/2014	G 152	223S 33	L4 314	L22 395

Table 1C: One hundred *M. genitalium* positive female population sample list collected from CAPRISA HIPPS cross sectional study

Sample No.	Collection Date	*gyrA*	23S rRNA	L4	L22
73	01/15/2015	G 153	23S 234	L4 315	L22 396
74	06/09/2015	G 154	23S 235	L4 316	L22 397
75	09/02/2014	G 155	23S 236	L4 317	L22 398
76	02/06/2015	G 156	23S 237	L4 318	L22 399
77	05/19/2015	G 157	23S 238	L4 319	L22 400
78	04/30/2015	G 158	23S 239	L4 320	L22 401
79	07/21/2014	G 159	23S 240	L4 321	L22 402
80	04/20/2015	G 160	23S 241	L4 322	L22 403
81	06/22/2015	G 161	23S 242	L4 323	L22 404
82	11/06/2014	G 162	23S 243	L4 324	L22 405
83	05/22/2015	G 163	23S 244	L4 325	L22 406
84	05/11/2015	G 164	23S 245	L4 326	L22 407
85	01/29/2015	G 165	23S 246	L4 327	L22 408
86	02/18/2015	G 166	23S 247	L4 328	L22 409
87	05/04/2015	G 167	23S 248	L4 329	L22 410
88	05/07/2015	G 168	23S 249	L4 330	L22 411
89	01/20/2015	G 169	23S 250	L4 331	L22 412
90	03/26/2015	G 170	23S 251	L4 332	L22 413
91	03/03/2015	G 171	23S 252	L4 333	L22 414
92	08/11/2014	G 172	23S 253	L4 334	L22 415
93	11/04/2014	G 173	23S 254	L4 335	L22 416
94	06/11/2015	G 174	23S 255	L4 336	L22 417
95	10/21/2014	G 175	23S 256	L4 337	L22 418
96	10/01/2014	G 176	23S 257	L4 338	L22 419
97	09/24/2014	G 177	23S 258	L4 339	L22 420
98	07/18/2014	G 178	23S 259	L4 340	L22 421
99	04/23/2015	G 179	23S 260	L4 341	L22 422
100	9/24/2014	G 180	23S 261	L4 342	L22 423

APPENDIX D: ETHICAL CLEARANCE FOR THE STUDY

1. Approval of the study: The study was approved by University of KwaZulu-Natal (UKZN), Biomedical research ethics committee (BREC) reference no. BE685/18.



UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 February 2019

Ms L Mvuna (211541602)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
211541602@ukzn.ac.za

Protocol: Prevalence and molecular susceptibility testing of Mycoplasma genitalium in KwaZulu-Natal.
Degree: MMedSc **BREC REF: BE685/18**

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received 16 November 2018.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 28 January 2019 to BREC correspondence dated 26 November 2018 has been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have been met and the study is given **full ethics approval** and may begin as from 11 February 2019. Please ensure that site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from **11 February 2019.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **noted** by a full Committee at its next meeting taking place on **12 March 2019.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

[redacted]

Professor V Rambiritch
Chair: Biomedical Research Ethics Committee

Supervisor: mlisanak@ukzn.ac.za Co Supervisor: singhra@ukzn.ac.za
Co-investigator: Ayesha.kharsany@caprisa.org Postgrad admin: Dudhraj1p@ukzn.ac.za

Biomedical Research Ethics Committee
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1910 - 2010
100 YEARS OF ACADEMIC EXCELLENCE
Founding Campuses: Edgewood, Howard College, Medical School, Pietermaritzburg, Westville

2. Re-certification of the study: Re-certification of the study was approved by UKZN BREC reference no. BE685/18.

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28 January 2020

Ms L Mvuna (211541602)
School of Laboratory Medicine and Medical Sciences
211541602@ukzn.ac.za

Dear Ms Mvuna

Protocol: Prevalence and molecular susceptibility testing of Mycoplasma genitalium in KwaZulu-Natal. Degree: MMedSc
BREC REF: BE685/18

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	11 February 2020
Expiration of Ethical Approval:	10 February 2021

I wish to advise you that your application for Recertification received on 17 January 2020 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 10 March 2020.

Yours sincerely

[redacted]
Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

Supervisor: mlisanak@ukzn.ac.za Co Supervisor: singhraj@ukzn.ac.za Co-investigator: Ayesha.kharsany@caprisa.org Postgrad admin: Dudhrajhp@ukzn.ac.za

APPENDIX E: Access letter to use the samples from CAPRISA HIPPS cohort Study.

CAPRISA
CENTRE FOR THE AIDS PROGRAMME OF RESEARCH IN SOUTH AFRICA
Generating Knowledge - Impacting Health

Doris Duke Medical Research Institute (2nd floor), 719 Umbilo Road, Private Bag X7, Congella, 4013, Durban, South Africa
tel: +27 31 2604555 | **fax**: +27 31 2604549 | email: **caprisa@caprisa.org** | www.caprisa.org

15 January 2019
Govan Mbeki Building
University of KwaZulu-Natal
Private Bag X 54001
Durban
4000

Dear Sir/Madam

Re: Letter of permission for Miss Londeka Mvuna to access clinical samples from the HIV incidence Provincial Surveillance System (HIPSS) study repository

As Principle investigator of the HIPSS study, I have provided permission for Ms Londeka Mvuna to access samples in the form of extracted DNA from the repository, in support for her BREC application. The DNA was extracted from vaginal swabs and urine, for her MMed Science studies. I am a collaborator on this project which answers some of the key questions, namely. Ms Mvuna's research project focuses on determining the prevalence of antimicrobial resistance among *Mycoplasma genitalium* among 15-49 year old men and women in KwaZulu-Natal. Furthermore, she will undertake her research to determine the genes associated with macrolide and fluoroquinolones resistance (L2, L4, *parC* and *gryA*) among (approximately 300) of *Mycoplasma genitalium DNA* positive samples.

It is important to note that participants in the HIPPS study were provided with detailed study related information for study participation and for long term sample storage of clinical samples for future testing, which reads as follows … .
"If you agree to take part in the HIPSS study, there may be some remaining blood, urine and vaginal swab samples (females) known as samples, taken from you during the study that might be useful for future research. You are being asked to agree to the storage of the left over samples for possible future research that will include additional testing. This is research that will be conducted in the future that may or may not be related to the HIPSS study.
This consent form gives you information about the collection, storage, and use of your samples for possible future research. The study staff will talk to you about this information. Please ask if you have any questions. If you agree to the storage of your samples for possible future research, you will be asked to note this on this consent form. You will get a copy of this form to keep. for retrospective testing."

The HIPSS study was approved by BREC with the reference number BF269/13. Kindly note that all samples for this study will not include any participant identifying information and samples will be linked to behavioural data using the barcodes. Kindly contact me should you require any further information.

Sincerely,

AYESHA BM KHARSANY
Principle investigator HIPSS study.
Honorary Associate Professor / Senior Scientist
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