Identification of mutations in genes associated with metronidazole resistance and susceptibility in *Trichomonas vaginalis*

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Dissertation submitted in partial fulfilment of the requirements for the degree:

Master of Medical Science in the

School of Clinical Medicine,

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South Africa

“Our biggest fear is not that we are inadequate.

Our deepest fear is that we are powerful beyond measure.”

-Marianne Williamson-

DEDICATION

This dissertation is dedicated to my late father who taught me to be an independent and determined person, to my dearest mother for her prayers and enduring encouragement throughout my master’s degree, and to my siblings and friends for their constant support.

I appreciate you all.

ACKNOWLEDGEMENTS

First, and foremost, I would like to thank the Almighty God for the strength, courage, patience and wisdom that enabled me to complete this master's degree.

I would also like express my deep and sincere gratitude to the following:

- To my supervisor, Professor Nathlee Abbai, thank you for your guidance, support, motivation and the opportunity to conduct research under your supervision. It was a great privilege working with you.
- To the Department of Clinical laboratory Medicine, thank you for making me feel welcomed and for your guidance when I conducted my experiments.
- To Inqaba Biotechnical Industries, for helping with the sequencing of samples.
- My sincerest gratitude goes to the National Research Foundation, for their support and financial assistance.

DECLARATION

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.

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As the candidate's supervisor, I have read the thesis and have given our approval for submission for examination.

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Date: 26 August 2021

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SYMBOLS AND ABBREVIATIONS

Symbol and Abbreviation	Description
%	Percentage
°C	Degree Celsius
µl	Microlitre
AO	Acridine Orange
ATP	Adenosine Triphosphate
Bp	Base pair
BLAST	Basic Local Alignment Search Tool
BREC	Biomedical Research Ethics Committee
BSA	Bovine serum albumin
BV	Bacterial vaginosis
$C_3H_3N_3O_2$	5-nitroimidazole
CD4	Cluster of Differentiation 4
CDC	Centre for Disease Control
CLIA	Clinical Laboratory Improvement Amendments
CPLM	Cysteine-peptone-liver-maltose
DM	Diamonds Media
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
ELISA	Enzyme-Linked Immunosorbent Assays
FDA	Food and Drug Administration
HIV	Human Immunodeficiency Virus
MIC	Minimum Inhibitory Concentration
MTZ	Metronidazole
NAATs	Nucleic Acid Amplification Tests
NC	Negative control
NCBI	National Centre for Biotechnology Information
Ntr	*Nitroreductases*
PAS	Periodic Acid-Schiff
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PC	Positive control
PEM	Plastic envelop method
PFOR	Pyruvate-ferredoxin oxidoreductase
PID	Pelvic inflammatory disease
POC	Point-of-care
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive oxygen species
SCM	School of Clinical Medicine
SDS	Sodium Duodecyl Sulfate
SNPs	Single Nuclear Polymorphisms
SOD	Superoxide Dismutase
STI	Sexually Transmitted Infections
TE	T_{10} E_1

TMA	Transcription Mediated Amplification
WHO	World Health Organization

ABSTRACT

Trichomonas vaginalis is the most common non-viral, sexually transmitted pathogen worldwide. Although, metronidazole cure rates are high for *T. vaginalis* infection, resistance has been reported. Mutations in the *nitroreductase* genes of *T. vaginalis* have also been implicated in metronidazole resistance. Therefore, the aim of this study was to detect mutations in the *nitroreductase* genes and link the mutations to metronidazole resistance patterns in *T. vaginalis* isolated from South African pregnant women, a currently under-researched area.

Vaginal swabs were collected from 362 pregnant women recruited from the King Edward VIII hospital antenatal clinic in Durban from October 2018 to March 2019. The swabs were cultured in Diamonds TYM medium to obtain pure isolates of *T. vaginalis*. Pure isolates were sub-cultured and subjected to metronidazole susceptibility assays. The susceptibility assays were conducted under aerobic and anaerobic conditions. DNA was extracted from the pure isolates to perform the polymerase chain reaction (PCR) assays for the detection of the *nitroreductase* genes and the *PFOR* gene. The PCR amplicons were sequenced using the Sanger approach in order to identify mutations associated with resistance.

A total of 21/362 (5.8%) pregnant women tested positive for *T. vaginalis* infection. Of the 21 *T. vaginalis* isolates tested for anaerobic metronidazole susceptibility, 9.5% (2/21) had an MIC of 4 µg/ml (resistant), 38.1% (8/21) had an MIC of 2 µg/ml (intermediate) and 52.4% (11/21) had an MIC ≤ 1 µg/ml (susceptible). For the *ntr2* gene, susceptible and resistant isolates carried mutations which were absent in the intermediate isolates. The susceptible isolates carried mostly insertion mutations and the resistant isolate carried substitution mutations. Some deletion mutations were also observed in the *ntr2* gene. For the *ntr3* gene, two substitution mutations were observed in the intermediate isolate only. In the *ntr4* gene, substitution mutations were only observed in the susceptible and resistant isolate. For the *ntr5* gene, substitution and insertion mutations were observed in the resistant isolate and not in the intermediate or susceptible isolates. For the *ntr6* gene, insertion and deletion mutations were observein the intermediate isolate and a single deletion mutation in the resistant isolate. For the *PFOR* gene, a single substitution mutation was observed in the intermediate and resistant isolate.

In this study, mutations in the *ntr2, ntr3, ntr4, ntr5* and *ntr6* genes were observed across metronidazole susceptibility profiles. Previous studies have not identified mutations in the *ntr2*, *ntr3* and *ntr5* genes, so there is not enough data to support the functions of those genes and their association with metronidazole resistance. Future studies aimed at identifying the function of these mutations are needed.

CHAPTER ONE

INTRODUCTION

Trichomonas vaginalis is a common non-viral sexually transmitted pathogen that causes the sexually transmitted infection (STI), Trichomoniasis (Petrin et al., 1998). *T. vaginalis* (46%) is more prevalent compared to *Neisseria gonorrhoeae* (1%), *Chlamydia trachomatis* (26%), and *Syphilis* (4%) (Mgone et al., 2002). *T. vaginalis* mainly targets the squamous epithelium of the human genital tract, occupying the lower genital tract in women and the prostate and urethra in men (Krieger et al., 1988). Individuals who are infected show a variety of symptoms such as vaginal discharge, genital burning or itching, dysuria and pain during urination or sexual intercourse. However, some individuals infected with *T. vaginalis* may be asymptomatic (Sutton et al., 2007). Infections caused by *T. vaginalis* during pregnancy have been associated with pelvic inflammatory disease (PID), low birth weight infants, preterm delivery, and premature rupture of membranes (Silver et al., 2014). In men *T. vaginalis* may result in epididymitis, prostatitis, and infertility (Krieger, 1995). Age, sexual behaviour, number of sexual partners, other sexually transmitted diseases (STDs), menstrual process, diagnostic methods, social and economic conditions are all the risk factors that influence the likelihood of infection (Menezes et al., 2016). The prevalence is high among low-income, gynaecologic, and STD clinic patients (Menezes et al., 2016).

Trichomoniasis has been reported to have over 276.4 million reported infections worldwide (Ramjee, Abbai, & Naidoo, 2015). Out of the 276 million cases, 25 million cases have been reported in pregnant women (Salakos et al., 2018). Pregnant women infected with *T. vaginalis* have a higher risk of preterm delivery since *T. vaginalis* causes the premature rupture of membranes. A previous study demonstrated that pregnant women infected with *T. vaginalis* have a 30% risk of preterm delivery or delivering infants with low birth weight (Cotch et al., 1997). *T. vaginalis* infection also contributes to the increase in mother-to-child transmission of Human immunodeficiency virus (HIV) infection (Price et al., 2018). In women, the increased transmission of infection may be due to a decrease in the lining of the cervico-vaginal epithelium, as well as the influx of CD4 lymphocytes and macrophages at the site of infection, providing a flood of HIV susceptible or HIV infected cells (Price et al., 2018). In 2016, a systematic review of 75 studies conducted on the prevalence of STIs in pregnant women found that the prevalence of *T. vaginalis* was around 3.9-24.6% in Latin America and Southern Africa (Joseph Davey et al., 2016).

T. vaginalis infection is challenging to diagnose clinically due to its heterogeneous nature and the problems with diagnostic testing (Garber, 2005). The most widely used method of detection is wet-mount microscopy. Compared to other diagnostic assays, wet-mount microscopy is 40-60% sensitive in some asymptomatic women (Nathan et al., 2015). Motile trichomonads have been observed to be highly specific, which has proven useful when discovering methods of treatment (Van Der Pol, 2016).

The correct diagnosis of trichomoniasis is a vital link in the prevention and treatment of the disease, and it is of great importance to prevent the spread of the disease. Currently, wet mount microscopy, polymerase chain reaction (PCR), culture, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) are the most common techniques used to detect *T. vaginalis* (Asmah, 2018). Wet-mount microscopy is not recommended for the screening of asymptomatic women. Wet-mount microscopy is easy to use and cost-effective; however, it lacks sensitivity and contributes to the under-diagnosis of the disease (Garber, 2005). The broth culture technique has been the best method for detecting *T. vaginalis* over the past four decades (Garber, 2016). Diamonds TYI medium is the standard broth which only requires an inoculum size in the range of 102 organisms/ml. Culture techniques have not been available because they are expensive. However, they effectively determine the epidemiology of *T. vaginalis*, especially in STI clinics that are far from clinical laboratories (Garber, 2016).

One of the important clinical characteristics of *T. vaginalis* is its emerging resistance to metronidazole (Kulda, 1999). Metronidazole is an antibiotic that is used to treat anaerobic bacterial infections, protozoal infections, and microaerophilic bacterial infections (Cudmore et al., 2004). It was first used to treat *Trichomonas vaginalis* infections, but it was later found to be useful against other protozoal infections such as amebiasis and giardiasis (Lofmark et al., 2010). In the United States, metronidazole resistance was reported in 4.3 to 9.6% of *T. vaginalis* clinical isolates (Snipes et al., 2000). In South Africa, metronidazole resistance was detected in 6% of women attending an antiretroviral clinic (Rukasha et al., 2013). *T. vaginalis* mechanisms of resistance to metronidazole is associated with the down-regulation of enzymes such as pyruvate ferredoxin oxidoreductase (PFOR) and ferredoxin, which have the potential to reduce metronidazole (Wright et al., 2010). PFOR is an enzyme that is found in the hydrogenosomes (membrane-bound organelles) of *T. vaginalis*, which produce adenosine triphosphate (ATP) and hydrogen (Wright et al., 2010). In addition, other studies have found an association between mechanisms of resistance and reduced thioredoxin reductase activity and free flavins, both of which have been shown to reduce metronidazole (Kissinger, 2015; Leitsch et al., 2012). Carlton et al. (2007) identified 11 *nitroreductase* genes in *T. vaginalis*, one of which could reduce metronidazole (Carlton et al., 2007). Mechanisms of metronidazole resistance are not well elucidated, resulting in a surge in the reports of metronidazole resistance in *T. vaginalis* clinical isolates. Therefore, there is a greater need for studies that will investigate the mechanisms of metronidazole resistance.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of *Trichomonas vaginalis*

Trichomoniasis is the most prevalent curable STI caused by a single-celled, anaerobic, flagellated protozoan parasite known as *Trichomonas vaginalis*. In 2016, the World Health Organization (WHO) estimated *T. vaginalis* cases to be around 156 million worldwide (Rowley et al., 2019). WHO reported that *T. vaginalis* is found on every continent and across all ethnicities, as shown in Figure 1 (Rowley et al., 2019). There are approximately 3.7 million people infected with trichomoniasis in the United States, with an estimated 1.1 million new cases every year (Satterwhite et al., 2013). However, a higher prevalence of 42.8 million infections are reported in African countries (de Waaij et al., 2017).

In addition, Africans, African Americans, and Garifunas (Descendants of West African, Central African, Island Carib, and Arawak people) showed higher rates (ten times higher) of *T. vaginalis* infection when compared to Caucasian women (Shafir et al., 2009). Ginocchio et al. (2012) studied the prevalence of *T. vaginalis* and co-infection with *C. trachomatis* and *N. gonorrhoeae* in the United States. In that study among racial groups, *T. vaginalis* prevalence was highest for blacks (20.2%), followed by whites (5.7%) and Hispanics/Latinos (5.0%) (Ginocchio et al., 2012). Mgone et al. (2002) conducted a study in women from Papua New Guinea. They reported a high prevalence rate for *T. vaginalis* ranging from 21% in pregnant women to 42.6% for women in the general population.

A cross-sectional study conducted among women in rural Mopani District, South Africa, observed a 20% vaginal prevalence and a 1.2 % rectal prevalence of *T. vaginalis* (de Waaij et al., 2017). Geographically, the prevalence of *T. vaginalis* in pregnant women varies; *T. vaginalis* prevalence ranged from 3.9–24.6 percent in low- to middle-income countries (i.e., Latin America and Southern Africa) according to a 2016 systematic analysis of 75 studies of STI prevalence in pregnant women (Joseph Davey et al., 2016). *T. vaginalis* was found to be present in 20% of HIV-positive pregnant women in South Africa (Price et al., 2018), with high rates of incident infection in pregnant women (9.2/100 person-years) in both South Africa and Zimbabwe according to recent research (Teasdale et al., 2018).

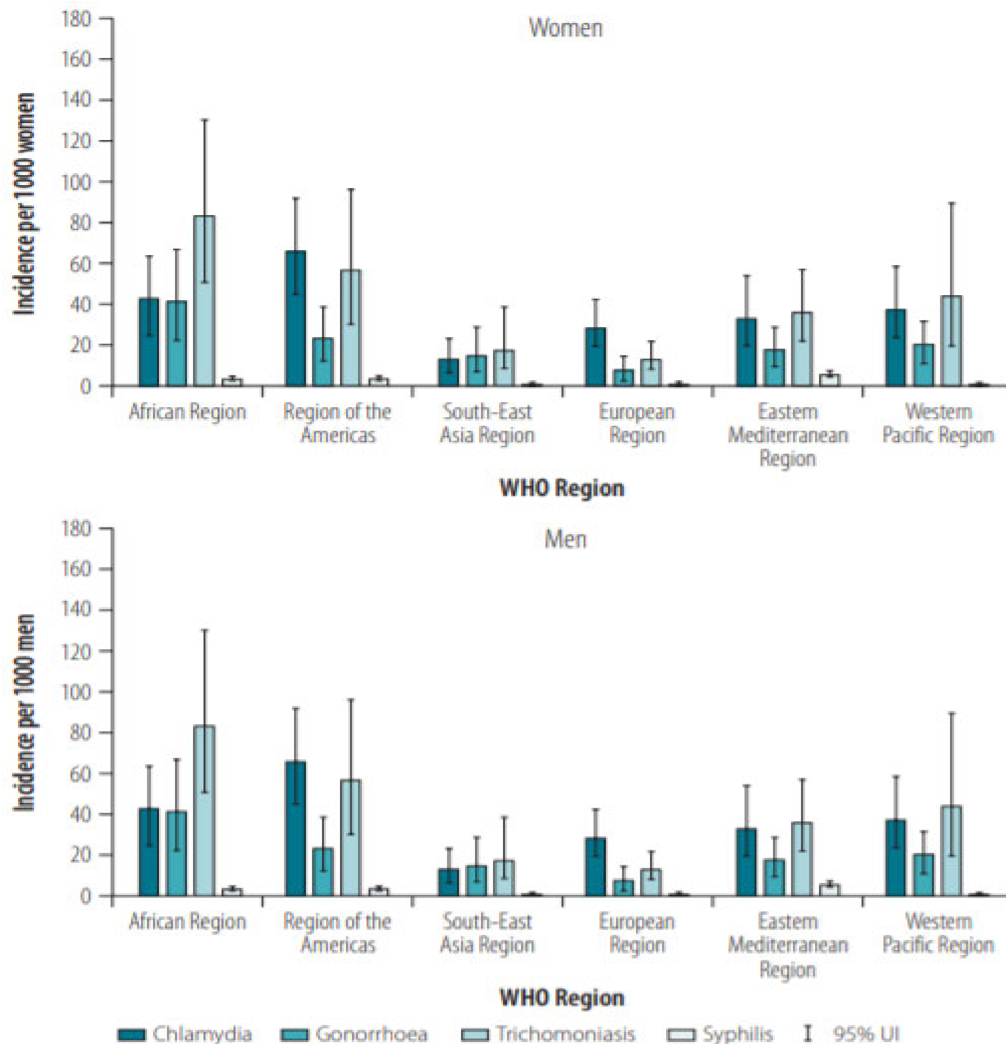

Figure 1: Prevalence of *T. vaginalis* across WHO regions (Rowley et al., 2019).

According to Newman et al. (2015), the global prevalence of trichomoniasis among women was about 5.0 percent (4.0-6.4%), whereas it was supposed to be around 0.6 percent (0.4-0.8%) among men. The presence of zinc in the prostatic fluid helps to explain why males have a lower infection rate. Zinc has been shown to have anti-trichomonal properties *in vitro*, leading to *T. vaginalis* suppression *in vivo* (Krieger & Rein, 1982).

2.2 History

Alexandre Donné, a French physician and microscopist at the Faculty of Paris, first discovered the protozoa *T. vaginalis* in 1836. Donné observed motile microorganisms in the odorous, foamy discharge of women who presented with genital irritation and vaginal discharge (Sood & Kapil, 2008). Donné (1836) published the first systematic definition of *T. vaginalis* infection (Donné, 1836), citing an unpublished morphologic description of the organism by Dujardin (Despommier et al., 1995). Dujardin thought of it as a hybrid of traits from both monas and tricodes classes, hence the name *Tricomonas*

vaginale, which later changed to the current term "*Trichomonas vaginalis*" by Ehrenberg (Ehrenberg, 1838). However, research on *T. vaginalis* only began in the 20th century. In the 1960s and 1970s, the studies conducted focused on biochemical experiments and microscopic analysis to understand the growth characteristics and behaviour of the organism (Sood & Kapil, 2008). It was not until the 1980s that immunological methods and techniques of molecular biology became available and used to study the microorganism's pathogenesis and immunology (Sood & Kapil, 2008).

2.3 Morphology

T. vaginalis is a pear-shaped, micro-aerotolerant, urogenital flagellate pathogen. It varies in shape and size, with a length of 10 µm and a width of 7 µm (Honigberg & King, 1964). *T. vaginalis* possess five flagella, four of which are situated at the anterior portion (Petrin et al., 1998) (Figure 2). The fifth flagellum lies within the parasite's undulating membrane. The microorganism proceeds in a quivering motion through the anterior flagella and the undulating membrane. After it attaches to vaginal epithelial cells, the microorganism takes on a more ameboid-like appearance (Wartoń & Honigberg, 1979). Both actin fibres and tubulins make up the cytoskeleton of the parasite *T. vaginalis*. Different forms of tubulin have been discovered inside trichomonad cells, according to researchers (Petrin et al., 1998). *T. vaginalis* actin isolates are reported to move more slowly than actin isolates from muscles. The nucleus is located in an anterior region of the cell surrounded by a porous nuclear membrane (Masha et al., 2019). An axostyle, a tiny hyaline rod-like structure seen on the parasite *T. vaginalis*, is another feature discovered on the parasite. This unique feature extends across the parasite's posterior end, allowing it to attach to vaginal epithelial cells (Masha et al., 2019; Petrin et al., 1998).

Figure 2: A representation of *T. vaginalis* in broth culture (Petrin et al., 1998)

2.4 Growth requirements, nutrition and metabolism

T. vaginalis is an obligate parasite, unable to synthesize several macromolecules, including purines, pyrimidines, and several lipids (Petrin et al., 1998). These nutrients are obtained by phagocytosis of host and bacterial cells or from vaginal secretions (Heine & McGregor, 1993; Huggins & Preti, 1981). *T. vaginalis* culture media must contain most of the required macromolecules, vitamins, and minerals in order for the microorganism to grow. Serum is essential for trichomonad growth since it contains

lipids, fatty acids, amino acids, and trace metals (Diamond, 1957). Iron is also required for growth in order to keep ferredoxin and PFOR activity at maximum levels (Gorrell, 1985).

2.5 Pathogenesis of *T. vaginalis*

T. vaginalis is an obligate parasitic microorganism that infects the genital tract's squamous epithelium. It resides in the lower female genital tract and the male urethra and prostate, where it replicates. This parasite feeds by phagocytosing vaginal epithelial cells, bacteria, and erythrocytes (Burch et al., 1959). Adhesion plays a significant role in the pathogenesis of *T. vaginalis* (Adegbaju & Morenikeji, 2008). Several adhesion molecules identified on the surface of the flagellated parasite are known to facilitate the attachment of *T. vaginalis* to human cells. Antibodies attach to adhesion molecules and cause the reduction of parasite adhesion and cytopathic effects on the host cells (Engbring & Alderete, 1998). In addition, contact of *T. vaginalis* to mammalian cells results in the upregulation of adhesion molecules and *T. vaginalis* becomes flat in shape when attaching itself to the host cell (Engbring & Alderete, 1998). The parasite cannot live in the external environment since there is no cyst type, however, *T. vaginalis* can survive up to 3 hours in a damp environment outside the human body (Burch et al., 1959). The only recognized host is humans, and transmission is mainly through sexual contact. Nonsexual transmission is possible, but it is uncommon (Crucitti et al., 2011).

2.6 Reproduction and life cycle

T. vaginalis targets the squamous epithelium of the human urogenital tract (Kissinger, 2015). The incubation period is usually between four and twenty-eight days (Petrin et al., 1998). In women, *T. vaginalis* colonizes the female lower genital tract, in men, the prostate, and the urethra, where replication occurs by binary fission (Kissinger, 2015). *T. vaginalis* does not have a cystic stage and only occurs as a trophozoite. The tiny ovoid flagellates replicate by longitudinal binary fission, which prevents the nuclear membrane from vanishing (Pereira-Neves et al., 2003) (Figure 3).

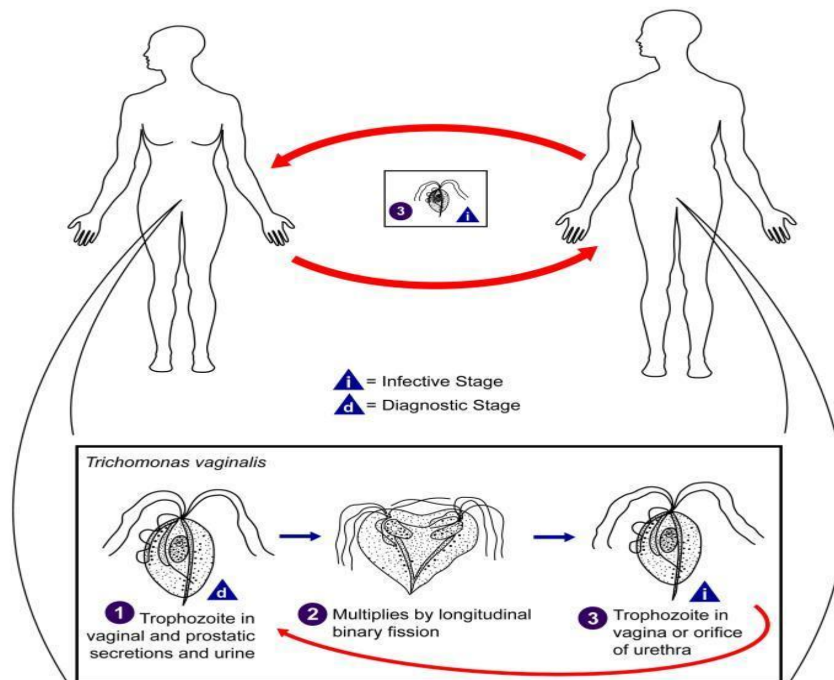

Figure 3: The life cycle of *T. vaginalis* in males and females (Pereira-Neves et al., 2003)

2.7 Transmission

T. vaginalis is transmitted by sexual intercourse. The infection can last for months or even years in women but usually lasts for less than ten days in men (Krieger, 1995). The parasitic microorganism cannot live in external conditions. However, it can survive for more than three hours outside the human body in a wet environment (Burch et al., 1959). Nonsexual transmission occurs when trichomonads enter the vaginal canal via infected douche nozzles, specula, or toilet seats (Whittington, 1957). After several hours of exposure to air and swimming pool water, live *T. vaginalis* were observed in urine and sperm (Catterall & Nicol, 1960). Adu-Sarkodie (1995) reported the possibility of *T. vaginalis* transmission within a family in Ghana from a mother to her children through the sharing of towels (Adu-Sarkodie, 1995). In a study conducted by Buvé et al. (2001), a 40% prevalence among women aged 15–49 years who never had sexual intercourse was reported (Buvé et al., 2001). These findings could not be explained by under-reporting of sexual activity because that would indicate that every woman aged 15 to 19 had had her first sexual encounter.

2.8 Clinical manifestations of *T. vaginalis*

Past studies have shown that 85% of women and 77% of men infected with *T. vaginalis* are asymptomatic (Petrin et al., 1998). A third of these women start developing symptoms within six months (Petrin et al., 1998). Symptomatic women usually have an odorous yellow/grey-green vaginal discharge, dysuria, vulvar irritation, vaginal itching, and lower abdominal pain (Price et al., 2018). In

men, the symptoms include urethral discharge and dysuria, prostatitis, and pruritis (Price et al., 2003). The standard vaginal pH is 4.5, but when infected with *T. vaginalis*, the pH increases to greater than 5.0 (Petrin et al., 1998). In addition, *T. vaginalis* infection is responsible for causing vaginal micro-abrasions, thereby increasing the risk of HIV acquisition (Van Der Pol, 2016).

2.9 Detection of *T. vaginalis*

2.9.1 Microscopic techniques

The most widely used diagnostic test is wet mount microscopy (Nathan et al., 2015). In a study conducted by (Radonjic et al., 2006), the sensitivity of wet mount microscopy was 66% and the specificity was 100% (Radonjic et al., 2006). Despite this method being simple and cheap, it has its disadvantages, such as the sample must be quickly examined and recorded since *T. vaginalis* degrades under external conditions (Radonjic et al., 2006). During the degradation process, trichomonads become less motile, retract the flagella and changes shape by becoming rounder, making it difficult to differentiate from structures as shown in Figure 4(1) (Radonjic et al., 2006). It is not advisable to use wet mount microscopy to screen asymptomatic women since it has a sensitivity of 40-60% in these women (Van Der Pol, 2016). All of the samples which are found negative by this method, should be cultured for a proper diagnosis (Cevahir et al., 2002).

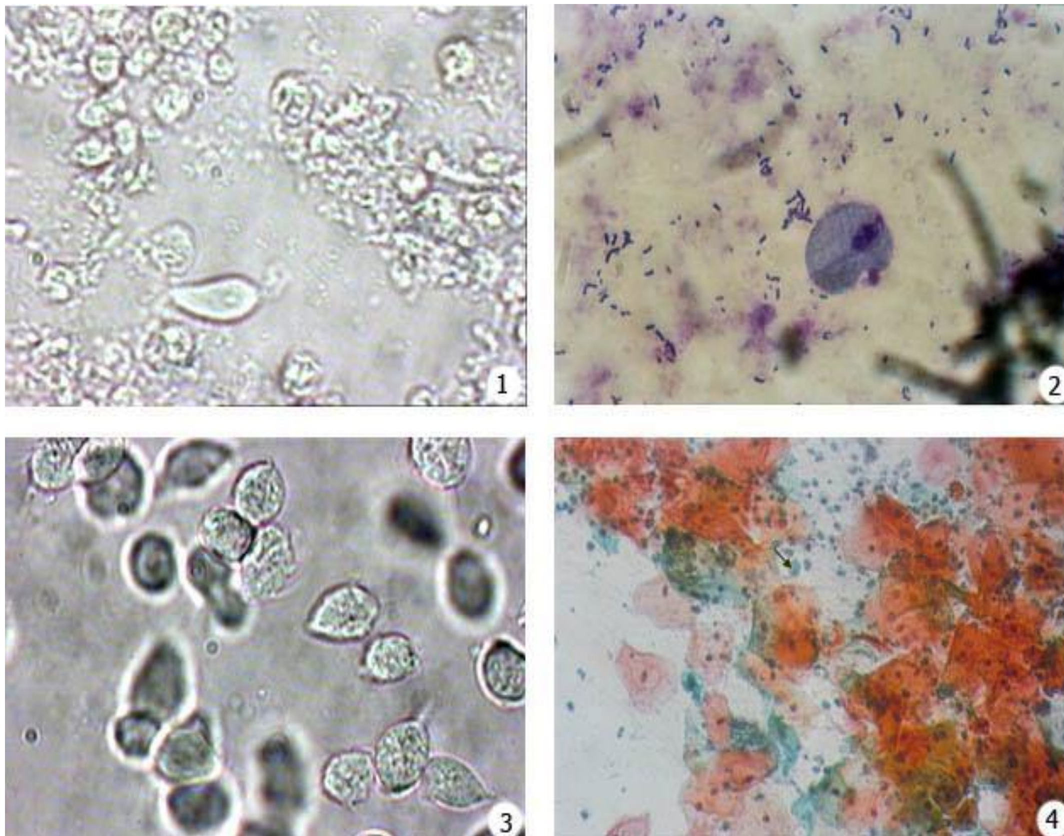

Figure 4: (1) *T. vaginalis* observed using microscopy 400X magnification; (2) *T. vaginalis* in Giemsa 1000X magnification; (3) *T. vaginalis* in cultivation cysteine-peptone-liver-maltose (CPLM) in 400X

magnification; (4) *T. vaginalis* in Periodic Acid-Schiff (PAS) stain in 100X magnification (Karaman et al., 2008).

2.9.2 Culture techniques

Diamond's TYI liquid broth media, cysteine-peptone-liver-maltose (CPLM) shown in Figure 4 (3), Feinberg and Whittington's medium, and self-contained culture systems (InPouch TV kit) are among the media available for culture (Whittington, 1957). Culture has a sensitivity range of 75%-96% and a specificity range of up to 100% (Nye et al., 2009). Since urinary culture is less sensitive, vaginal secretions are the preferred specimen for culture in women (Nye et al., 2009). Urethral swabs, urine sediments, and semen are culturable samples for men (Lawing et al., 2000; Mohamed et al., 2001). However, culture diagnosis has limitations (Fouts & Kraus, 1980). To diagnose *T. vaginalis* in cultures, an incubation period of 2 to 7 days is typically used, and during this time, infected patients may continue to transmit the infection (Van Gerwan & Muzny, 2019). Clinicians also have limited access to resources for culture such as incubators (Van Gerwan & Muzny, 2019). A plastic envelope method (PEM) was developed to improve the acceptability of culture-based diagnosis by allowing immediate analysis and culture in one self-contained device has been developed (Beal et al., 1992).

2.9.3 Staining techniques

Staining techniques were introduced to advance the sensitivity of direct microscopy, since culture methods were relatively slow and wet mount preparation lacked sensitivity (Petrin et al., 1998). Staining techniques such as acridine orange (AO), Giemsa, Fontana-Masson silver stain, Periodic Acid-Schiff (PAS) (Figure 4), and Leishman stain have been used for the diagnosis of *T. vaginalis* (Nagesha, et al., 1970). Papanicolaou (Pap) staining has proven useful in the diagnosis of trichomoniasis and is used in gynaecologic screening for cytological abnormalities, mostly in populations with a high prevalence of STIs (Birnbaum & Kraussold, 1975). However, staining techniques are limiting since *T. vaginalis* is not always in its usual pear-shaped form with flagella (Petrin et al., 1998).

2.9.4 Molecular techniques

Recombinant DNA technology has been used in trichomoniasis diagnostic studies for the past decade (Garber, 2005). The United States Food and Drug Administration (US FDA) has approved the Affirm VPIII point-of-care DNA hybridization probe assay (Becton, Dickinson & Co.; USA) for *T. vaginalis* diagnosis in women (Kissinger, 2015). *Gardnerella vaginalis, T. vaginalis* (Kissinger, 2015) (Brown et al., 1999; Petrin et al., 1998), and *Candida albicans* are all detected simultaneously from vaginal specimens using the Affirm VP III nucleic acid probe test. In about 45 minutes, the results are available (Garber, 2005; Kissinger, 2015). Due to the existence of DNA from dead trichomonads after treatment, it has been suggested that this test could be correlated with false positives (Garber, 2005). The sensitivity and specificity of the Affirm VP III test are around 83% and 97%, respectively (Bachmann et al., 2011; Garber, 2005; Kissinger, 2015).

2.9.5 Nucleic Acid Amplification Testing (NAAT)

Many NAAT-based approaches, such as transcription-mediated amplification (TMA) and polymerase chain reaction (PCR) have been implemented. In 2011, the *Trichomonas* APTIMA test, a TMA assay approved by the US FDA for the detection of vaginal trichomoniasis (Schwebke et al., 2018). This test is highly sensitive and precise, and it can be done on vaginal swabs (self-collected or collected by a clinician), semen, or liquid endocervical specimens in women (in Thin Prep media) (Huppert et al., 2011). Wet mount, culture, and the Affirm VPIII assay are all less sensitive when compared to the *Trichomonas* APTIMA test (Andrea & Chapin, 2011; Hollman et al., 2010; Roth et al., 2011). The *Trichomonas* APTIMA test tends to be the most sensitive tool for detecting trichomoniasis in asymptomatic women (Hollman et al., 2010). If validated by Clinical Laboratory Improvement Amendments (CLIA) regulations, the APTIMA test can be used to test urine or urethral swabs from men, although penile-metal swabs are favoured over urine samples since they increase detection rates (Workowski & Bolan, 2015). Since there is no evidence that *T. vaginalis* causes anorectal infection, NAATs should not be used to diagnose *T. vaginalis* anorectal infection (Francis et al., 2008), (Workowski & Bolan, 2015). The Xpert® TV assay (Cepheid, Sunnyvale, CA) was the first *T. vaginalis* NAAT to be accepted by the FDA for use in female urine, endocervical swabs, and patient and clinician-collected vaginal specimens, as well as male urine (Schwebke et al., 2018). The Xpert® TV assay has a diagnostic sensitivity and specificity of 99.5–100% and 99.4–99.9% for female genital specimens and 97.2–99.9% for male urine specimens, respectively (Schwebke et al., 2018). The Xpert® TV assay produces results within 60–90 minutes after being obtained and put in the testing platform, allowing for point-of-care (POC) diagnosis and management (Badman et al., 2016).

2.9.6 Antibody-Based techniques

T. vaginalis is known to have eight different serotypes (Ackers, 1990). However, immunoblot analysis reveals a wide range of antigenic markers (Garber et al., 1986). Anti-trichomonal antibodies have been detected using a variety of methods, including agglutination, complement fixation, indirect hemagglutination, gel diffusion, fluorescent antibody, and enzyme-linked immunosorbent assay (ELISA) (Jaakmees et al., 1966; Mason et al., 1976). The nature of the antigen or pathogen, its live or inactivated form, its local concentration, and the frequency and length of immune system stimulation influence the serum or local antibody response to a pathogen (Petrin et al., 1998). An antibody response cannot be detected in some cases, either because the system is not sensitive enough to detect low levels of specific antibodies or because the serum humoral response has not yet been elicited (Petrin et al., 1998). Current and previous infections may not be distinguished because trichomonal antibodies can persist for a long time after treatment (Petrin et al., 1998).

2.10 Risk factors for *T. vaginalis* infection

Several factors contribute to the high risk of *T. vaginalis* infection. These factors include unprotected sex, past trichomoniasis infection, multiple sex partners, poor hygiene, menstrual cycle, age (>20 years),

the use of contraceptives, and having a history of STIs (Gatti et al., 2017). Another study conducted in San Francisco, United States, reported that *T. vaginalis* infection is also associated with rectal intercourse (anal sex) (Bax et al., 2011). A multivariate analysis was also conducted which identified two factors that could contribute to the high prevalence of vaginal *T. vaginalis*. This study observed the association of *T. vaginalis* with a single relationship status (de Waaij et al., 2017). In South Africa, de Waaij et al. (2017), reported that *T. vaginalis* infection is associated with being HIV positive, the use of hormonal contraceptives, and being unmarried. Nyemba et al. (2020) also reported risk factors for STIs during pregnancy and these included, younger maternal age, increased gestational age, HIV status and being unmarried/not cohabitating (Nyemba et al., 2020). Mabaso et al. (2020) reported a high prevalence of *T. vaginalis* infection among pregnant women who were not married (91.5%), who had a regular sexual partner (80.9%), and who were not living with their partner (57.4%). Their study also showed a 59.6% prevalence of *T. vaginalis* infection in asymptomatic women.

2.11 Prevalence of *T. vaginalis* in pregnant women

In pregnant women, *T. vaginalis* is associated with low birth weight, preterm delivery, and premature rupture of membranes (von Glehn et al., 2017). In Nigeria and Tanzania, the prevalence of *T. vaginalis* ranges from 1.0 to 2.0% in antenatal populations (Kamga et al., 2019; Olowe et al., 2014). In South Africa, prevalence rates for women attending antenatal clinics range from 10 to 52% (Price et al., 2018), (Dessai et al., 2020; Mabaso et al., 2020). The prevalence rates of *T. vaginalis* was found to be: 1% in rural Vietnam (Lan et al., 2008), 0.37% in Belgium (Depuydt et al., 2010), and 2.9% in China (Huang et al., 2007). In Brazil, pregnant women have a prevalence of 7.7% for *T. vaginalis* (Gatti et al., 2017). De Waaij et al. (2017) investigated the prevalence of *T. vaginalis* infection in Mopani District, South Africa, and found the prevalence rates to be very high in rural areas (95% CI 17.0% to 23.4%). Another study on women attending a STD clinic in Mumbai, India, reported a prevalence of 14.3% for pregnant women (Sugathan, 2016).

2.12 Genome of *T. vaginalis*

The first draft genome around two-thirds of the 160-megabase of *T. vaginalis*, was published in 2007 (Carlton et al., 2007). The genome contains about 60,000 genes spread across six chromosomes, is made up of repeats and transposable elements (Carlton et al., 2007). *Trichomonas vaginalis* viruses (TVVs) are found in some strains of *T. vaginalis*, with some strains harboring up to four different TVV species at the same time (Goodman et al., 2011). Because of its broad usage by researchers in investigations of virulence, biochemistry, cell and molecular biology, and its capacity to develop axenically without being infected with dsRNA viruses, the *T. vaginalis* isolate G3 from a patient from Kent, England, was chosen as the genome reference strain (Carlton et al., 2007).

2.13 Genetic variation in *T. vaginalis*

Organisms undergo mutations due to normal cellular operations or interactions with the environment, causing genetic variation or polymorphism (Liu & Cordes, 2004). Understanding the transmission dynamics, strain virulence, and pathophysiology of *T. vaginalis* infection, is vital for safeguarding patients, particularly female patients, from major disease and reproductive health risks. *T. vaginalis* genetic diversity and population structure, on the other hand, are mostly unknown (Vanácová et al., 1997). Typing methods such as antigenic characterization, isoenzyme analysis, repetitive sequence hybridization, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis, and sequence polymorphism have been employed to type *T. vaginalis* isolates to try and find correlations between genotypes and biologically relevant phenotypes or geographical distribution (Crucitti et al., 2008). Single nucleotide polymorphisms (SNPs) (base insertion, deletion and substitution) and microsatellite loci are genetic markers useful in molecular population genetics (Conrad et al., 2011). Mao & Liu (2015) conducted a study in Henan, China, to determine the genetic diversity of *T. vaginalis* isolates using 18S rRNA halotypes. They analysed their results using a phylogenetic tree as shown in figure 5. SNPs can influence the activity of a protein when they occur in a coding sequence and result in an amino acid change (also known as a non-synonymous SNP or mutation). Translation rates or mRNA half-life may be changed if the mutation is synonymous (that is, it does not change the nature of the amino acid) (Mendell & Dietz, 2001). A premature stop codon caused by a mutation might result in the synthesis of a shortened protein (Nicholson et al., 2010). If the mutation results in a premature stop codon, nonsense-mediated decay can result in a shortened protein product or a near-null phenotype (Nicholson et al., 2010).

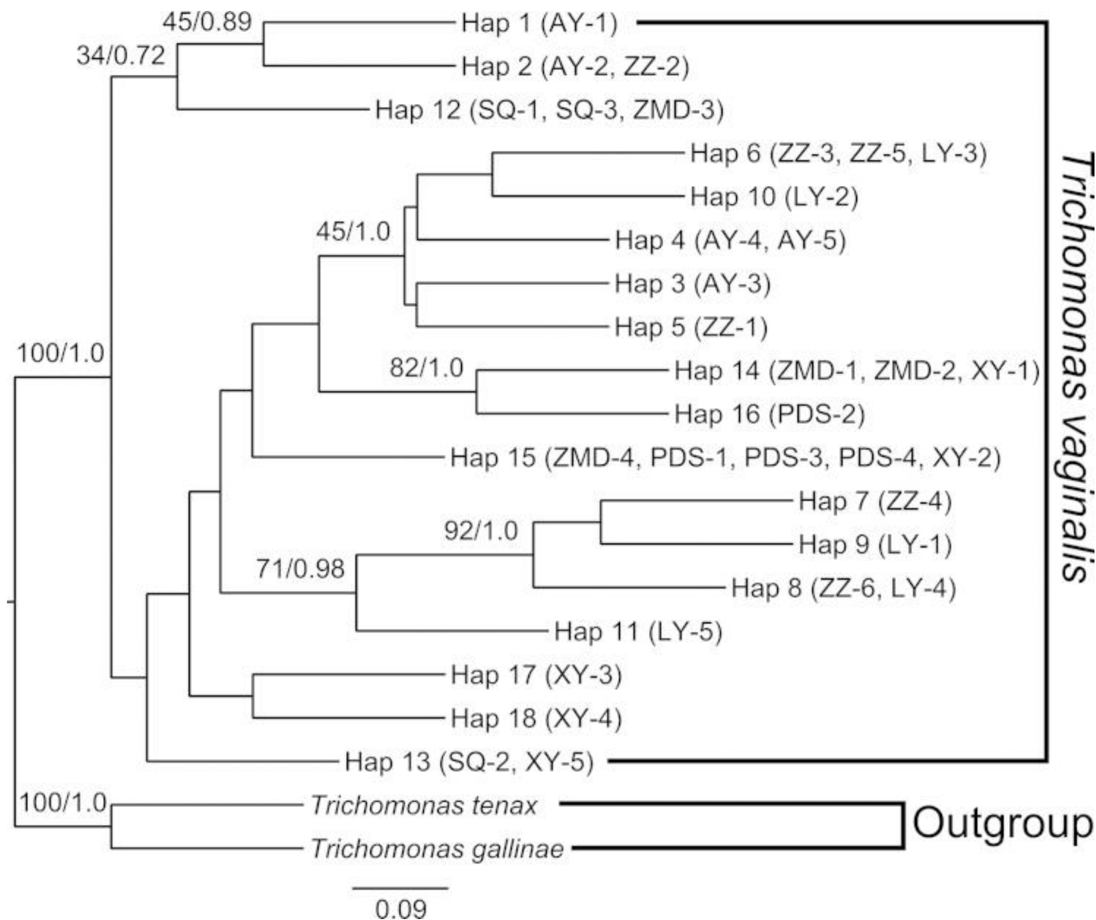

Figure 5: A phylogenetic analysis of *T. vaginalis* isolates from Henan province inferred by maximum parsimony and bayesian inference based on 18S *rRNA* sequences, using *T. gallinae* and *T. tenax* as the outgroup (Mao & Liu, 2015).

2.14.1 Nitroreductases and nitroimidazoles

Nitroreductases are a group of proteins that help to reduce nitro-containing substances (Christopher D. Gwenin, Kalaji, Williams, & Kay, 2011). These enzymes' versatility makes them useful in a wide range of biological and medical applications. They are used in environmental decontamination (bioremediation) (van Dillewijn et al., 2008), cancer therapy, treatment of parasitic diseases and explosives detection (Gwenin et al., 2008). There are two types of NTRs: bacterial NTRs and flavin reductases figure 6 (Zenno et al., 1994). Flavin reductases have flavin mononucleotide as a cofactor which uses the ping-pong bi-bi kinetic mechanism to catalyse (Gwenin et al., 2011; Roldán et al., 2008). Bacterial NTRs split into aerobic (oxygen insensitive) and anaerobic (oxygen sensitive) (Thomas & Gwenin, 2021). The function of NTRs and not well elucidated, but scientists have proposed that they play a role in detoxification as they are responsible for reducing a wide range of compounds (Roldán et al., 2008). NTRs may also help in responding to oxidative stress which protects against oxidative

damage (Liochev, Hausladen, & Fridovich, 1999). Since NTRs are able to affect mutagenic, toxic and carcinogenic properties of nitroaromatics, nitrofuran compounds have been utilized to create nitroimidazoles, a class of antimicrobials (Ang et al., 2017).

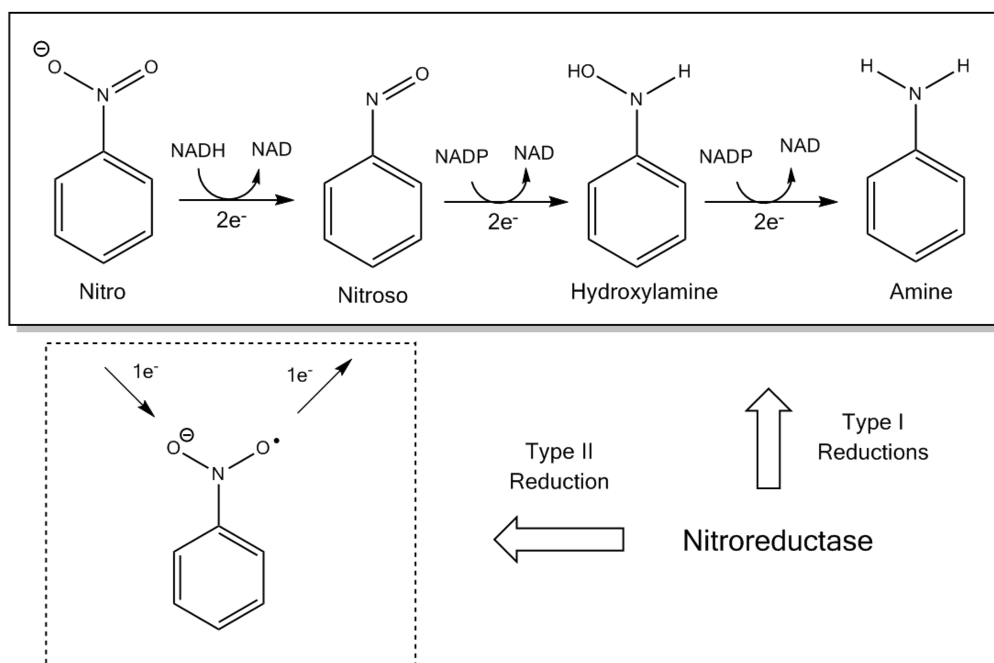

Figure 6: The nitro group reduction process and the respective electron transfers required (Powell, 2020)

Nitroimidazoles are a class of antimicrobial drugs have a wide range of activity against parasites, mycobacteria, and anaerobic Gram-positive and Gram-negative bacteria (Ang et al., 2017). They play a significant role in the fight against antibiotic resistance. They are prodrugs that have to reduce the nitro group before they can show effectiveness (Thomas & Gwenin, 2021)

2.14.2 Metronidazole

Individuals suffering from trichomoniasis are treated with metronidazole, an antimicrobial that belongs to the family of 5 nitroimidazoles ($C_3H_3N_3O_2$) drugs (Cudmore et al., 2004). Metronidazole may have a 95% success rate in treating *T. vaginalis* infection together with tinidazole and secnidazole (Cudmore & Garber, 2010), however, tinidazole and secnidazole are not yet available in South Africa. A 2 mg oral single dose of metronidazole or tinidazole regimens, and 400-500 mg oral dose of metronidazole twice a day for seven days, are recommended by the WHO and Centre for Disease Control (CDC) to treat *T. vaginalis* infection (Workowski & Berman, 2011). Patients who are undergoing treatment should abstain from alcohol consumption for 24 hours after the completion of metronidazole or 72 hours after tinidazole completion (Kissinger, 2015). If the patient remains untreated after single-dose

metronidazole therapy, a 7-day dose of metronidazole or single-dose tinidazole acts as a substitute (Kissinger, 2015).

Metronidazole is administered as a prodrug and the mechanism of action is summarized as follows: (i) molecules gain entry into the cell using passive diffusion, (ii) the nitro group is reduced to reactive oxygen species and (iii) free radicals act upon DNA or protein within the cell (Ang et al., 2017) this process is shown in figure 5. The hydrogenosome has a significant role in drug activation (Kulda, 1999; Müller, 1993).The redox potential of the compound and the number of electrons transferred are responsible for the reduction of products inside the cell (Edwards, 1993). Metronidazole is recommended for the following qualities: it acts as gynaecological prophylaxis, it is easy to administer (oral, topical, intra-vaginal or intravenous routes), has minimal side effects and is cost-effective (Lofmark et al., 2010).

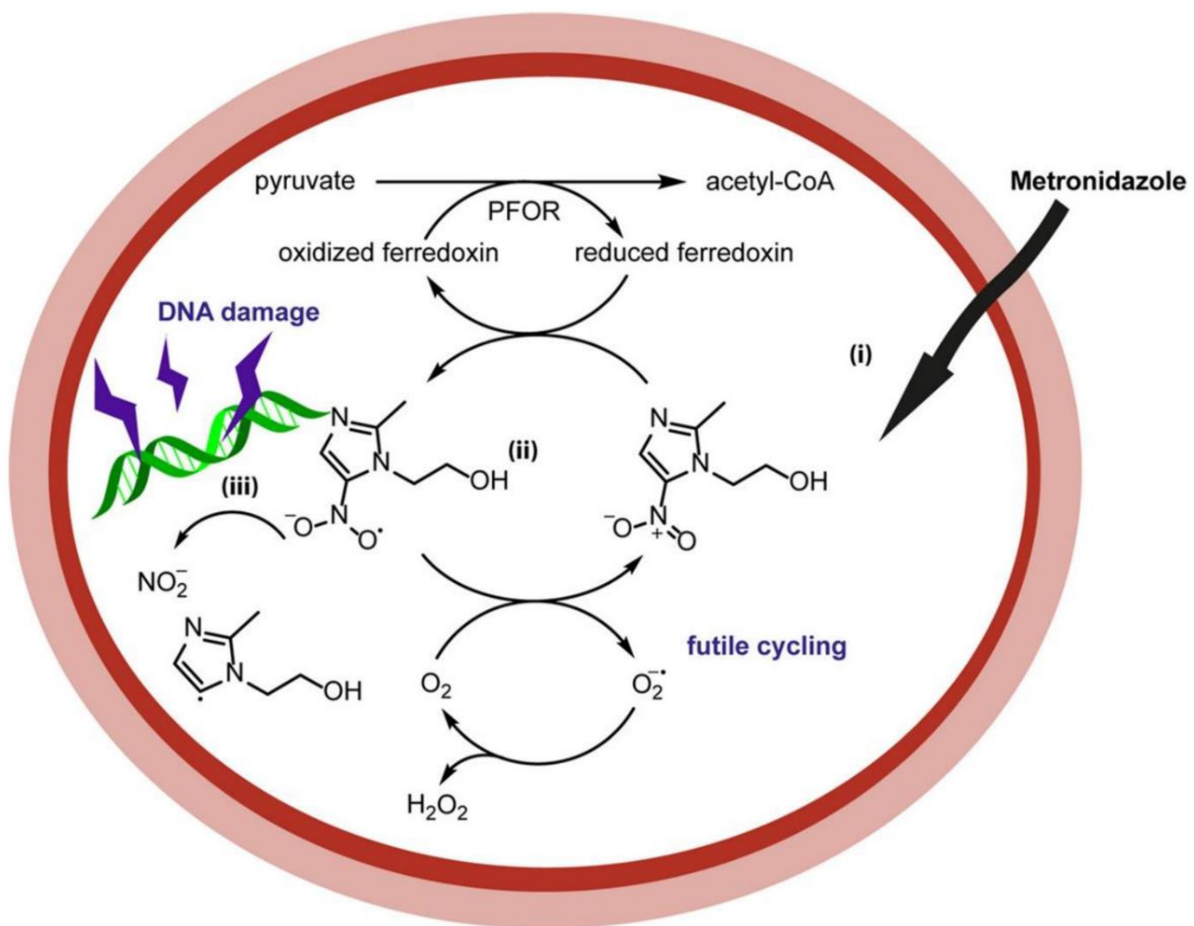

Figure 7: Mechanism of metronidazole action (Edwards, 1993)

2.15 Emerging patterns of resistance to metronidazole and the resistance mechanisms

The occurrence of *T. vaginalis* repeat infections ranges from 5% to 31% and have outcomes that are similar to primary infections (Kissinger et al., 2008; Krashin et al., 2010). Although the rate of *T. vaginalis* infections are unbelievably high, the sources are not yet understood (Kissinger, 2015). A

potential reason is drug resistance (Kissinger, 2015). Several studies of metronidazole-resistant *T. vaginalis* isolates (Dunne et al., 2003) have been published, with resistance classified as aerobic or anaerobic. The formation of resistance occurs in stages, with aerobic resistance coming first. Clinical isolates from patients who have failed to respond to therapy develop resistance (Upcroft & Upcroft, 2001). Mabaso & Abbai (2021) reported the following findings on metronidazole susceptibility under anaerobic conditions: 9.5% of *T. vaginalis* resistant isolates had a MIC of 4 μg/ml, 38.1% intermediate isolates had a MIC of 2 μg/ml, and 52.4% isolates had a MIC $\leq$ 1 μg/ml which were susceptible (Mabaso & Abbai, 2021).

There are several mechanisms of metronidazole resistance that have been reported (Dunne et al., 2003). Anaerobic mechanisms include the change in hydrogenosome structure, the decreased activity of the (PFOR), increased oxygen scavenging abilities (Superoxide dismutase (SOD), catalase/peroxidase). Aerobic resistance is caused by ferredoxin's interaction with intracellular oxygen, which results in reduced levels of intracellular ferredoxin (Meri et al., 2000). These mechanisms result in the occurrence of pyruvate fermentation in the cytosol instead of the hydrogenosome, inhibiting nitroimidazole action (Meri et al., 2000). Metronidazole gains entrance via passive diffusion into the cell and the organelle then competes with the terminal enzyme hydrogenase, an electron acceptor from ferredoxin (Kulda, 1999). The major hydrogenosomal enzyme, PFOR, generates the electrons required for drug reduction in a susceptible strain of *T. vaginalis* (Rasoloson et al., 2001; Cudmore et al., 2004). PFOR catalyzes pyruvate's oxidative decarboxylation to acetyl-CoA and CO2 (Rasoloson et al., 2001; Cudmore et al., 2004). The ferredoxin accepts the electrons released in the PFOR process, which is then re-oxidized by the enzyme. The drug is activated when there is electron transfer to all the important nitro-group, thereby forming a toxic nitro-radical also known as free radicals or reactive oxygen species (ROS) (Upcroft & Upcroft, 2001). These radicals result in chromosomal breakage and apoptosis in bacteria (Dunne et al., 2003).

Furthermore, the presence of metronidazole causes damage to the production of molecular hydrogen, which in turn increases intracellular hydrogen peroxide (Brown et al., 1999; Kulda, 1999). Under aerobic conditions, oxygen causes the conversion of radicals back to metronidazole. Therefore, when susceptibility assays are conducted aerobically, drug inhibitory concentrations tend to be higher than those that are conducted anaerobically (Upcroft & Upcroft, 2001). In addition, there have been studies conducted on the emergence of metronidazole resistant *T. vaginalis* (Coelho, 1997). Resistance arises when there are multiple metronidazole treatment courses for either trichomoniasis or any other infection (Dunne et al., 2003). Aerobic resistance arises when metronidazole competes with oxygen for electrons, which is suggested to damage oxygen scavenging species enabling the increase in unnecessary recycling of metronidazole (Dunne et al., 2003). During aerobic resistance, oxygen reduces the levels of metronidazole toxicity by re-oxidizing nitroradical anions before they cause damage to the parasite, reverting metronidazole to an inactive form (Bradic et al., 2017). Studies suggest that metronidazole

resistant strains of *T. vaginalis* have insufficient levels of the ferredoxin protein, which is important for the drug's activation (Quon, d'Oliveira, & Johnson, 1992). In a study conducted by Paulish-Miller et al. (2014), the researchers identified stop codons in genes that encode two putative *nitroreductases* associated with metronidazole resistance (Paulish-Miller et al., 2014). The *T. vaginalis* genome encodes for 11 putative *nitroreductase* genes, of which more than one of those genes could reduce metronidazole (Paulish-Miller et al., 2014).

The presence of stop codons in *ntr4*$_{TV}$ and *ntr6*$_{TV}$ was associated with metronidazole resistance (Snipes et al., 2000). Other causes of treatment failure, such as non-compliance, vomiting after oral metronidazole administration, re-infection from an infected partner, infection from a new partner, poor drug absorption, and inadequate drug transfer to the site of infection, should all be considered when evaluating *T. vaginalis* resistance (Masha et al., 2019).

2.16 Rationale

Previous studies have identified the possible genes that contribute to metronidazole resistance via biochemical assays. However, our understanding of metronidazole resistance in pregnant women with *T. vaginalis* infection is limited. This lack of data has sparked our interest in identifying mutations that may contribute to drug resistance or susceptibility. In addition, the detection and mutation scanning of these genes is under-investigated in pregnant women. Our study attempted to fill this gap in research. This study aimed to detect mutations associated with metronidazole resistance and susceptibility in *T. vaginalis s*trains isolated from South African pregnant women attending King Edward Hospital. We achieved the aim by amplifying genes associated with metronidazole resistance and sequencing the positive amplicons. The sequencing of these specific genes enabled us to detect specific mutations associated with resistance or susceptibility.

2.17 Hypothesis

Metronidazole resistance and susceptibility are associated with specific mutations in *nitroreductase* and *PFOR* genes.

2.18 Aims

To detect and identify mutations in genes associated with metronidazole resistance and susceptibility in *T. vaginalis* isolates, isolated from pregnant women attending King Edward VIII hospital.

2.19 Objectives

1. To perform metronidazole susceptibility assays on clinical isolates of *T. vaginalis* isolated from pregnant women
2. To amplify and sequence the *nitroreductase* and *PFOR* genes from the clinical isolates
3. To perform mutation scanning of the PCR amplicons across metronidazole susceptible, intermediate and resistant isolates

4. To link clinical factors such as abnormal vaginal discharge and previous treatment of STIs with patterns of resistance

2.20 Outputs

A research article titled: "Detection of mutations in the *nitroreductase* genes of *Trichomonas vaginalis* across metronidazole susceptibility patterns"- by Tumelo Mzenda, Nonkululeko Mabaso and Nathlee Abbai, has been submitted for publication in the Journal of Parasitology (#PARE-D-21-00322).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethics Statement

The Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (BREC/00001930/2020) approved the current study (see appendix A).

3.2 Study design and population

This study was a retrospective laboratory-based study that used stored isolates of *T. vaginalis*. The isolates were obtained from pregnant women attending the antenatal clinic at the King Edward VIII hospital in Durban, South Africa, during October 2018 to March 2019. The women were asked to provide self-collected vaginal swabs. Instructions on how to collect the swabs were provided to the women. Upon collection the swabs were immediately placed in Diamond's TYM medium (Diamond, 1957). Diamond's TYM medium was supplemented with amikacin (4µg/ml), amphotericin B (5µg/ml), ampicillin (1mg/ml), chloramphenicol (1µg/ml), ciprofloxacin (2µg/ml) and vancomycin (5µg/ml). The tubes were transported within 1 hour of inoculation to the School of Clinical Medicine Research Laboratory, at the University of KwaZulu-Natal for processing.

3.3 Diagnosis and propagation of *T. vaginalis* clinical isolates

Upon arrival at the laboratory, the culture tubes were incubated at 37° C for 7 days. Cultures were examined daily from day 2 to 7 by wet mount microscopy. A specimen was considered positive for *T. vaginalis* infection if cells with jerk motility were observed. A sample was considered negative if no trophozoites were observed on day 7. A sub-culture of positive samples was performed by transferring 500µl of the culture into 5ml fresh Diamond's TYM medium supplemented with amikacin, amphotericin B, ampicillin, chloramphenicol, ciprofloxacin and vancomycin at 48-hour intervals until non-contaminated axenic cultures were obtained. The pure isolates were stored at -80°C until further use. A total of 21/362 (5.8%) pregnant women tested positive for *T. vaginalis* infection. Twenty-one isolates were available for analysis in this study.

3.4 Revival of *T. vaginalis* stored isolates

The vials of stored axenic cultures were removed from -80°C storage and placed for 2-4 minutes in a water bath set at 37°C, until completely thawed. The entire contents of each vial were immediately inoculated into Diamond's media, which was pre-warmed to 37°C. The inoculated media were placed in a 37°C incubator with 5% CO_2. After 24 hours, the cultures were examined by wet mount microscopy at 400X magnification. If enough growth was observed, 500µl of the culture was transferred aseptically into 5ml of Diamond's TYI medium. If insufficient numbers of viable trichomonads were present, the culture was topped-up to 10 mL with fresh Diamond's media. This was done to dilute excess dimethylsulfoxide (DMSO). Microscopic examination was thereafter performed after 48 and 72 hours

of incubation to monitor growth. If growth was observed, a subculture was made into fresh antibiotic-free Diamond's medium.

3.5 Metronidazole susceptibility assay

Metronidazole susceptibility assays were performed in 96 well flat-bottomed microtiter plates under aerobic and anaerobic incubation conditions. Two-fold serial dilutions of metronidazole were made using Diamond's TYM medium. The resulting concentrations of metronidazole ranged from 3.1 µg/ml to 200 µg/ml for the aerobic assay and 0.25 µg/ml to 16 µg/ml for the anaerobic assay. *T. vaginalis* cultures were then standardized to an inoculum of $1x10^5$ and $1.5x10^4$ trichomonads/well for the aerobic and anaerobic assays, respectively. The *T. vaginalis* ATCC 50148 strain was used as a control strain and untreated cultures of the respective isolates were used as growth controls. For the aerobic conditions, the plates were incubated at 37°C for 48 hours. Under anaerobic conditions, plates were incubated in air-tight anaerobic jars containing Oxoid™ AnaeroGen™ 2.5L gas packs (ThermoFisher Scientific, United States) and Oxoid™ Resazurin Anaerobic indicator strips (ThermoFisher Scientific, United States), at 37°C for 48 hours. *T. vaginalis* motility and growth were assessed using the inverted microscope at 400X magnification. *T. vaginalis* growth and motility were scored according to the scoring criteria described by Upcroft & Upcroft (2001). Trophozoite numbers were scored 1+ (0-10 motile parasites- not more than 20% coverage of well surface and significantly less active), 2+ (20 to 50% coverage of the well surface and some trophozoite motility), 3+ (more than 50 % coverage of the well surface, almost confluent growth with much motility), and 4+ (confluent growth with full motility) (Upcroft & Upcroft 2001). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of metronidazole in which a score of 1+ was observed after 48 hours of incubation. Breakpoints suggested by Upcroft & Upcroft (2001) were used for the anaerobic assay. MIC ≤ 1 µg/ml was considered susceptible, MIC = 2 µg/ml was considered intermediate (low-level resistance), and MIC ≥4 µg/ml was considered resistant (Upcroft & Upcroft 2001). For the aerobic assay, MIC ≤ 25 µg/ml was considered susceptible, MIC of 50 to 100µg/ml was considered intermediate (low-level resistance), and MIC of 200µg/ml was considered moderate-level resistant (Ghosh et al., 2018). All experiments were performed in triplicate for each *T. vaginalis* isolate.

3.6 *T. vaginalis* Genomic DNA extraction

T. vaginalis DNA was extracted from all 21 isolates using the phenol-chloroform method (Shaio, Lin, & Liu, 1997). The culture was centrifuged (Eppendorf micro centrifuge 5415C, United States) twice at 1500 xg for 10 minutes, and the supernatant was discarded after every spin. The cells were washed twice in phosphate buffered saline (PBS), pH 7.4 using centrifugation at 1500 xg for 10 minutes and the supernatant was discarded after each wash. Approximately, 500 µl of lysis buffer (300 µl sodium citrate, 90 ml sodium chloride, 400 µl SDS and 200 µl proteinase K) was added, the sample was mixed and transferred into a clean tube. The mixture was then incubated in a water bath at 65°C for 30 minutes, and then cooled to room temperature. DNA was purified by adding equal volumes of phenol chloroform

(1:1; vol/vol) and centrifuged at 1500 xg for 10 minutes. The aqueous phase (clear top phase) was transferred into a clean tube. An equal volume of chloroform was added and centrifuged at 1500 xg for 10 minutes. The aqueous phase was transferred into a clean tube. DNA extracts were then precipitated with 2 volumes of 95% ethanol (vol/vol) and 0.1 volume of 3 M sodium acetate (pH 5.2). The DNA pellets were washed with 70% (vol/vol) ethanol, air-dried at room temperature and dissolved in 50 µl of T_{10} E_1 (TE) buffer. The concentration and purity of the extracted DNA was measured using the NanoDrop Spectrophotometer (ThermoFisher Scientific, United States).

3.7 Confirmation of *T. vaginalis* by *18S ribosomal RNA* PCR

A forward primer S1 (5'-TCCCGGATAATTGAAACGGA-3') and a reverse primer S2 (5'-GAATGTGATAGCGAAATGGG-3') were used to amplify a region of approximately 413-bp within the 650-bp repeat region of the 18S rRNA. These primers were modified from a previous study conducted by Paulish-Miller et al. (2014). The primers PCR was performed in a total volume of 50 µl. The reaction mixture contained 16 µl of nuclease-free PCR water, 25 µl of the DreamTaq PCR Master Mix (ThermoFisher Scientific, United States), 2 µl of each primer and 1 µg of template DNA. The cycling conditions were initial denaturation at 94ºC for 3 minutes followed by 40 cycles at 94ºC for 45 seconds, 50ºC for 40 seconds, 72ºC for 1 minute and a final extension at 72ºC for 7 minutes. PCR amplification was performed in a T100 thermocycler (BioRad, United States). PCR products were analyzed by electrophoresis on a 1% agarose gel in 0.5 X TBE buffer, stained with novel juice (Bio-Helix Co., Ltd) at 80 V and viewed under UV illumination (Gene Genius System).

3.8 PCR amplification of the *T. vaginalis nitroreductase* and *PFOR* genes

Five *nitroreductase* genes and a *PFOR* gene were PCR amplified from 21 isolates to identify polymorphisms associated with metronidazole resistance. The ATCC 50148 strain was used as a positive control and no template DNA indicated the negative control. PCR was performed in a total volume of 50 µl with each reaction containing 25 µl of DreamTaq PCR Master Mix, 1µl (0.1-1.0 µM) of each primer, 2 µl of template DNA and 21 µl of nuclease-free PCR water. Cycling conditions were as follows: initial denaturation at 95ºC for 2 minutes followed by 30 cycles at 95ºC for 1 minute, annealing was at a temperature specific for each gene primer for 1 minute, extension at 72ºC for 2 minutes; and a final extension cycle at 72ºC for 5 minutes. Primers used to perform the PCR amplification reactions are listed in Table 1. PCR products were analyzed by gel electrophoresis on a 1% agarose gel in 0.5X TBE buffer at 80 V and viewed under UV illumination (Gene Genius System).

Table 1: Primers used in PCR amplification of the *nitroreductase* and *PFOR* genes (Paulish-Miller et al., 2014).

Primers	Sequence (5'-3')	Amplicon size (bp)
tvntr2		594
Forward	CCAAAGGCACAAATAAACAATTTT	
Reverse	TCTATACAAATTATGAACCATCAT	
tvntr3		549
Forward	ATGAGTGTTCTCAAGTGCATCCAA	
Reverse	TTAGTCAATATGAGTAATCTTTCC	
tvntr4		549
Forward	ATGAGTGTCCTTAAGTGCATCCAA	
Reverse	TTAGTCGGCATAAACTACCTTAGA	
tvntr5		943
Forward	AAGGAATAAGATGAAATAATAAAA	
Reverse	TTGGATATAGATATCCGCAGAAAT	
tvntr6		743
Forward	CATTGAATTTATTCGTTCAAAATT	
Reverse	TTATTCAATGTATGTAACCTTTCT	
TV PFOR		~200
Forward	CTGCAAGCTCCTTACACAGC	
Reverse	AAGAGGGAGTTAGCCCAAGC	

3.9 DNA sequencing and mutation analysis

Sanger DNA sequencing was performed on the *nitroreducatase* and *PFOR* PCR amplicons. The sequencing was conducted using the BrilliantDye™ Terminator v3.1 Cycle Sequencing on an ABI3500XL genetic analyser. The sequencing was performed at Inqaba Biotechnical Industries (Hatfield, Pretoria, South Africa). The ABI sequencing files were edited on CHROMAS (Technelysium, Queensland, Australia). The forward and reverse sequences were aligned using the DNAMAN software (Lynnon Biosoft, California, United States). The identity of the edited sequences was confirmed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). The edited sequences from susceptible, intermediate and resistant isolates were aligned using Clustal W (https://www.genome.jp/tools-bin/clustalw). The alignments were performed in order to identify mutations across the resistance profiles.

CHAPTER FOUR

RESULTS

4.1 Confirmation of *T. vaginalis* by *18S ribosomal RNA* PCR

A total of 21/362 (5.8%) pregnant women tested positive for *T. vaginalis* infection. The expected size for the *18S rRNA* gene product (413 bp) was present in all 21 isolates tested. The PCR was used to confirm the identity of the isolates before proceeding to the mutation analysis. To further confirm the identity of the isolates, the PCR products were sequenced using the Sanger approach. The results of the sequencing revealed that the isolates showed 100% identity to the *Trichomonas vaginalis* G3 strain (XM_001292180.1).

4.2 Metronidazole susceptibility patterns

The *in vitro* aerobic metronidazole MICs ranged from 3.1 to 50 µg/ml and the mean MICs ± standard deviation was 7.73 ± 11.08. Of the 21 *T. vaginalis* isolates tested for anaerobic metronidazole susceptibility, 9.5% (2/21) had an MIC of 4 µg/ml (resistant), 38.1% (8/21) had an MIC of 2 µg/ml (intermediate) and 52.4% (1/21) had an MIC ≤ 1 µg/ml (susceptible) (Figure 5). The MIC of the *T. vaginalis* ATCC 50148, control strain was 1µg/ml for the anaerobic assay and 25µg/ml for the aerobic assay, which was within the expected range (Muller et al. 1988). One *T. vaginalis* isolate (TV 128), was unable to be revived after storage for performing the aerobic assays. Of the 20 *T. vaginalis* isolates tested for aerobic metronidazole susceptibility, 95% (19/20) had a MIC of ≤ 25 µg/ml, and 5% (1/20) had a MIC of 50 µg/ml (Figure 8). Anaerobic metronidazole MICs ranged from 0.25 to 4 µg/ml, and the mean MICs ± standard deviation was 1.63 ± 0.95.

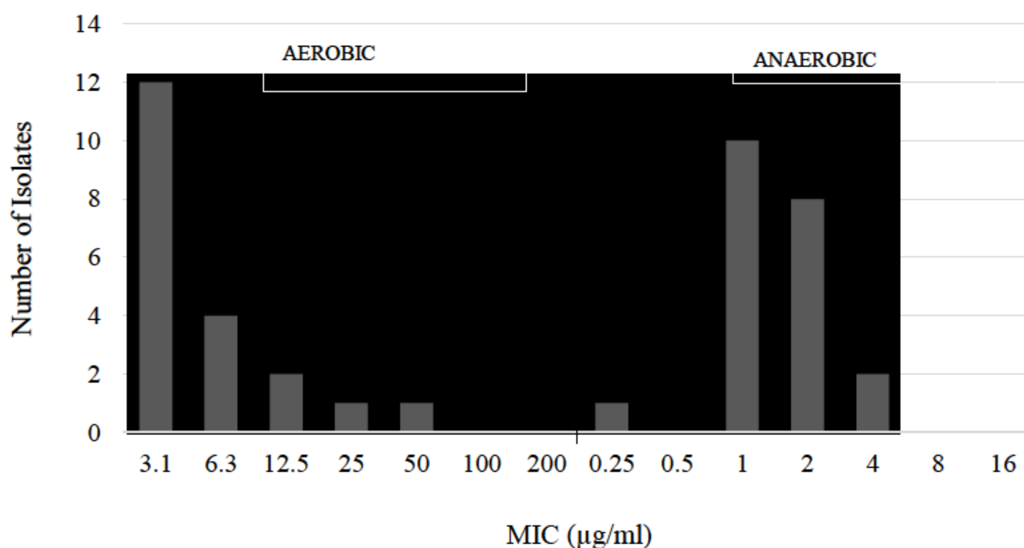

Figure 8: The distribution of metronidazole aerobic and anaerobic MICs for *T. vaginalis* clinical isolates. Aerobic metronidazole susceptibility is shown on the left (3.1 – 200µg/ml) and anaerobic metronidazole susceptibility is on the right (0.25 – 16µg/ml).

4.3 Clinical and behavioural factors associated with metronidazole susceptibility patterns

Table 2 describes the characteristics of the study population according to metronidazole susceptibility patterns.

Table 2: Characteristics of the study population according to metronidazole susceptibility patterns.

Metronidazole anaerobic susceptibility	Intermediate (N=8)	Resistant (N=2)	Susceptible (N=11)	p-value	Overall (N=21)
Has a regular sex partner				0.305	
No	0 (0%)	0 (0%)	3 (27.3%)	Fisher's	3 (14.3%)
Yes	8 (100%)	2 (100%)	8 (72.7%)		18 (85.7%)
Co habiting with sex partner				1.000	
No	6 (75.0%)	2 (100%)	7 (63.6%)	Fisher's	15 (71.4%)
Yes	2 (25.0%)	0 (0%)	4 (36.4%)		6 (28.6%)
Age 1st sex				0.500	
<15	0 (0%)	0 (0%)	1 (9.1%)	Fisher's	1 (4.8%)
15_20	7 (87.5%)	1 (50.0%)	9 (81.8%)		17 (81.0%)
21_25	1 (12.5%)	1 (50.0%)	1 (9.1%)		3 (14.3%)
Lifetime number of sex partners				0.741	
>4	3 (37.5%)	0 (0%)	3 (27.3%)	Fisher's	6 (28.6%)
1	0 (0%)	0 (0%)	2 (18.2%)		2 (9.5%)
2_4	5 (62.5%)	2 (100%)	6 (54.5%)		13 (61.9%)
Condom use				0.701	
Never	3 (37.5%)	0 (0%)	5 (45.5%)	Fisher's	8 (38.1%)
Rarely	0 (0%)	0 (0%)	1 (9.1%)		1 (4.8%)
Sometimes	5 (62.5%)	2 (100%)	5 (45.5%)		12 (57.1%)
Trimester				0.773	
2nd	2 (25.0%)	1 (50.0%)	2 (18.2%)	Fisher's	5 (23.8%)
3rd	6 (75.0%)	1 (50.0%)	9 (81.8%)		16 (76.2%)
Current vaginal discharge				0.273	
No	5 (62.5%)	0 (0%)	8 (72.7%)	Fisher's	13 (61.9%)
Yes	3 (37.5%)	2 (100%)	3 (27.3%)		8 (38.1%)
Treated for sexually transmitted infections in the past				0.246	
No	7 (87.5%)	1 (50.0%)	6 (54.5%)	Fisher's	14 (66.7%)
Yes	1 (12.5%)	1 (50.0%)	5 (45.5%)		7 (33.3%)
Having symptoms of sexually transmitted infections in the past 3 months before enrolment				1.000	
No	4 (50.0%)	1 (50.0%)	5 (45.5%)	Fisher's	10 (47.6%)
Yes	4 (50.0%)	1 (50.0%)	6 (54.5%)		11 (52.4%)

Behavioural factors

With reference to having a regular sex partner, 100% of the women infected with *T. vaginalis,* had displayed intermediate and resistant profiles to metronidazole when compared to 72.7% who were susceptible to metronidazole. The association between metronidazole susceptibility patterns and having a regular sex partner was not shown to be significant, p=0.305. Across the metronidazole susceptibility patterns (intermediate: 75.0%; resistant: 100%; susceptible: 63.6%), a higher proportion of women reported not cohabiting with their sex partner. Cohabitation status was not shown to be significantly associated with metronidazole susceptibility patterns, p=1.000. Overall, the majority of the women (81.0%) infected with *T. vaginalis*, had experienced their first sex between the ages of 15-20 years. Of these women, 87.5% had an intermediate profile, 50.0% had a resistant profile and 81.8% had a susceptible profile towards metronidazole, however this was not significant (p=0.500). In addition, 61.9% of the infected women reported having between 2-4 lifetime number of sexual partners with the majority being resistant to metronidazole (100%), followed by 62.5% who had an intermediate profile, and 54.5% who had a susceptible profile. There was no significant association between lifetime number of sexual partners and metronidazole susceptibility patterns, p=0.741. There was no significant association between condom use and metronidazole susceptibility patterns, p=0.701. Of the majority of the women who reported "sometimes" using condoms (57.1%), 62.5% had an intermediate profile, 100% had a resistant profile and 45.5% had a susceptibility profile for metronidazole (Table 3).

Clinical factors

The majority of the infected women were in the third trimester of pregnancy (76.2%). Of these women, 75.0% had an intermediate profile, 50.0% had a resistant profile and 81.8% had a susceptible profile towards metronidazole, however, trimester of pregnancy was not significantly associated with metronidazole susceptibility patterns (p=0.773). Of the 38.1% of the women who presented with symptoms of abnormal vaginal discharge, 37.5% had an intermediate profile, 100% had a resistant profile and 27.3% had a susceptible profile towards metronidazole, however, having an abnormal vaginal discharge was not significantly associated with metronidazole susceptibility patterns (p=0.273). Only 33.3% of the women had reported being treated for STIs in the past, 12.5% had an intermediate profile, 50.0% had a resistant profile and 45.5% had a susceptible profile towards metronidazole. This association was not significant, p=0.246. In addition, 52.4% of the infected women had indicated that they had experienced symptoms of STIs in the 3 months prior to enrolment in this study. Of the previously symptomatic women, 50.0% had an intermediate profile, 50.0% had a resistant profile and 54.5% had a susceptible profile towards metronidazole, this association was also not statistically significant, p=1.000 (Table 3).

4.4 Detection of mutations in *nitroreductase* and *PFOR* genes

A fragment size of 594 bp corresponding to the *ntr2* gene was amplified across 17/21 (80.9%) of the isolates (Figure 9a). Figure 7b shows the presence of mutations in the *ntr2* gene detected across *T. vaginalis* metronidazole susceptible, intermediate and resistant isolates. From the alignment it was observed that the resistant and intermediate isolates carried mutations which were absent in the susceptible isolate.

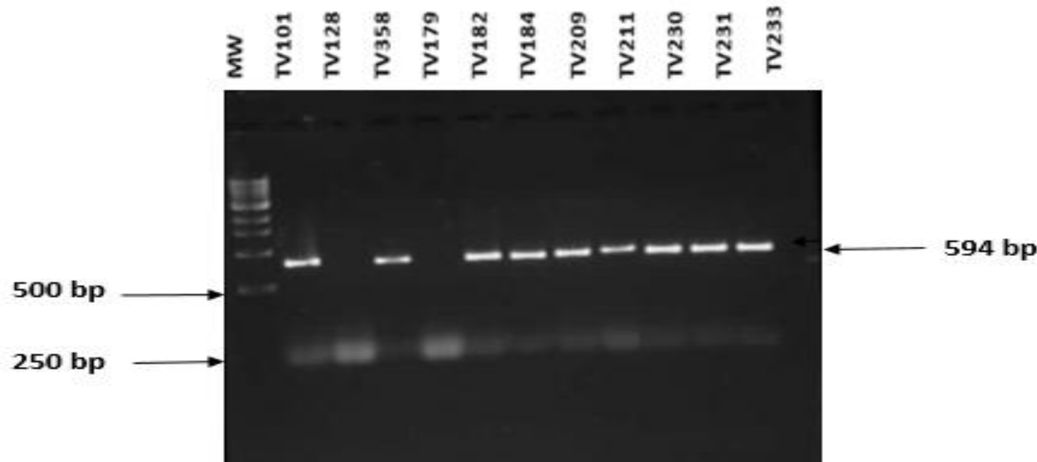

Figure 9a: Agarose gel electrophoresis showing positive amplicons generated for the *ntr2* gene. A fragment size of 594 bp was observed. Lane 1 represents the molecular weight marker (ThermoFisher Scientific) and the remaining lanes represent selected *T. vaginalis* isolates. Blanks on the gel indicate samples that did not amplify.

The following mutations (were observed for the *ntr2* gene in the selected resistant isolate when compared to the intermediate and susceptible isolate: T $\rightarrow$ G, T $\rightarrow$ A, C $\rightarrow$ A, T $\rightarrow$ C, A $\rightarrow$ T (Figure 9b). In addition, the susceptible isolate had some insertion mutations: G nucleotide, T nucleotide and an A nucleotide highlighted in red. The resistant isolate also had some insertion mutations highlighted in green (Figure 9b).

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CLUSTAL 2.1 multiple sequence alignment


Ntr2-182        AGTTACAAGGGATATGAAATCACTTGG-ATGTTCTCAATCTGAGTGATAGTATAGTGAAA
Ntr2-184        AGTTACAAGGGATATGAAATCACTTGGGAGGTTCTCAATCTGAGTGATAGTATAGTGAAA
Ntr2-253        AGGTACAAGGGATATGAAATCACTTGG-ATGTTCTCAATCTGAGTGATAGTATAGAGAAA

                ** *********************** * ************************ ****

Ntr2-182        TGTTCTAATCTCTCATTTGTAATCATATTTGCACTTGAATATTA-AAAAAAATCATTAAA
Ntr2-184        TGTTCTAATCTCTCATTTGTAATCATATTTGCACTTGAATATTA-AAAAAAATCATTAAA
Ntr2-253        TGTTATAATCTCTCATTTGTAATCATATTTGCACTTGAATATTAGAAAAAAATCATTAAA
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Ntr2-182        GTATAATATTTATTATGTTT-AGTAATAGTGGACCTTAGCTTTGATTGGCTTTGGTGGAA
Ntr2-184        GTATAATATTTATTATGTTTTAGTAATAGTGGACCTTAGCTTTGATTGGCTTTGGTGGAA
Ntr2-253        GTATAATATTTATTATGTTT-AGTAATAGTGGACCTCTGCTTAGATTGGCTTAGGTGGAA
                ******************** ***************  **** ********* *******

Ntr2-182        -TTGTTGGATGCTCGACACCCTTTGCAA-CTGCGACAGCCATAATGATATCTCCCTTCTT
Ntr2-184        ATTGTTGGATGCTCGACACCCTTTGCAAACTGCGACAGCCATAATGATATCTCCCTTCTT
Ntr2-253        -TTGTTGGATGCTCGACACCCTTTGCAA-CTGCGACAGCCATAATGATATCTCCATTCTC

                 *************************** ************************* ****

Ntr2-182        TACTGGAAGTGTATCAGAGAGATCTGAGTACATTACAGCGCCAACTGGCATTGTGACATA
Ntr2-184        TACTGGAAGTGTATCAGAGAGATCTGAGTACATTACAGCGCCAACTGGCATTGTGACATA
Ntr2-253        TACTGGAAGTGTATCAGAGAGATCTGAGTACATTACAGCGACAACTGGCATTGTGACATA

                **************************************** *******************
Ntr2-182        ACCAAGGCTGACAGCTGCAAGCATGATTGACTCTGCCATAATGCCAGCATCAACATACAC
Ntr2-184        ACCAAGGCTGACAGCTGCAAGCATGATTGACTCTGCCATAATGCCAGCATCAACATACAC
Ntr2-253        ACCAAGGCTGACAGCTGCAAGCATGATTGACTCTGCCATAATGCCAGCATCAACATACAC
                ************************************************************

Ntr2-182        GTATCTTTCGTCAGCACGCTCGTTTTTAACAACCATGAAGAATGCTTGGCAGTCACATGT
Ntr2-184        GTATCTTTCGTCAGCACGCTCGTTTTTAACAACCATGAAGAATGCTTGGCAGTCACATGT
Ntr2-253        GTATCTTTCGTCAGCACGCTCGTTTTTAACAACCATGAAGAATGCTTGGCAGTCACATGT
                ************************************************************

Ntr2-182        TATACAGTTCTTAACGCCAAGTTCTTCATATCTTTTCTTGAAAAATGGAACTGCTTTTGG
Ntr2-184        TATACAGTTCTTAACGCCAAGTTCTTCATATCTTTTCTTGAAAAATGGAACTGCTTTTGG
Ntr2-253        TATACAGTTCTTAACGCCAAGTTCTTCATATCTTTTCTTGAAAAATGGAACTGCTTTTGG
                ************************************************************

Ntr2-182        ATCAACTGATTCAAGAGACTTCTGCTCTATTTTAGCAGCAATTTCTGGGTTTTGGATTAC
Ntr2-184        ATCAACTGATTCAAGAGACTTCTGCTCTATTTTAGCAGCAATTTCTGGGTTTTGGATTAC
Ntr2-253        ATCAACTGATTCAAGAGACTTCTGCTCTATTTTAGCAGCAATTTATGGGTTTTGGATTAC
                ******************************************** ***************

Ntr2-182        ATAAACATCACATCCTTGGAAGCCACATGCAGATGGAGCCATCAATGCATGGTTTGCAAT
Ntr2-184        ATAAACATCACATCCTTGGAAGCCACATGCAGATGGAGCCATCAATGCATGGTTTGCAAT
Ntr2-253        ATAAACATCACATCCTTGGAAGCCACATGCAGATGGAGCCATCAATGCATGGTTTGCAAT
                ************************************************************

Ntr2-182        GGCTTCGATGTGTTCCTTTGGGATGACATAGTTATGATCATATTCTCTGATTGTTCTTCT
Ntr2-184        GGCTTCGATGTGTTCCTTTGGGATGACATAGTTATGATCATATTCTCTGATTGTTCTTCT
Ntr2-253        GGCTTCGATGTGTTCCTTTGGGATGACATAGTTATGATCATATTCTCTGATTGTTCTTCT
                ************************************************************


Ntr2-182        TGCTTCGATTGAGGAGAGAACGTCCATACTGAAGACTCTTTCTAAAAATGAACTGATTTT
Ntr2-184        TGCTTCGATTGAGGAGAGAACGTCCATACTGAAGACTCTTTCTAAAAATGAACTGATTTT
Ntr2-253        TGCTTCGATTGAGGAGAGAACGTCCATACTGAAGACTCTTTCTAAAAATGAACTGATTTT
                ************************************************************

Ntr2-182        TATTTGTGAATGCATTGGATAAATTAAGAACTCCTGCACATTGAAATACTTTCAAATTGA
Ntr2-184        TATTTGTGAATGCATTGGATAAATTAAGAACTCCTGCACATTGAAATACTTTCAAATTGA
Ntr2-253        TATTTGTGAATGCATTGGATAAATTAAGAACTCCTGCACATTGAAATACTTTCAAATTGA
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Figure 9b: Clustal W multiple alignment of the *ntr*2 gene. Sample 182 represents the intermediate isolate, 184 the susceptible and 253 the resistant isolates. Substitution mutations present in the resistant and susceptible isolates are highlighted in yellow, insertion mutations in the resistant isolates only are highlighted in green. Insertion mutations in the susceptible isolates only are highlighted in red.

Mutations were highlighted as per the figure and does not necessarily mean all the genes have the same mutation.

For the *ntr3* gene, the expected band size was 549 bp. PCR amplification was conducted on 11/21 (52.3%) of the isolates and only five of these isolates showed clear bands (Figure 10a). Absence of the PCR amplicon or band indicated that the isolate does not contain the gene or that non-specific binding had occurred.

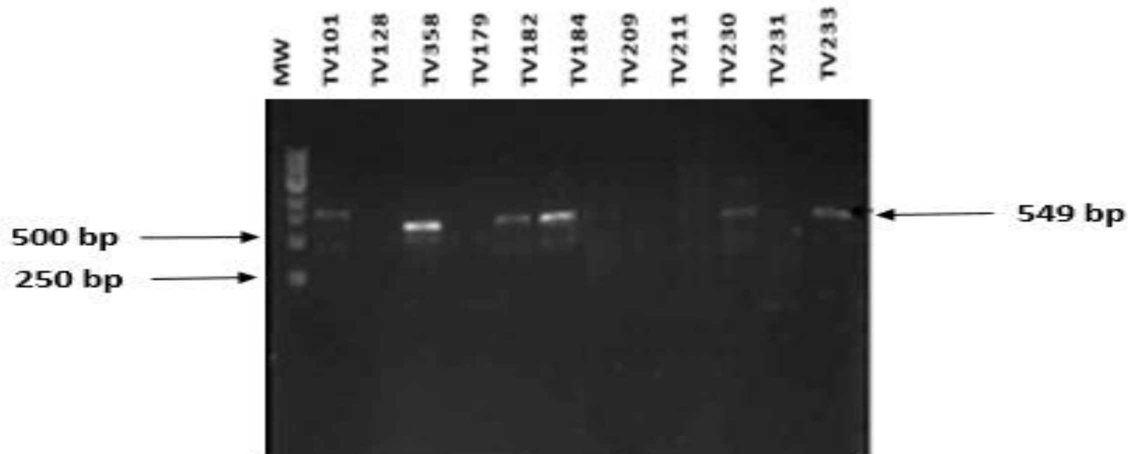

Figure 10a: Agarose gel electrophoresis showing positive amplicons generated for the *ntr3* gene. A fragment size of 549 bp was observed. Lane1 represents the molecular weight marker (Gene ruler 100 bp, ThermoFisher Scientific) and the remaining lanes represent selected *T. vaginalis* isolates. Blanks on the gel indicate samples that did not amplify.

Figure 10b shows the presence of mutations in the *ntr3* gene detected across *T. vaginalis* metronidazole susceptible, intermediate and resistant isolates. For the *ntr3* gene, two substitution mutations were observed in the intermediate isolate only: G $\rightarrow$ A and T $\rightarrow$ C highlighted in yellow (figure 10b).

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CLUSTAL 2.1 multiple sequence alignment

Ntr3-101        TGCAGGTTTAACAGCTCCTTCTTCAATGGATATTCAGGGAGTCGATATTGTCGTTGTCAC
Ntr3-231        TGCAGGTTTAACAGCTCCTTCTTCAATGGATATTCAGGGAGTCGATATTGTCGTTGTCAC
Ntr3-270        TGCAGGTTTAACAGCTCCTTCTTCAATGGATATTCAGGGAGTCGATATTGTCGTTGTCAC
                ************************************************************

Ntr3-101        AAATAAAGAAAAGTTAGCTCAGATCTCTGATGCAACATTCAAGAGACTTCCTGAACCAGG
Ntr3-231        AAATAAAGAAAAGTTAGCTCAGATCTCTGATGCAACATTCAAGGGACTTCCTGAACCAGG
Ntr3-270        AAATAAAGAAAAGTTAGCTCAGATCTCTGATGCAACATTCAAGGGACTTCCTGAACCAGG
                *******************************************  ****************

Ntr3-101        ACAAAAGCATTTCCAGCAACGTATCAATGAATGGGGTGTTAAGAATCCTGTCACATGCGA
Ntr3-231        ACAAAAGCATTTCCAGCAACGTATCAATGAATGGGGTGTTAAGAATCCTGTCACATGCGA
Ntr3-270        ACAAAAGCATTTCCAGCAACGTATCAATGAATGGGGTGTTAAGAATCCTGTCACATGCGA
                ************************************************************

Ntr3-101        TGCACCAGTTCTTTACCTCTTTGTCAAGAATGAGAGAGCTCATAAGGACTACGTTCAAAT
Ntr3-231        TGCACCAGTTCTTTACCTCTTTGTCAAGAATGAGAGAGCTCATAAGGACTACGTTCAAAT
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Ntr3-270        TGCACCAGTTCTTTACCTCTTTGTCAAGAATGAGAGAGCTCATAAGGACTACGTTCAAAT
                ************************************************************


Ntr3-101        CGATTGTGGTTTGATTGTTGAGTCAATGATTTTGCTTGCAGCTGATATGGGTTATTCCAC
Ntr3-231        CGATTGTGGTTTGATTGTTGAGTCAATGATTTTGCTTGCAGCTGATATGGGTTATTCCAC
Ntr3-270        CGATTGTGGTTTGATTGTTGAGTCAATGATTTTGCTTGCAGCTGATATGGGTTATTCCAC
                ************************************************************


Ntr3-101        AATGACAATTGGTGCTGTTGCACTCTCTGATCTTTCTGAGGTCCTTAACATTCCTAAGGG
Ntr3-231        AATGACAATTGGTGCTGTTGCACTCTTTGATCTTTCTGAGGTCCTTAACATTCCTAAGGG
Ntr3-270        AATGACAATTGGTGCTGTTGCACTCTTTGATCTTTCTGAGGTCCTTAACATTCCTAAGGG
                ************************** *********************************

Ntr3-101        AGGTGTTATTGTCGGCCTTGCAATGGGCAAAGCTGCTCCAGATTTGAAGCTCCCAGAAAG
Ntr3-231        AGGTGTTATTGTCGGCCTTGCAATGGGCAAAGCTGCTCCAGATTTGAAGCTCCCAGAAAG
Ntr3-270        AGGTGTTATTGTCGGCCTTGCAATGGGCAAAGCTGCTCCAGATTTGAAGCTCCCAGAAAG
                ************************************************************

Ntr3-101        ACAAATCAAGGGAAAGATTACTCATATTGACTAAA
Ntr3-231        ACAAATCAAGGGAAAGATTACTCATATTGACTAAA
Ntr3-270        ACAAATCAAGGGAAAGATTACTCATATTGACTAAA
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Figure 10b: Clustal W multiple alignment of the *ntr3* gene. Sample 101 represents the intermediate isolates, 231 the susceptible and 270 the resistant isolates. Substitution mutations present in the intermediate isolates are highlighted in yellow, there were no further mutations observed in the susceptible and the resistant isolates

For *ntr4* gene, the expected fragment size of 549 bp was obtained. PCR amplification was successful in 18/21 (85.7%) of the isolates, however, our gel image was trimmed and only shows 9/18 isolates (Figure 11a).

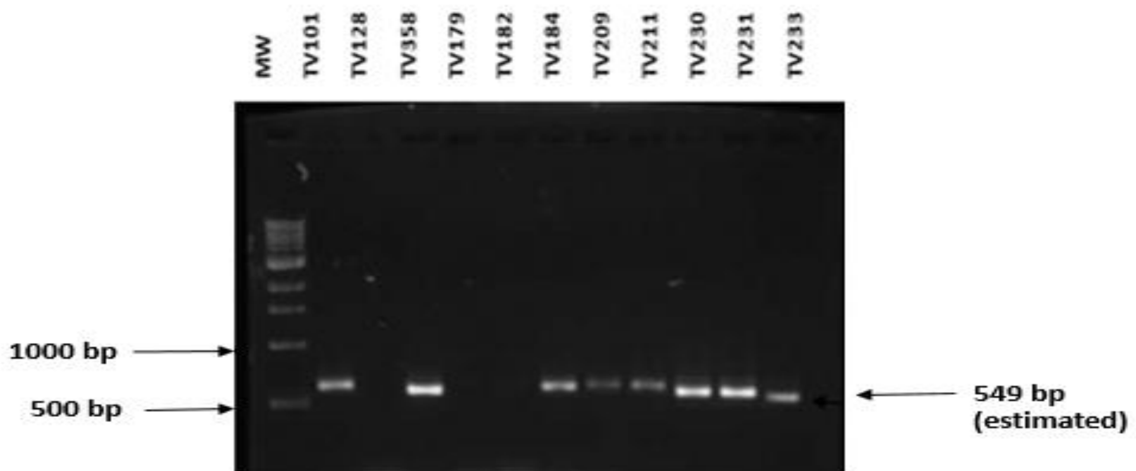

Figure 11a: Agarose gel electrophoresis showing positive amplicons generated for the *ntr4* gene. A fragment size of 549 bp was observed. Lane 1 represents the molecular weight marker (Gene ruler 100 bp ladder, ThermoFisher Scientific) and the remaining lanes represent selected *T. vaginalis* isolates. Blanks on the gel indicate samples that did not amplify.

The following mutations were observed in the *ntr4* gene for the resistant isolate when compared to the intermediate and susceptible isolates: A → G, C → G and T→ G, which are highlighted in yellow as shown Figure 11b. In addition, the following substitution mutations were observed in the susceptible isolate: A → G and G → C, which are highlighted in red.

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CLUSTAL 2.1 multiple sequence alignment

Ntr4-230        GGGCGAAGAAGTTCCAAAGGCAGATATTGATCTCATCGCA
Ntr4-266        GGGCGAAGAAGTTCCAAAGGCAGATATTGATCTCATCGCA
Ntr4-253        GGGCGAAGAAGTTCCAAAGGCAGATATTGATCTCATCGCA
                ****************************************

Ntr4-230        AACAGTGGCTTAACTGCTCCATCTTCTATGGATATCCAAGGTGTCGACATCTACGTCGTC
Ntr4-266        AACAGTGGCTTAACTGCTCCATCTTCTATGGATATCCAAGGTGTCGACATCTACGTCGTC
Ntr4-253        AACAGTGGCTTAACTGCTCCATCTTCTATGGATATCCAAGGTGTCGACATCTACGTCGTC
                ************************************************************

Ntr4-230        AGAGGCCAAGAAAAGCTTGCCAAGATTGAAGAAGCTACACTCAAGGCCCTTCCAGAATAC
Ntr4-266        AGAGGCCAAGAAAAGCTTGCCAAGATTGAAGAAGCTACACTCAAGGCCCTTCCAGAATAC
Ntr4-253        AGAGGCCAAGAAAAGCTTGCCGAGATTGAAGAAGCTACACTCAAGGCCCTTCCAGAATAC
                ********************* **************************************

Ntr4-230        GCCACAAAGTACTTCGTTAATCGTCATGAACAGCTTCATGTTAAGAACGTTATCACCTGC
Ntr4-266        GCCACAAAGTACTTCGTTAGTCGTCATGAACAGCTTCATGTTAAGAACGTTATCACCTGC
Ntr4-253        GCCACAAAGTACTTCGTTAATCGTCATGAACAGCTTCATGTTAAGAACGTTATCACCTGC
                ******************* ****************************************


Ntr4-230        GATGCTCCAGTTCTTTTCGTCTTAGTTAAGAATGAGAGAGCTCACAAAGATTACTATCAT
Ntr4-266        GATGCTCCAGTTCTTTTCGTCTTAGTTAAGAATGAGAGAGCTCACAAAGATTACTATCAT
Ntr4-253        GATGCTCCAGTTCTTTTCGTCTTAGTTAAGAATGAGAGAGCTCACAAAGATTACTATCAT
                ************************************************************


Ntr4-230        ATCGATTGCGGTCTCATCGTCGAATCAATGATTTTGCTTGCCCAAGATATGGGATACAGC
Ntr4-266        ATCGATTGCGGTCTCATCGTCGAATCAATGATTTTGCTTGCCCAAGATATGGGATACAGC
Ntr4-253        ATCGATTGCGGTCTCATCGTCGAATCAGTGATTTTGCTTGCCCAGGATATGGGATACAGC
                *************************** **************** ***************

Ntr4-230        ACAATGTGCATCGGTGCTATCGGTATGGCTGATCTTTCTGAAGTTCTTGGTATTCCAAAG
Ntr4-266        ACAATGTGCATCGGTGCTATCGGTATGGCTGATCTTTCTGAAGTTCTTGGTATTCCAAAG
Ntr4-253        ACAATGTGCATCGGTGCTATCGGTATGGGTGATCTTTCTGAAGTTCTTGGTATTCCAAAG
                **************************** *******************************

Ntr4-230        GATGCTGCTATTATGGGTCTTGCCATGGGTAAAGCTGCCCCAGAACAGGATCTTCATAAA
Ntr4-266        GATGCTGCTATTATGGGTCTTGCCATGGGTAAAGCTGCCCCAGAACAGGATCTTCATAAA
Ntr4-253        GAGGCTGCTATTATGGGTCTTGCCATGGGTAAAGCTGCCCCAGAACAGGATCTTCGTAAA
               ** **************************************************** ****

Ntr4-230         AGGGCAATCAAGTCTAAGGTAGTTTATGCCGACTAAATA
Ntr4-266         AGGCCAATCAAGTCTAAGGTAGTTTATGCCGACTAAATA
Ntr4-253         AGGGCAATCAAGTCTAGGGTAGTTTATGCCGACTAAATA
                *** ************ **********************
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Figure 11b: Clustal W multiple alignment of the *ntr4* gene. Sample 230 represents the intermediate isolate, 266 the susceptible and 253 the resistant isolates. Substitution mutations present in the resistant isolate are highlighted in yellow, substitution mutations present in the susceptible isolates are highlighted in red.

An estimated band size of 943 bp for the *ntr5* gene was amplified across 13/21 (61.9%) of the isolates (Figure 12a).

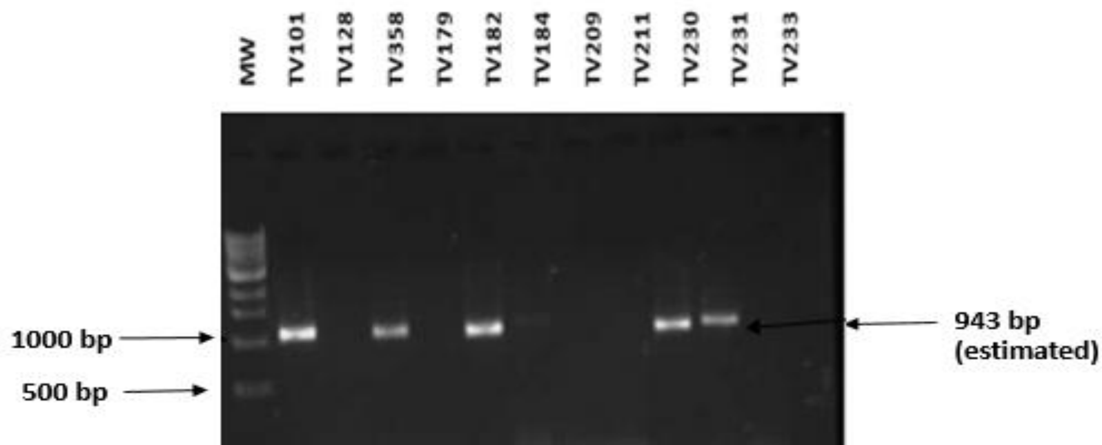

Figure 12a: Agarose gel electrophoresis showing positive amplicons generated for the *ntr5* gene. A fragment size of 943 bp was observed. Lane 1 represents the molecular weight marker (Gene ruler 100 bp ladder, ThermoFisher Scientific) and the remaining lanes represent selected *T. vaginalis* isolates. Blanks on the gel indicate samples that did not amplify.

For the *ntr5* gene, the following substitution mutations were observed in the resistant isolate when compared to the intermediate and the susceptible isolates: C →A, T → G, T → A, CT → TA and T→C as highlighted in yellow. The following insertion mutations were observed in the resistant isolate when compared to the intermediate and the susceptible isolates: insertion of a T nucleotide in the first set of sequence alignments, a C nucleotide insertion in and an A nucleotide, which are highlighted in green in the latter set of alignments (Figure 12b).

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CLUSTAL 2.1 multiple sequence alignment

Ntr5-230        AAATAATAAAATTTTTAATTTGAAAGTTTGATAAAATCTACTATG-GTTCAGAGAAAATT
Ntr5-358        AAATAATAAAATTTTTAATTTGAAAGTTTGATAAAATCTACTATG-GTTCAGAGAAAATT
Ntr5-253        AAATAATAAAATTTTTAATTTGAAAGTTTGATAAAATATACTATGTGGTAAGAGAAAATT
                ************************************* ******* * * **********

Ntr5-230        GCTATTCATATACCGTGAAATCTAAATTATGTAATAAATTAGAAAATCTTGTTTTGCAA
Ntr5-358        GCTATTCATATACCGTGAAATCTAAATTATGTAATAAATTAGAAAATCTTGTTTTGCAA
Ntr5-253        GCAATTCATATACCGTGAAATCTAAATTATGTAATAAATTAGAAAATCTTGTTTTGCAA
                 ** ********************************************************


Ntr5-358        TTTTCAATAATGTCCGTTTTTGATGCTATTGACACAAGAAGAACAATCAGACAATATGAT
Ntr5-230        TTTTCAATAATGTCCGTTTTTGATGCTATTGACACAAGAAGAACAATCAGACAATATGAT
Ntr5-253        TTTTCAATAATGTCCGTTTTTGATGCTATTGACACAAGAAGAACAATCAGACAATATGAT
                ************************************************************

Ntr5-230        CAATCCTTTGTTCCACCAAAAGAACACGTTAAAAAGATTGCAGAAGCAGCAATTAAATCA
Ntr5-358        CAATCCTTTGTTCCACCAAAAGAACACGTTAAAAAGATTGCAGAAGCAGCAATTAAATCA
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Ntr5-253        CAATCCTTTGTTCCACCAAAAGAACACGTTAAAAAGATTGCAGAAGCAGCAATTAAATCA
                ************************************************************

Ntr5-230        CCCACAGGCTACAACAGACAAGGAGTTGATTTACTTGTCGTTACAAACAAATACATTATC
Ntr5-358        CCCACAGGCTACAACAGACAAGGAGTTGATTTACTTGTCGTTACAAACAAATACATTATC
Ntr5-253        CCCACAGGCTACAACAGACAAGGAGTTGATTTACTTGTCGTTACAAACAAAGACATTATC
                *************************************************** ********

Ntr5-230        AACAACATGAATGATAAAGTATTCGATGCATTAGACGAAGATGCAAAGAATTATTTCCGC
Ntr5-358        AACAACATGAATGATAAAGTATTCGATGCATTAGACGAAGATGCAAAGAATTATTTCCGC
Ntr5-253        AACAACATGAATGATAAAGTATTCGATGCATTAGACGAAGATGCAAAGAATTATTTCCGC
                ************************************************************

Ntr5-230        ACAAGAAAGGAGCTTGGTCTTGAAAAGTTCATAACATGTGATGCACCAGTACTTTACGTC
Ntr5-358        ACAAGAAAGGAGCTTGGTCTTGAAAAGTTCATAACATGTGATGCACCAGTACTTTACGTC
Ntr5-253        ACAAGAAAGGAGCTTGGTCTTGAAAAGTTCATAACATGTGATGCACCAGTACTTTACGTC
                ************************************************************

Ntr5-230        TTGTATGCTAATGGAAAGACAGATGATGTCTATGCACGCTTAGATGCTGGAATCATGGCA
Ntr5-358        TTGTATGCTAATGGAAAGACAGATGATGTCTATGCACGCTTAGATGCTGGAATCATGGCA
Ntr5-253        TTGTATGCTAATGGAAAGACAGATGATGTCTATGCACGCTTAGATGCTGGAATCATGGCA
                ************************************************************

Ntr5-230        GAGTCTATTCTTCTTACAGCTACAAGCCTTGGCTATGCTACAATGCCAATTGGAACATTC
Ntr5-358        GAGTCTATTCTTCTTACAGCTACAAGCCTTGGCTATGCTACAATGCCAATTGGAACATTC
Ntr5-253        GAGTCTATTCTTCTTACAGCTACAAGCCTTGGCTATGCTACAATGCCAATTGGAACATTC
                ************************************************************


Ntr5-230        ATGATTGGCGACCTTTCTGAGTACGGAATTCCAAAGGACAAAGTCCTATTAGCAATTGCT
Ntr5-358        ATGATTGGCGACCTTTCTGAGTACGGAATTCCAAAGGACAAAGTCCTATTAGCAATTGCT
Ntr5-253        ATGATTGGCGACCTTTCTGAGTACGGAATTCCAAAGGACAAAGTCCTATTAGCAATTGCT
                ************************************************************

Ntr5-230        ATGGGCAAAGCTAGAGCTGGAATTGAAATTCCACCATGCGATAGAAATACAAAGATAACT
Ntr5-358        ATGGGCAAAGCTAGAGCTGGAATTGAAATTCCACCATGCGATAGAAATACAAAGATAACT
Ntr5-253        ATGGGCAAAGCTAGAGCTGGAATTGAAATTCCACCATGCGATAGAAATACAAAGATAACT
                ************************************************************

Ntr5-230        TACTTAGAATAAAAATAGAATTCTCTATTCATTATATAATATTTTATTCAGTCTTATAAA
Ntr5-358        TACTTAGAATAAAAATAGAATTCTCTATTCATTATATAATATTTTATTCAGTCTTATAAA
Ntr5-253        TACTTAGAATAAAAATAGAATTCTCTATTCATTATATAATATTTTATTCAGTCTTATAAA
                ************************************************************

Ntr5-230        CTTGTATTTTTGTTCACATATGTGAAAATACATCATTATAGCATAATCAAACAAATTTTT
Ntr5-358        CTTGTATTTTTGTTCACATATGTGAAAATACATCATTATAGCATAATCAAACAAATTTTT
Ntr5-253        CTTGTATTTTTGTTCACATATGTGAAAATACATCATTATAGCATAATCAAACAAATTTTT
                ************************************************************

Ntr5-230         -CTTTAGAATTCTTAAGAAAT-CTTAAAAAACT-GATATCGCTCCACCACCTCCTGAGCT
Ntr5-358         -CTTTAGAATTCTTAAGAAAT-CTTAAAAAACT-GATATCGCTCCACCACCTCCTGAGCT
Ntr5-253         TCTTTAGAATTCTTAAGTAATACGTAAAAAAATAAGATATCGAGCCACCACCTCCTGAGCT
                 *************** *** * *******   *******  *****************

Ntr5-230         TGAATCACTATATTTCTGCGGATATC
Ntr5-358         TGAATCACTATATTTCTGCGGATATC
Ntr5-253         TGAATCACTATATTTCTGCGGATACC
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Figure 12b: Clustal W multiple alignment of the *ntr5* gene. Sample 230 represents the intermediate isolate, 358 the susceptible and 253 the resistant isolates. Substitution mutations present in the resistant isolate are highlighted in yellow, insertion mutations present in the resistant isolate are highlighted in green.

For the *ntr6* gene, the expected band size of 743 bp. Amplification was successful on 17/21 (80.9%) of the isolates, however the gel only shows 9/21 isolates (Figure 13a). Isolates that did not amplify were omitted from the gel image.

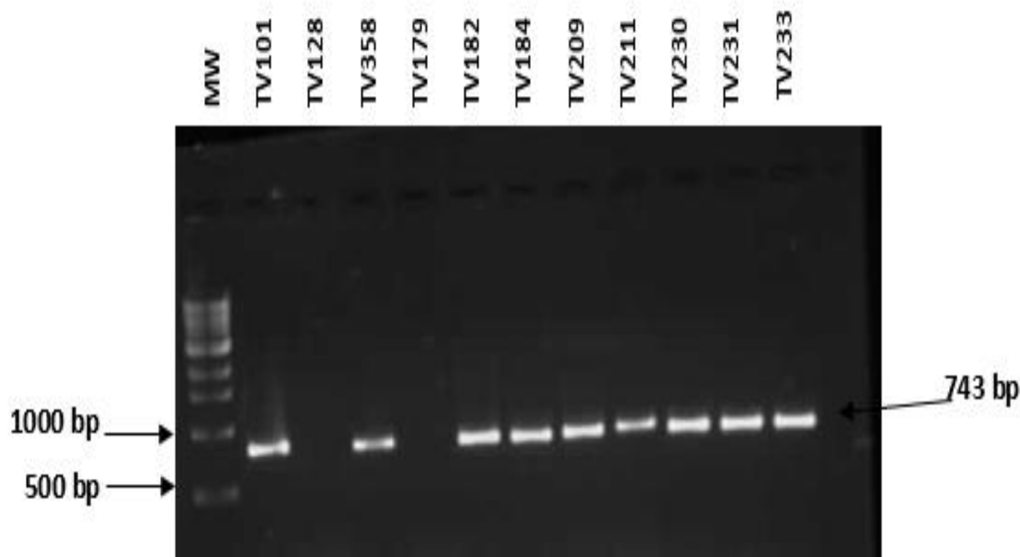

Figure 13a: Agarose gel electrophoresis showing positive PCR amplicons generated for the *ntr6* gene. A fragment size of 743 bp was observed. Lane 1 represents the molecular weight marker (Gene ruler 100 bp ladder, ThermoFisher Scientific) the remaining lanes represent selected *T. vaginalis* isolates. Blanks on the gel indicate samples that did not amplify.

For the *ntr6* gene, insertion and deletion mutations were observed in the intermediate isolate and a single deletion mutation in the resistant isolate (deletion of the G nucleotide) when compared to the susceptible isolate (Figure 13b).

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CLUSTAL 2.1 multiple sequence alignment

Ntr6-230          GAAATGGTTTGCCAACACCAATGCCAAGAAGAACTGATTTTGGTGGAAGACCAAGAAC
Ntr6-231          ATAATGGTTTGCCAACACCAATGCCAAGAAGAACTGATTTTGGTGGAAGACCAAGAAC
Ntr6-253          ATAATGGTTTGCCAACACCAATGCCAAGAAGAACTGATTTTGGTGGAAGACCAAGAAC
                  ********************************************************

Ntr6-230         TTCTTCTGTTTGTGGGCGGATAAGTGTACCAAGAGGAACTGTACCAAGTCCAAGATCATG
Ntr6-231         TTCTTCTGTTTGTGGGCGGATAAGTGTACCAAGAGGAACTGTACCAAGTCCAAGATCATG
Ntr6-253         TTCTTCTGTTTGTGGGCGGATAAGTGTACCAAGAGGAACTGTACCAAGTCCAAGATCATG
                 ************************************************************

Ntr6-230          GGCAGCCATCAAAACACTCATGGCGAGAATACCGGAATCAAGTTGTTGAATGGCTGGGGA
Ntr6-231          GGCAGCCATCAAAACACTCATGGCGAGAATACCGGAATCAAGTTGTTGAATGGCTGGGGA
Ntr6-253          GGCAGCCATCAAAACACTCATGGCGAGAATACCGGAATCAAGTTGTTGAATGGCTGGGGA
                  ************************************************************

Ntr6-230          TGCACGCTCATTCTTGACAAGAAGGAAAACAGCAGAGCAATCATAGAGAACTTCCTGCTT
Ntr6-231          TGCACGCTCATTCTTGACAAGAAGGAAAACAGCAGAGCAATCATAGAGAACTTCCTGCTT
Ntr6-253          TGCACGCTCATTCTTGACAAGAAGGAAAACAGCAGAGCAATCATAGAGAACTTCCTGCTT
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Ntr6-230      AACATGTGTCTGCTTTTGCATTCCAAGGTACATTTGTTGAGACTTCTCATCAAGGGAAGC
Ntr6-231      AACATGTGTCTGCTTTTGCATTCCAAGGTACATTTGTTGAGACTTCTCATCAAGGGAAGC
Ntr6-253      AACATGTGTCTGCTTTTGCATTCCAAGGTACATTTGTTGAGACTTCTCATCAAGGGAAGC
              ************************************************************

Ntr6-230      AAAAACAGCATCGTTAAGCTTTTGGAGTTTTTCCTTGTTTGTAACAACGACGAGATCTGT
Ntr6-231      AAAAACAGCATCGTTAAGCTTTTGGAGTTTTTCCTTGTTTGTAACAACGACGAGATCTGT
Ntr6-253      AAAAACAGCATCGTTAAGCTTTTGGAGTTTTTCCTTGTTTGTAACAACGACGAGATCTGT
              ************************************************************

Ntr6-230      TTCCTGAACATTCATAGCTGATGGAGAGTTGAAAGCTGCATCAACAATTTTCTCTAAGTC
Ntr6-231      TTCCTGAACATTCATAGCTGATGGAGAGTTGAAAGCTGCATCAACAATTTTCTCTAAGTC
Ntr6-253      TTCCTGAACATTCATAGCTGATGGAGAGTTGAAAGCTGCATCAACAATTTTCTCTAAGTC
              ************************************************************

Ntr6-230      TTCCTTTGGAATGACATAGTTTGGATCATAGCATCTGATTGTGCGTCTGGACTTGAGTTG
Ntr6-231      TTCCTTTGGAATGACATAGTTTGGATCATAGCATCTGATTGTGCGTCTGGACTTGAGTTG
Ntr6-253      TTCCTTTGGAATGACATAGTTTGGATCATAGCATCTGATTGTGCGTCTGGACTTGAGTTG
              ************************************************************

Ntr6-230      TGAGATAGACATTATCTAAAAATGAAGTCATATTTGAGATTTTTCTTTTCAATTAGTTTT
Ntr6-231      TGAGATAGACATTATCTAAAAATGAAGTCATATTTGAGATTTTTCTTTTCAATTAGTTTT
Ntr6-253      TGAGATAGACATTATCTAAAAATGAAGTCATATTTGAGATTTTTCTTTTCAATTAGTTTT
              ************************************************************

Ntr6-230      CCGAACAGTTTCTCCAAAATTTTCTTACAAAAATTTGAAAGGCTACTATCAAAAAAATTTA
Ntr6-231      CCGAACAGTTTCTCCAAAATTTTCTTACAAAAATTTGAAAGGCTACTATCAAAAAAATTTA
Ntr6-253      CCGAACAGTTTCTCCAAAATTTTCTTACAAAAATTTGAAAGGCTACTATCAAAAAAATTTA
              ************************************************************


Ntr6-230      TCCCCAATTTTTATCATAAACATATATTGCCTTCTTCTTCATTTTGCGG-AGATATTCGG
Ntr6-231      TCCCCAATTTTTATCATAAACATATATTGCCTTCTTCTTCATTTTGCGGGAGATATTCGG
Ntr6-253      TCCCCAATTTTTATCATAAACATATATTGCCTTCTTCTTCATTTTGCGG-AGATATTCGG

             ************************************************* **********

Ntr6-230      CAGGCAATAAATTTTGAACGAATAAAATTCAAT--
Ntr6-231      CAGGCAATAAATTTTGAACGAATAAAATTCAATGA
Ntr6-253      CAGGCAATAAATTTTGAACGAATAAAATTCAATGA
             *********************************
```

Figure 13b: Clustal W multiple alignment of the *ntr6* gene. Sample 230 represents the intermediate isolate, 231 the susceptible and 253 the resistant isolates. Mutations present in the resistant and the intermediate isolates are highlighted in yellow.

For the *PFOR* gene, the expected band size of approximately 200 bp was detected across 11/21 (47.65%) of the isolates (Figure 14a).

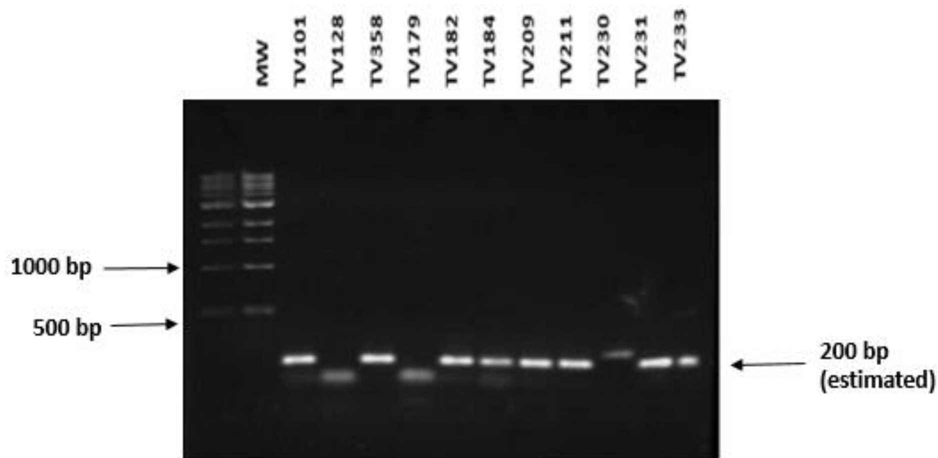

Figure 14a: Agarose gel electrophoresis showing positive amplicons generated for the *PFOR* gene. A fragment size of 200 bp was observed. Lane 1 represents the molecular weight marker (Gene ruler 100 bp ladder, ThermoFisher Scientific), and the remaining lanes represent selected *T. vaginalis* isolate

According to the multiple alignment, a single substitution mutation was observed in the intermediate isolate only, T→A (figure 14b).

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CLUSTAL 2.1 multiple sequence alignment


PFOR-182        ACGCTACAGGCTGCTCTCTCGTTTGGGGTGCTACATTCCCATTCAAC
PFOR-184        ACGCTACAGGCTGCTCTCTCGTTTGGGGTGCTACATTCCCATTCAAC
PFOR-253        ACGCTACAGGCTGCTCTCTCGTTTGGGGTGCTACATTCCCATTCAAC
                ***********************************************

PFOR-182        CCATTCACAACAAACGAGCGCGGACACGGTCCAGCTTGGGCTAACTCCCTCTTAA
PFOR-184        CCATACACAACAAACGAGCGCGGACACGGTCCAGCTTGGGCTAACTCCCTCTTAA
PFOR-253        CCATACACAACAAACGAGCGCGGACACGGTCCAGCTTGGGCTAACTCCCTCTTAA
                ******************************************************
```

Figure 14b: Clustal W multiple alignment of the *PFOR* gene. Sample 182 represents the intermediate isolate, 184 the susceptible and 253 the resistant isolate. Mutations present in the intermediate isolate are highlighted in yellow.

In this set of multiple sequence alignment, we observe a mutation present in the intermediate isolate only as shown in the second set of alignments position number 5.

CHAPTER FIVE

DISCUSSION

Globally, trichomoniasis is the most reported non-viral sexually transmitted infection and has the highest prevalence compared to other curable STIs (Menezes et al., 2016). Treatment with metronidazole has shown to be successful in most cases. Nonetheless, there has been an increase in metronidazole resistant *T. vaginalis* isolates (Bosserman et al., 2011). This study aimed to identify mutations that are associated with metronidazole resistance and susceptibility in South African pregnant women. Robinson discovered metronidazole resistance in 1962 after a patient infected with *T. vaginalis* did not respond to two doses of metronidazole treatment (Robinson, 1962). Metronidazole resistance differs depending on the geographical location and the population structure (Kissinger, 2015).

In this study, metronidazole resistance was detected in 9.5% of *T. vaginalis* isolates under anaerobic incubation conditions. In Egyptian non-pregnant women, a similar prevalence rate of metronidazole resistance (8.2%) was reported (Abdel-Magied et al., 2017). Another study in Brazil reported metronidazole resistance in 13.3% (4/30) of *T. vaginalis* strains. However, that study was conducted in *T. vaginalis* isolated from urine specimens and the susceptibility assay was performed under microaerophilic conditions (37°C, 5% CO_2) (da Luz Becker et al., 2015). In a South African study, metronidazole resistance was reported to be 6% in HIV-positive, non-pregnant South African women (Rukasha et al., 2013) . In that study, in which resistance was measured aerobically, 95% of the isolates were susceptible to metronidazole and only one isolate exhibited low-level resistance (MIC = 50µg/ml). Similarly, the detection of low level anaerobic metronidazole resistant *T. vaginalis* strains was shown to be 7% (Ghosh et al., 2018).

In this study, a higher proportion of women who were not cohabiting with their sex partners were infected with *T. vaginalis* when compared to women who were cohabiting. In a study conducted by Naidoo and Wand (2013), they reported that unmarried women or women who did not cohabit with their partners, had a higher risk of *T. vaginalis* infection (Naidoo & Wand, 2013). This was due to the fact the number of sexual partners increases ones chances of acquiring STIs. The association between metronidazole susceptibility patterns and clinical and behavioural factors was also determined. Across the metronidazole susceptibility patterns, a higher proportion of women reported not cohabiting with their sex partner. However, there was no significant association between cohabitation status and susceptibility patterns. A high proportion of the infected women reported having between 2-4 sexual partners in their lifetime. The majority of these women showed resistance to metronidazole, however, this association was not significant. Naidoo and Wand (2013) also found that women who had ≥ three lifetime partners had a higher prevalence of infection (Naidoo & Wand, 2013). In the present study, most infected women (76.2%) were in the third trimester of pregnancy. Of these women, 50.0% were

resistant to metronidazole, however, trimester of pregnancy was not significantly associated with metronidazole susceptibility patterns (p=0.773). However, a study conducted on Nigerian pregnant women, found that women in their first trimester showed significantly higher prevalence of trichomoniasis compared to women in their second and third trimesters ($p < 0.05$) (Oyeyemi et al., 2015).

According to Sood and Kapil (2008) and Donbraye et al. (2011), around 50% of women infected with *T. vaginalis* are asymptomatic (Sood & Kapil, 2008); (Donbraye et al., 2011). In this study, a small proportion of the infected women, 38.1% women presented with symptoms of abnormal vaginal discharge. Of these women, 100% had a resistant profile towards metronidazole, however, having an abnormal vaginal discharge was not significantly associated with metronidazole susceptibility patterns (p=0.273). In addition, only 33.3% of the women had reported being treated for STIs in the past and of those previously treated, 50.0% had a resistant profile towards metronidazole. However, there was no significant association between past treatment and susceptibility patterns (p=0.246).

Despite an increase in reports of metronidazole resistance in *T. vaginalis* isolates, the mechanisms and variables that contribute to metronidazole resistance development are still unknown. Several mechanisms of resistance have been proposed, including genetic changes in the target and drug activating genes (Butler et al., 2010). In this study, mutation detection of the five selected *nitroreductase* genes from *T. vaginalis* revealed the presence of mutations in the resistant and intermediate isolates which were absent in the susceptible isolates. The majority of nitroreductases investigated thus far are oxygen-insensitive and capable of reducing nitroaromatic compounds by consecutive two-electron reductions, yielding nitroso intermediates and hydroxylamine end products (Bryant et al., 1991). Initially we had sequenced nine genes, however, there were no mutations observed in the other genes. We then chose to report on the genes that had mutations. For the *ntr2* gene, susceptible and resistant isolates carried mutations which were absent in the intermediate isolates. The susceptible isolates carried mostly insertion mutations and the resistant isolate carried substitution mutations. Some deletion mutations were also observed in the *ntr2* gene. There was a substitution of T → G, T → A, C → A, T → C nucleotide and lastly A→ T nucleotide. In addition, the intermediate and the resistant isolates had a G, T and A nucleotide deletion. Previous studies have not identified these mutations present in this gene, thereby limiting our discussion. For the *ntr3* gene, substitution mutations G → A and T→ C were observed in the intermediate isolate. We cannot conclude that these mutations are associated with metronidazole resistance as they were present in the intermediate isolate but not the resistant isolate. In addition, there is no published data on mutations in the *ntr3* gene, associated with metronidazole resistance.

The following substitution mutations were observed in the *ntr4* gene of the resistant *T. vaginalis* isolate when compared to the intermediate and susceptible isolates; A → G, C → G and T → G. A previous study had observed a C to G substitution (C213G) in the *ntr4* gene (Paulish-Miller et al., 2014). However, in our study we did not determine the positions of the substitutions. We used colour coding to identify a specific mutation in each gene. Additional mutations not described by Paulish-Miller et al. (2014) were detected in our study isolates. The most frequent substitution mutation observed in the *ntr4* gene of the resistant isolate was A to G, which was present at five different positions of the gene. The association of this substitution with metronidazole resistance needs further investigation. We also detected substitution and insertion mutations in the *ntr5* gene of the resistant isolates. No previous studies have identified these mutations in the *ntr5* gene of *T. vaginalis* isolates. The *ntr6* gene insertion and deletion mutations were observed in the intermediate isolate and a single deletion mutation in the resistant isolate which was the deletion of the G nucleotide. A previous study, did not describe any deletions in the *ntr6* gene of the resistant isolates, however they did observe substitutions (Paulish-Miller et al., 2014). For the *PFOR* gene, we were only able to identify mutations in the intermediate isolate and not in the resistant or susceptible isolates. There were no previous studies, which had looked at mutations in the *PFOR* gene associated with metronidazole resistance, thereby limiting our discussion.

The discovery of these additional mutations in the *nitroreductase* genes requires further investigation since these mutations were detected in the intermediate and resistant isolates but absent in the susceptible isolate. The presence of these mutations in association with the resistant phenotype still needs to be confirmed. A previous study proposed that the *ntr6* gene may be associated with down-regulation in the B7258 and B7268M resistant laboratory pair and the down regulation of other genes (Bradic et al., 2017). Bradic et al. (2017) also proposed that metronidazole resistance in *T. vaginalis* is linked to the development of tolerance to high levels of oxygen providing protection against this antibiotic (Bradic et al., 2017).

A study conducted by Rasoloson et al. (2001) suggested that *T. vaginalis* bears mechanisms that enable it to survive in a high fluctuating oxygen environment making it pre-adaptable to metronidazole treatment (Rasoloson et al., 2001). Their study was further supported by the development of rapid aerobic resistance *in vitro* and the emergence of metronidazole clinical resistance when the antibiotic had only been introduced for 2 years. Metronidazole resistance may also take place in isolates containing intact *T. vaginalis ntr4* and *ntr6* genes, which shows that resistance may be caused by multiple resistance mechanisms (Paulish-Miller et al., 2014).

CHAPTER 6

CONCLUSION

In this study, mutations in the *ntr2, ntr3, ntr4, ntr5*, *ntr6* and *PFOR* genes were observed across metronidazole susceptibility profiles. Previous studies have not identified mutations in the *ntr2*, *ntr3* *ntr5*, and *PFOR* genes so there is not enough data to support the functions of those genes and their association with metronidazole resistance. Future studies, which are aimed at identifying the function of these mutations are urgently needed. This can serve as a future research area for our research group.

Study limitations

There is not enough recent data on the mechanisms of resistance of *T. vaginalis* to metronidazole, which limits our understanding of the extent to which the pathogen has evolved. Another limitation would be that we only investigated the presence of mutations in a selected number of genes. There may be other genes that also code for resistance, which were not analyzed in this study.

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APPENDICES

06 December 2020

Miss Tumelo Mzenda (219020884)
School of Clinical Medicine
Medical School

Dear Miss Mzenda,

Protocol reference number: BREC/00001930/2020
Project title: Identification of mutations in genes associated with metronidazole resistance and susceptibility in Trichomonas vaginalis
Degree: MMedSci

EXPEDITED APPLICATION: APPROVAL LETTER

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

The conditions have been met and the study is given full ethics approval and may begin as from 06 December 2020. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations dated 10th November 2020, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 06 December 2020. 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 09 February 2021.

Yours sincerely,

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
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