Characterization and drug resistance of *Trichomonas vaginalis* clinical isolates

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted by me in respect of a degree at any other University or tertiary institution.

Signed……..on this……day of……..2013

You have trusted Him in a few things, and He has not failed you. Trust Him now for everything, and see if He does not do for you exceeding abundantly above all that you could ever have asked or thought, not according to your power or capacity, but according to His own mighty power, that will work in you all the good pleasure of His most blessed will. You find no difficulty in trusting the Lord with the management of the universe and all the outward creation, and can your case be any more complex or difficult than these, that you need to be anxious or troubled about His management of it?

Hannah Whitall Smith

What then can I say but “lf God is for us, who can be against us?”

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AP	Adenosine proteins
ATP	Adenosine triphosphate
BPGA	1, 3 Bisphosphoglyceric acid
CCC	Cation chloride transporter
CDC	Centers for Disease Control and Prevention
cHSP	Cytoplasmic heat-shock protein
CO_2	Carbon dioxide
DNA	Deoxyribose nucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
FDA	Food and Drug Adminstration
FN	Fibronectin
GAP	Glyceraldehyde-3-phosphate
H_2S	Hydrogen sulphide
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HSP70	Heat Shock Protein 70
IC	Internal control
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IGS	Intergenic spacer region
IL	lnterleukin
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-8	Interleukin-10
Kb	Kilobase
KDa	Kilodalton
LF	Lytic factor
LPG	Lipophosphoglycan
Mb	Megabase
MIC	Minimum inhibitory concentration
NAAT	Nucleic acid amplification test
NaOAc	Sodium acetate
NGU	Non-gonococcal urethritis
NH_3	Ammonia
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate

PFGE	Pulsed-field gel electrophoresis
PKS	Protein kinase coding genes
PMNs	Polymorphonuclear leukocytes
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
STI	Sexually transmitted infection
TNF-α	Tumor necrosis factor-alpha
TGF-β	Transforming growth factor-beta
TYM	Tryptone Yeast Maltose
UPGMA	Unweighted pair group method with arithmetic mean
USA	United States of America
UV	Ultraviolet light
µl	Microlitre
µm	Micrometre
V	Voltage
VEC	Vaginal epithelial cell
WHO	World Health Organization

LIST OF ARTICLES IN PREPARATION FOR SUBMISSION AND CONFERENCE CONTRIBUTIONS

PUBLICATIONS

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Characterization and drug resistance of *Trichomonas vaginalis* clinical isolates

by

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SUMMARY

Sexually transmitted infections (STIs) are a major cause of acute illnesses, infertility, long term disability and death with far reaching health, social and economic consequences. *Trichomonas vaginalis* is the causative organism of trichomoniasis which classically presents in women as a malodorous green-yellowish discharge accompanied by itching and burning. In men infection can cause non-gonococcocal urethritis and chronic prostatitis. Complications of *T. vaginalis* include preterm delivery, low birth weight, predisposition to HIV infection and cervical cancer.

Previous studies in South Africa have focused mostly on *T. vaginalis* detection with reported rates of prevalence of *T. vaginalis* infections ranging between 20% to 49%. Despite estimates showing *T. vaginalis* being the most prevalent sexually transmitted disease worldwide, very little is known about the genetic diversity of *T. vaginalis* clinical isolates. Furthermore, the degree of metronidazole resistance in a particular setting needs to be investigated, since this has implications on the treatment regimen of trichomoniasis.

The purpose of this study was: i) To detect *T. vaginalis* in HIV positive female patients from the Anti-Retroviral clinic of the Tshwane District Hospital, Pretoria using three methods, namely microscopy, culture and PCR; ii) To characterize *T. vaginalis* isolates using both the random amplified polymorphic DNA (RAPD) assay and the intergenic spacer region-polymerase chain reaction-restriction fragment length polymorphism (IGS-PCR RFLP) assay

and iii) To phenotypically determine metronidazole resistance of the *T. vaginalis* isolates and to compare the results with random amplified polymorphic DNA (RAPD) assay results.

Self-collected vaginal swabs from 380 women were included in the first part of the study. *Trichomonas vaginalis* was detected using: wet mount microscopy, culture (modified Diamond's medium) and molecular detection using a commercial kit, *Trichomonas vaginalis* 240/250 IC (Sacace Biotechnology, Italy). The genetic relatedness of 92 culture positive *T. vaginalis* isolates was determined. Five primers (TV1, TV2, TV3, TV5 and TV6) were used for the RAPD assay. The PCR-IGS-RFLP products were digested with five enzymes, namely: *Alu*I, *Hinf*I, *Rsa*I, *Sau*3AI and *Tsp*509. A 24 h and 48 h interval microtiter assay was used to detemine the metronidazole antimicrobial susceptibility of 30 *T. vaginalis* isolates.

A total of 8% (30/380) of specimens were positive for *T. vaginalis* using microscopy, 24% (92/380) of specimens were positive using culture and 31% (118/380) of the specimens were positive using the commercial PCR kit *Trichomonas vaginalis* 240/250 IC (Sacace Biotechnology, Italy). RAPD assay analysis showed a high level of genetic diversity between the different *T. vaginalis* isolates. The dendrogrammes obtained from the RAPD markers grouped the 92 *T.vaginalis* isolates into between nine to 24 clusters with a 70% similarity, while the PCR-IGS RFLP assay results for the isolates were genetically indistinguishable. The minimal inhibitory concentration (MIC) for metronidazole was between 0.06 to 25 µg/ml. Only 6% (2/30) of the *T. vaginalis* isolates were resistant. The dendrogrammes constructed in the second part of the study did not group the metronidazole resistant isolates together in one cluster. No link between resistance and a specific *T. vaginalis* genotype could be indicated.

This study proved that PCR is a more sensitive diagnostic tool for the detection of *T. vaginalis* to improve the diagnosis of trichomoniasis. A high prevalence of *T. vaginalis* in HIV positive women in South Africa was observed. The RAPD assay proved to be useful in discriminating between the different *T. vaginalis* isolates, while the IGS-PCR RFLP assay was not a suitable marker. In future, other *T. vaginalis* genes, such as the ferredoxin and beta-tubulin genes could be investigated to determine the genetic variability of *T. vaginalis* isolates. Although metronidazole is the only antimicrobial drug used for treatment of trichomoniasis in South Africa, a low prevalence of *in vitro* resistance was found. This study emphasized the importance of *in vitro* antimicrobial drug susceptibility testing to ensure continunous screening for possible cases of metronidazole resistance and to monitor MIC changes.

CHAPTER 1

1.0 Introduction

Sexually transmitted infections (STIs) and their sequelae are among the top five reasons why people in developing countries seek medical treatment (WHO, 2011). Sexually transmitted infections cause illnesses that have far-reaching health, social and economic consequences (WHO, 2001). Amongst women of child-bearing age (15 to 49 years), STIs excluding the human immunodefiency virus (HIV), are second only to complications in pregnancy and child-birth as a cause of morbidity and mortality (WHO, 2001). High rates of asymptomatic infections and an unwillingness to seek treatment for genital disease, may lead to underreporting (Cudmore and Garber, 2010; WHO, 2011).

According to the World Heath Organization (WHO), the total number of new cases of the four main STIs was estimated to be 448 milllion, broken down as: trichomoniasis 58% (258 million), *Chlamydia* infections 22% (101 million), gonorrhoea approximately 19.6% (88 million) and syphilis 2.4% (11 million) worldwide (WHO, 2011). This makes an infection with *Trichomonas vaginalis* (*T. vaginalis*), the causative agent of trichomoniasis, the most common non-viral STI in the world, with the bulk of the infections occuring in developing countries (Gehring and Efferth, 2009; Cudmore and Garber, 2010). The prevalence of *T. vaginalis* is estimated to be about 170 million to 190 million cases globally and 32 million of these cases are from Sub-Saharan Africa (WHO, 2001; Cudmore and Garber, 2010; Dwivedi *et al*., 2012).

Trichomonas vaginalis is a single-celled flagellated to pear-shaped sexually transmitted protozoan (Calton *et al*., 2007; Lazenby, 2011). The protozoan is the sole member of the lineage *Trichomonadidae* known to cause disease in humans (Harp and Chowdhury, 2011). Most cases of trichomoniasis are asymptomatic ranging between 70% to 100% in men and 30% to 85% in women, worldwide (Johnston and Mabey, 2008; Kissinger *et al*., 2008; Lazenby, 2011). In women, *T. vaginalis* infects the lower urogenital tract causing external vaginal and cervical ulceration (Lusk *et al*., 2010). Typical symptoms include that of purulent, frothy foul-smelling yellow-greenish secretions, which is linked with pruritis, dysuria (painful urination) and dyspareunia (pain during sexual intercourse) (Kissinger *et al*., 2008; Lusk *et al*.,

2010; Harp and Chowdhury, 2011). Women with trichomoniasis may experience several complications associated with adverse pregnancy outcome; preterm or premature labour, low birth weight, premature rupture of membranes, as well as a greater risk of tubal infertility and atypical pelvic inflammatory disease (Wright *et al*., 2010; Andrea and Chapin, 2011; Lazenby, 2011). In symptomatic men, *T. vaginalis* causes urethritis, prostatitis, balanoprostatitis and epididymo-orchitis (Sood and Kapil, 2008; Harp and Chowdhury, 2011). *Trichomonas vaginalis* also affects the bladder, urethra, paraurethral glands and the urinary tract (Ulogu *et al*., 2007; Harp and Chowdhury, 2011). Individuals infected with *T. vaginalis* have a 1.5% to 5% increased risk of HIV acquisition and transmission (Shafir *et al*., 2009; Mavedzenge *et al*., 2010; Lazenby, 2011). Although the associated risk in numbers is modest, the increase is significant considering the large burden of *T. vaginalis* infection worldwide (Shafir *et al*., 2009; Mavedzenge *et al*., 2010; Lazenby, 2011).

Sub-Saharan Africa is by far the worst affected area by the HIV/AIDS epidemic, with 25.4 million people living with HIV (UNAIDS/WHO, 2004; Uneke *et al*., 2007). The HIV/AIDS epidemic is affecting the female population the most severely in Sub-Saharan countries, with women making up to 59% of all living with HIV (UNAIDS/WHO, 2004; Uneke *et al*., 2007; UNAIDS/WHO, 2010). Trichomoniasis in HIV positive patients has neither been the main focus of a rigorous study nor of active control programmes in the Sub-Saharan African countries, including South Africa. This is likely due to the fairly mild nature of the disease (Uneke *et al*., 2007). In South Africa, data on the prevalence and detection of *T. vaginalis* is well documented (Hoosen *et al*., 2003; Mhlongo *et al*., 2010). However, data on the molecular characterization of *T. vaginalis* is limited.

Customarily wet-mount microscopy was used as a technique for identifying *T. vaginalis* (Garber, 2005). Culturing of *T. vaginalis* is the current gold standard for diagnosis of *T. vaginalis* (Garber, 2005). Molecular based tests for *T. vaginalis* detection have been developed and these consist of: i) PCR assays targeting a number of genes in *T. vaginalis* (eg, 18S ribosomal units and ferredoxin), ii) Affirm^{TM} VP111 Microbial ldentification Test (Becton Dickinson, New Jersey, USA) and the nucleic acid amplification tests (NAAT) (APTIMA TV, GenProbe,San Diego, USA), which is a fully automated nucleic acid amplification test.

Strain typing techniques are valuable tools to study the epidemiology of infectious organisms (Rojas *et al*., 2004; Meade *et al*., 2009). Molecular typing gives information on the type and

the level of genetic diversity of organisms in a particular population (Conrad *et al*., 2011). Strain typing for *T. vaginalis* can be used to learn the routes of transmission, pathogenicity and drug resistance (Swygard *et al*., 2004). Random amplified polymorphic DNA (RAPD) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) are two of the most promising molecular typing techniques that have proven to be useful in the typing of *T. vaginalis* (Crucitti *et al*., 2008). The use of RAPD and PCR-RFLP strain typing techniques would give valuable information on the type and level of genetic diversity of the *T. vaginalis* strains in a specific region.

Metronidazole is the drug of choice for the treatment of *T. vaginalis* infection, while other drugs, such as the 5-nitroimidazoles (tinidazole, ornidazole and secnidazole) can also be used (Upcroft and Upcroft, 2001; Gehring and Efferth, 2009). Metronidazole antimicrobial drug resistance amongst the *T. vaginalis* protozoa is an escalating problem worldwide with relatively few available substitute antimicrobial drugs (Dunne *et al*., 2003). It is estimated that 2.5% to 9.6% of all occurrences of trichomoniasis exhibit some degree of resistance to metronidazole (Schwebke and Barrientes, 2006; Cudmore and Garber, 2010). It is crucial to determine the extent of metronidazole resistance in *T. vaginalis* isolates in a specific clinical setting, since it has implications for the treatment regimen of infected patients.

The purpose of this study was to detect *T. vaginalis* using wet mount microscopy, culture (the gold standard), PCR and to characterize *T. vaginalis* isolates using the RAPD and IGS-PCR RFLP assays. The study also determined metronidazole resistance of *T. vaginalis* clinical isolates obtained from the Anti-Retroviral clinic at Tshwane District Hospital, Pretoria, South Africa.

The objectives of this study were:

- To collect 100 positive vaginal swabs from symptomatic HIV positive women presenting with vaginal discharge and pain during urination
- To extract genomic DNA using a commercial DNA extraction kit (Sacace Biotechnology, Italy)
- To perform conventional PCR to confirm the presence of *T. vaginalis* using a conventional PCR commercial kit (*Trichomonas vaginalis* 240/250 IC (Sacace Biotechnology, Italy).

- To determine the genetic relatedness of *T. vaginalis* strains IGS region using PCR-RFLP and a RAPD assay
- To construct dendrogrammes to determine the genetic relatedness
- To phenotypically detect metronidazole resistance in *T. vaginalis*
- To analyze data

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CHAPTER 2

Literature review

2.1 Introduction

The urogenital pathogen *T. vaginalis* is a flagellated oval to pear-shaped organism that differs in size from approximately 5 µm to 20 µm in length and 5 µm to 10 µm in width (Benchimol, 2004). Donne discovered and named *T. vaginalis* in 1836 after observing the protozoan in the genital discharge of men and women (Aslan *et al*., 2005; Diamantis *et al*., 2009). The most frequent sites of infection in women are the exterior of the vaginal epithelium, the Bartholin's glands, the urethra, Skene's ducts and less frequently in the bladder (Smith and Ramos, 2010). In males the sites of infection are mostly the urethra and prostate and less likely the preputial sac, the seminal vesicles and epididymis (Smith and Ramos, 2010).

Trichomonas vaginalis has a genome size of 160 Mb, which is extremely repetitive with analogous repeats and transposable elements (Calton *et al*., 2007; Clark *et al*., 2010). The protozoan is a mandatory parasite as it lacks the ability to synthesize many macromolecules *de novo*, particularly nucleotides and many lipids, including cholesterol (Ginger, 2006; Sood and Kapil, 2008; Harp and Chowdhury, 2011). *Trichomonas vaginalis* uses carbohydrates as a main energy source, although it can also use a variety of amino acids as energy substrates (Calton *et al*., 2007; Sood and Kapil, 2008).

Trichomonas vaginalis infections have been encountered on every continent, in every climate with no seasonal variability (Cudmore *et al*., 2004). It has been found in all racial groups and all socioeconomic strata (Cudmore *et al*., 2004; Sood and Kapil, 2008). Epidemiological studies looking at age-related ratio occurrences indicated an increase of trichomoniasis with age (peak occurence 20 to 45 years), in direct contrast to both *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (peak occurence at age 15 to 25 years) (Niccolai *et al*., 2000; Soper, 2004). Many mechanisms are thought to be involved in the pathogenic potential of *T. vaginalis* and this include the ability of the protozoa to evade the immune system and the surface of the trichomonads, which is a mosaic of adhesions, proteins and carbohydrates, which play a major role in the adherence to the host (Calton *et al*., 2007; Sood and Kapil, 2008; Clark *et al*., 2010). *Trichomonas vaginalis* eludes the immune system *via* complement-mediated obliteration, molecular imitation and covering itself with host plasma proteins (Yadav *et al*.,

2005; Fichorova, 2009; Singh *et al*., 2009; Harp and Chowdhury, 2011). The typical appearance of *T. vaginalis* in women is that of purulent, frothy foul smelling yellow-green secretions, which is linked with pruritus (irritation of the skin at the exit of the rectum), dysuria (pain during urination) and dyspareunia (pain during sexual intercourse) (Harp and Chowdhury, 2011). In symptomatic men *T. vaginalis* causes urethritis (inflammation of the urethra), balanoprostatitis (inflammation of the fore skin and prostate gland) and epididymo-orchitis (inflammation of the epididymis and testis) (Sood and Kapil, 2008; Harp and Chowdhury, 2011).

A reliable, accurate, convenient and inexpensive method for diagnosis of *T. vaginalis* is crucial for the treatment and control of trichomoniasis (Harp and Chowdhury, 2011). Traditionally wet mount microscopy was used as the technique for the identification of *T. vaginalis* (Garber, 2005). Culture is the current gold standard for diagnosis of *T. vaginalis* and is recommended after a negative wet mount microscopy result (Pattullo *et al*., 2009). However, in many clinical settings, culture is not available as it requires additional resources (Pattullo *et al*., 2009; Schwebke *et al*., 2011). New point of care tests for the detection of *T. vaginalis* are available and have been validated in developed countries and include the rapid antigen test and the nucleic acid amplification tests (NAAT) (Pattullo *et al*., 2009; Schwebke *et al*., 2011).

Attempts to develop a classification system based on studies of proteins, polysaccharides and isoenzyme profiles of *T. vaginalis*, have so far been unsuccessful (Singh, 1997; Crucitti *et al*., 2011). The development of DNA-based techniques offers new perspectives and molecular methods for the study of the genetic diversity of parasites (Rojas *et al*., 2004). These methods include PCR-asrestriction fragment length polymorphism (PCR-RFLP), DNA microsatellites and random amplification of polymorphic DNA (RAPD) (Kaul *et al*., 2004; Rojas *et al*., 2004; Upcroft *et al*., 2006a, Meade *et al*., 2009, Conrad *et al*., 2011).

Currently, metronidazole is the drug of preference, while other 5-nitroimidazoles (tinidazole, ornidazole and secnidazole) can also be used for treatment of *T. vaginalis* (Upcroft *et al*., 2006b; Gehring and Efferth, 2009). Medical resistance of *T. vaginalis* is defined as cases of failure to cure the infection after at least two successive courses of treatment with metronidazole (Upcroft *et al*., 2006b; Sood and Kapil, 2008). It is estimated that 2.5% to 9.6% of all occurrences of trichomoniasis exhibit some degree of resistance to metronidazole

(Schwebke and Barrientes, 2006; Cudmore and Garber, 2010). Vaccines are being examined as a method of prevention of acquiring *T. vaginalis* (Cudmore and Garber, 2010).

2.2 History of *T. vaginalis*

The first trichomonad species, *Trichomonas tenax*, was desribed in 1773 by Miller, who found it in dental plaque (Campbell, 2001). *Trichomonas tenax* is a commensal of the human oral cavity and has been linked with gingivitis (Athari *et al*., 2007). Donne described it as a morphological blend (a combination of characteristics) from two organisms *Tricodes* and *Monas,* thus he named the protozoa *Trico-monas* (Table 2.1) (Campbell, 2001).

Table 2.1: Scientific history of *T. vaginalis* from its discovery in 1836 until the first draft sequence genome (Sood and Kapil, 2008; Harp and Chowdhury, 2011)

Year	Event
1836	Donne A identified *T. vaginalis* as motile microorganisms in the purulent, frothy, leukorrhea of women presenting with vaginal discharge and genital irritation
1916	Holne O used the term 'trichomoniasis' to describe the clinical condition of trichomoniasis, wherein *T. vaginalis* colonizes the vaginal mucosa
1934 to 1939	Pocaccini L identified and classified *T. vaginalis* as a venereal disease in a group of Italian soldiers serving in the Eastern ltalian Army in Ethiopia
1940	Trussel RE found that *T. vaginalis* produced vaginitis in 9 of 20 women by culturing using Diamond's media
1959	Clark DH and Solomos E developed routine culture examination for *T. vaginalis*
1960s to 1970s	Biochemical tests and microscopic examination to understand the growth characteristics and behaviour of *T. vaginalis*
1980s to 2000s	lmmunological and molecular biology techniques are used to study the pathogenesis and immunology of *T. vaginalis*
2007	Joint project on *T. vaginalis* genome sequencing from the institute for Genomic Research (TIGR), the Center for the Advancement of Genomics (TCAG), the JC Venter Science foundation, the Joint Technology Center and the Institute for Biology Energy Alternatives (IBRA) where whole-genome shotgun methodology was used (contains 1.4 million shotgun reads assembled into 17 290 scaffolds at ~7.2X coverage) to sequence the genome of a *T. vaginalis* strain (~160 Mb genome)

The protozoan was compared to a "Monas" because of its trumpet like appearance and to a "Tricode" because it has cilia (Tricodes and Monas) (Campbell, 2001). Although Donne in 1836 assigned it to the epithet *vaginalis*, Donne pointed out that it is found in the genital secretions of males and females (Campbell, 2001; Valadkhani *et al*., 2008).

2.3 Scientific classification of *T. vaginalis*

Trichomonas vaginalis is part of the *Parabasalids* family in the *Excavata* domain (Table 2.2), a higly diverged lineage within the eukaryotic supergroup of micro-aerophilic eukaryotes that lack mitochondria and peroxisomes but contain unusual organelles called hydrogenosomes (Calton *et al*., 2007). The *Diplomonads* is another member of the *Excavata* and is most closely related to the *Parabasalids* (Gerbod *et al*., 2004; Schwebke and Burgess, 2004).

Table 2.2: Scientific classification of *T. vaginalis* (Schwebke and Burgess, 2004)

Rank	Scientific Name
Domain	*Eukaryota*
Unranked	*Excavata*
Phylum	*Zoomastigina*
Class	*Parasabalia*
Order	*Trichomonadia*
Genus	*Trichomonas*
Species	*T. vaginalis*
Binomial Name	*T. vaginalis*

One famous species of the *Diplomonads* is *Giardia lamblia,* commonly known as ‘‘Beaver Fever’’, which causes severe diarrhoea (Gerbod *et al*., 2004; Schwebke and Burgess, 2004). Morphological and molecular phylogenetic data divide parabasalids into two groups: the hypermastigs, which are gut symbionts of wood-feeding cockroaches and termites (eg the *Trichonymphida*) and the trichomonads (symbiotic, parasitic or free-living flagellates eg the *Trichomonadidae*) (Gerbod *et al*., 2004; Carpenter and Kneeling, 2007). *Trichonympha* are protists that inhabit the termite digestive systems assisting them to digest (Gerbod *et al*., 2004; Carpenter and Kneeling, 2007).

Four trichomonad species are currently found in humans: *Trichomonas tenax*, in the oral cavity, *Pentatrichomonas hominis* and *Dientamoeba fragilis*, in the intestinal tract and *T. vaginalis* in the genitourinary tract (Duboucher *et al*., 2006). Apart from a few pathogenic species (eg *T. vaginalis* and *Dientamoeba fragilis*), trichomonads are harmless commensals residing in the alimentary tract of various hosts (Figure 2.3) (Duboucher *et al*., 2006). *Trichomonas tenax*, a neighbouring taxon of *T. vaginalis* is found in patients with poor oral hygiene.

Table 2.3: List of trichomonads living in a variety of hosts

Species	Natural host location	References
Trichomonas vaginalis	Human urogenital tract	(Duboucher *et al.,* 2003)
Pentatrichomonas hominis	Human intestinal tract	(Jougnwutiwes, 2000)
Trichomonas tenax	Human oral cavity	(Mallat *et al.,* 2004)
Tetratrichomonas gallinarum	Bird's intestinal tract	(Kutisova *et al.,* 2005)
Tritrichomonas foetus	Parasite of pigs	(Gookin *et al*., 2010)
Tritrichomonas augusta	Lizard's intestinal tract	(Gookin *et al*., 2010)
Tritrichomonas foetus	Bovids's urogenital tract	(Duboucher *et al.,* 2006)

Trichomonas tenax is usually considered a harmless commensal of the human mouth; however, it can be involved in pulmonary trichomoniasis (Schwebke and Burgess, 2004). *Dientamoeba fragilis* is recognized as a common cause of chronic diarrhoea, whereas *T. vaginalis* is the causative agent of trichomoniasis (Johnston *et al*., 2004; Stark, 2006; Duboucher *et al*., 2006). *Tritrichomonas foetus* causes bovine trichomoniasis, which causes infertility, embryonic death and abortion in cattle (Johnston *et al*., 2004; Stark, 2006; Duboucher *et al*., 2006).

2.4 Characteristics and morphology of *T. vaginalis*

Trichomonas vaginalis is the most researched parasite of all trichomonads (Petrin *et al*., 1998; Benchimol, 2004). *Trichomonas vaginalis* can differ broadly in size and shape, depending on the environment's physicochemical conditions that change the appearance of the parasite (Figure 2.1) (Petrin *et al*., 1998; Benchimol, 2004; Ali and Nozaki, 2007). In favourable conditions (pure culture) the shape is more uniform, being pear shaped or oval with visible flagella. However, during unfavourable growth conditions, the trichomonad can round up and internalize its flagella (Benchimol, 2004; Ryan *et al*., 2010). The parasitic protozoan drives itself with four whip-like flagella that overhang from its front end, a fifth flagellum towards the rear (Benchimol, 2004; Ryan *et al*., 2010).

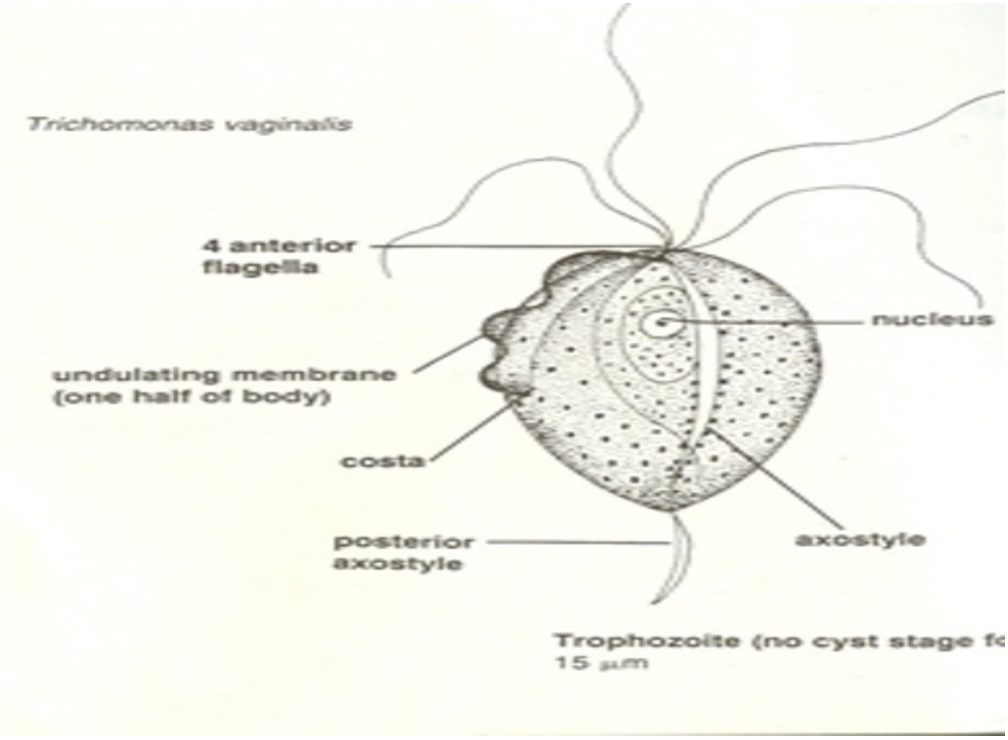

Figure 2.1: Diagrammatic representation of the general morphological structure of *T. vaginalis* (Ryan *et al*., 2010)

Trichomonas vaginalis has a cell membrane, but does not have a cell wall and half of the cell body has an undulating membrane, which helps drive nutrients into the cytosome (mouth-like structure) of *T. vaginalis* (Ryan *et al*., 2010). A clearly noticeable nucleus is located at the protozoan's centre (Aslan *et al*., 2005). *Trichomonas vaginalis* like other trichomonads is

known to exist as trophozoites and lacks the cyst stage (Sutcliffe *et al*., 2006). The reproduction of *T. vaginalis* is by mitotic division of the trichomonads occurring by longitudinal binary fission (Sutcliffe *et al*., 2006). Binary fission occurs every 8 to 12 hours under optimal conditions (Cudmore *et al*., 2004).

2.4.1 Physiology and metabolism of *T. vaginalis*

Trichomonas vaginalis is a primitive eukaryotic organism similar in many aspects to other eukaryotes; however, it differs from primitive eukaryotes in its energy metabolism and shows similarity to primitive anaerobic bacteria (Calton *et al*., 2007; Sood and Kapil, 2008). The protozoan *T. vaginalis* uses carbohydrates and amino acids as energy sources (Figure 2.2) (Calton *et al*., 2007; Sood and Kapil, 2008).

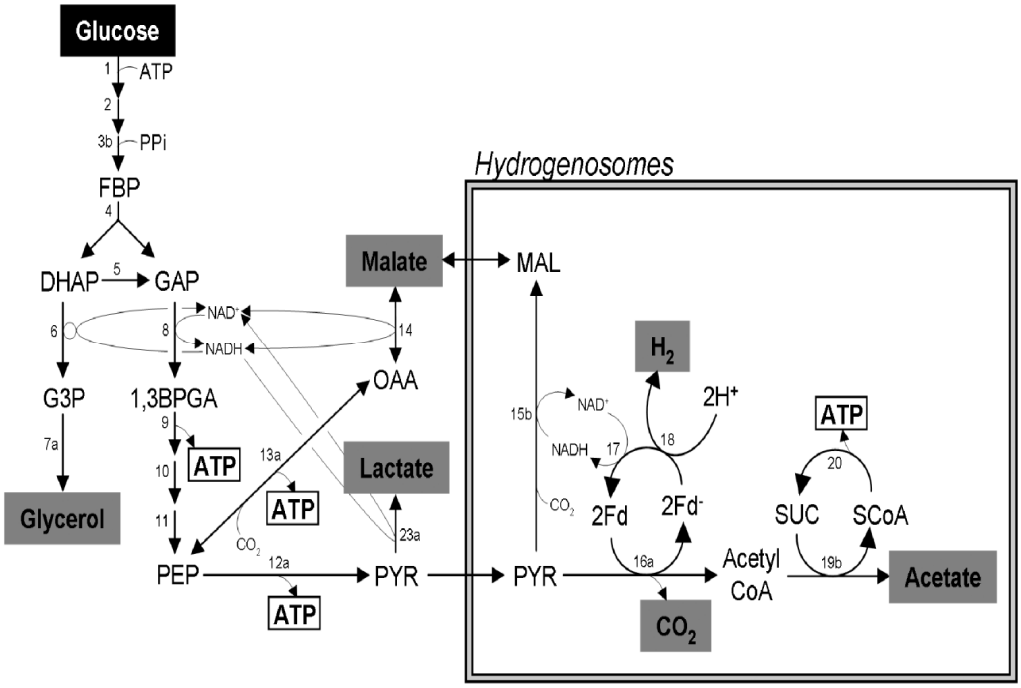

Figure 2.2: **Schematic representation of *T. vaginalis'* carbohydrate metabolism. Excreted end products of the glucose metabolism are in black characters on a grey background. ATP molecules produced by substrate level phosphorylation are boxed. DHAP-Dihydroxyacetone phosphate, GAP-Glyceraledehyde-3-phosphate, BPGA-1,3 Bisphosphoglycerate, PEP-Phosphoenolpyruvate, ATP-Adenosine trisphosphate (Calton *et al*., 2007)**

Trichomonas vaginalis uses carbohydrates as a main energy source *via* a fermentative metabolic pathway under aerobic and anaerobic conditions (Calton *et al*., 2007). The carbohydrate metabolic pathway occurs in the cytoplasm and hydrogenosomes (Sood and Kapil, 2008).

Metabolic products include acetate, lactate, malate, glycerol and carbon dioxide (Upcroft and Upcroft, 2001). In addition to carbohydrates, the protozoan is able to use a variety of amino acids as energy substrates (Figure 2.3) (Calton *et al*., 2007).

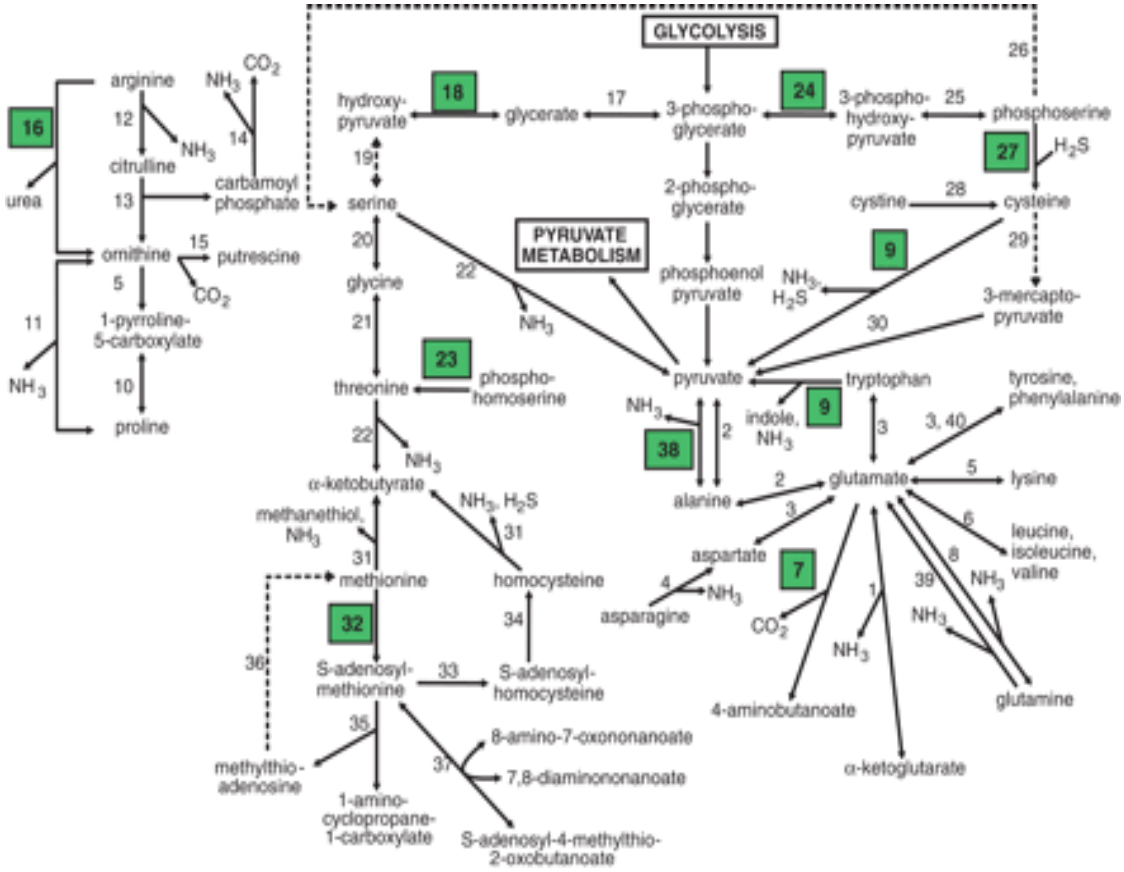

Figure 2.3: **Schematic representation of *T. vaginalis'* amino acid metabolism. Broken lines represent enzymes for which no gene was identified in the genome sequence, although the activity would appear to be required. Green boxes indicate enzymes encoded by candidate lateral gene transfer (LGT) genes (Calton *et al*., 2007)**

Amino acid metabolic pathways are more likely to be catabolic but may be reversible to allow the parasite to synthesize glutamate, aspartate, alanine, glutamine and glycine (Harp and Chowdhury, 2011). *Trichomonas vaginalis* uses a broad range of transport capabilities that facilitate transport of complex carbohydrates, fatty acids, nucleotides and amino acids through the members of the cation-chloride co-transporter (CCC) family proteins (Fichorova *et al*., 2006). *Trichomonas vaginalis* has to live within other organisms where it acquires all its nutritional requirements (Harp and Chowdhury, 2011). Essential nutrients are acquired from the genital secretions or through phagocytosis of bacterial cells (Ginger, 2006). Studies indicated that *de novo* lipid biosynthesis in *T. vaginalis* is confined to the major phospholipid

(phosphatidylethanoamine), whereas other lipids, including cholesterol are acquired from exogenous sources (Calton *et al*., 2007).

2.4.2 Genomics of *T. vaginalis*

The genome of *T. vaginalis,* the first of the *Parabasalid* was published in 2007 (Calton *et al*., 2007). The *T. vaginalis* isolate G3 from a patient from Kent, England, was chosen as the genome reference strain due to its widespread use by investigators in studies of virulence, biochemistry, cell and molecular biology and its ability to grow axenically without being infected with a dsRNA viruses (Clark *et al*., 2010). The *T. vaginalis* genome sequence was generated using the whole-genome shot gun methodology (Calton *et al*., 2007). The genome draft of *T. vaginalis* revealed an exceptionally large genome size of 160 Mb, nearly 10 times larger than predicted earlier (Singh *et al*., 2012). The genome of *T. vaginalis* expanded in size over years (Calton *et al*., 2007; Clark *et al*., 2010). The increase in genome size, which led to the large cell size could have been preferred in order to increase the surface area for phagocytosis of bacteria, erythrocytes and other host cells (Pereira-Nerves and Benchimol, 2004; Gomez *et al*., 2010).

There are six chromosomes in *T. vaginalis* and a core set of 60 000 protein-coding genes were identified (Calton *et al*., 2007; Clark *et al*., 2010). This means *T. vaginalis* has one of the highest coding capacities among eukaryotes (Calton *et al*., 2007; Clark *et al*., 2010). *Trichomonas vaginalis* has highly similar repeats and transposable elements with at least 65% of the genome being repetitive (Malik *et al*., 2008). The most common of the 60 repeat families were classified into four groups, namely: virus-like repeats, retrotransposon-like repeats, transposon-like repeats and unclassified repeats (Malik *et al*., 2008). A number of genes (99 883) were predicted from the genome sequence using gene finding algorithms. Thirty-eight thousand two hundred and thirteen were reclassified as repeats by screening with the 60 repeat families (Vanacova *et al*., 2005; Calton *et al*., 2007). The remaining 59 681 gene models were considered the core set of putative protein coding genes (Calton *et al*., 2007). Transfer RNAs for all 20 amino acids were identified and approximately 250 rRNA units were found on small contigs (Calton *et al*., 2007). All rRNA units appeared to be located on only one of the six *T. vaginalis* chromosomes (Calton *et al*., 2007).

2.4.3 *Trichomonas vaginalis* infected with small double-stranded RNA viruses and *Mycoplasma hominis*

Many *T. vaginalis* isolates are infected by a double-stranded RNA virus (dsRNA), designated *T. vaginalis* virus (TVV). *Trichomonas vaginalis* virus has been found in the cytoplasm closely associated with the Golgi complex or adjacent to the plasma membrane (Sood and Kapil, 2008). The population may include viruses of different sizes (33 to 200 nm) and shapes (filamentous, cylindrical and spherical particles) (Benchimol, 2004; Goodman *et al*., 2011). Up to four distinct TVVs can stably infect a clinical isolate of *T. vaginalis* (Goodman *et al*., 2011). The presence of double-stranded RNA (dsRNA) viruses, in trichomoniasis was found to correlate with a variation in the expression of certain surface antigens and loss of dsRNA accompanied loss of antigen expression (Sood and Kapil, 2008). However, the precise role of dsRNA viruses in the pathogenesis of trichomoniasis remains to be determined (Sood and Kapil, 2008).

Trichomonas vaginalis is also infected with *Mycoplasma hominis* (Van Belkum *et al*., 2001; Xiao *et al*., 2008). *Mycoplasma hominis* is a bacterial species, which is frequently found in the lower genital tract and has been implicated in pelvic inflammatory disease as well as pregnancy complications, such as preterm delivery and low-birth weight infants (Butler *et al*., 2010). *Trichomonas vaginalis* isolates and human epithelial cells are susceptible to *M. hominis* infection (Butler *et al*., 2010). Several studies have reported on the coexistence of *M. hominis* with *T. vaginalis* (Van Belkum *et al*., 2001; Fang *et al*., 2006; Xiao *et al*., 2006; Xiao *et al*., 2008). Approximately 50% of clinical isolates of *T. vaginalis* are infected with *M. hominis* (Fang *et al*., 2006). Different *T. vaginalis* isolates are infected by different *M. hominis* strains, which are postulated to exert different effects on *T. vaginalis,* such as pathogenicity and drug resistance (Xiao *et al*., 2008). However, a study by Butler *et al*. (2010) reported no correlation between the relationship of *M. hominis* and *T. vaginalis* with an increase in metronidazole resistance.

2.5 Epidemiology of *T. vaginalis*

Trichomonal infection has a cosmopolitan distribution and has been found in all racial groups, in both developed and developing countries (Soper, 2004; Sood and Kapil, 2008). It has been recognized that trichomoniasis plays a significant role in HIV transmission dynamics

(Mavedzenge *et al*., 2010). Risk factors associated with increased susceptibility to HIV infection include having multiple sexual partners, having unprotected sexual intercourse, poverty and vaginal douching (Sutton *et al*., 2007; Kissinger *et al*., 2008).

The worldwide prevalence of *T. vaginalis* in different regions ranges between 4.95% to 18.1% in women and 0.49% to 3.83% in men (Table 2.4) (WHO, 2011). A high prevalence of *T. vaginalis* has been observed among prison inmates in the United States of America, prevalence rates vary between 26% (45/205) to 46.9% (100/213) (Shuter *et al*., 1998; Willers *et al*., 2008).

Table 2.4: Prevalence of *T. vaginalis* in men and women in different WHO regions (WHO, 2011)

Region	Percentage (%)	
	Women	Men
Africa	18.12	3.82
Europe	6.22	0.62
North and South America	14.8	1.43
East Mediterranean	5.58	0.56
South-East Asia	5.58	0.56
Western Pacific	4.95	0.49

Epidemiological studies that focused on age-related ratio occurrences have established that trichomoniasis is more common in older women in contrast to other non-viral STIs (*C. trachomatis* and *N. gonorrhoeae*) where infection is more prevalent in younger women (Niccolai *et al*., 2000). In a study done by Gaydos and colleagues between July and December 2011 that followed 7 593 female patients from twenty-seven States in the USA, the trichomoniasis infection rate in women in their 50s was 13% (Gaydos *et al*., 2011). A prevalence of 11% was observed in women in their 40s, while the infection rate was 8.5% in women aged 18 to 19 years (Gaydos *et al*., 2011). The difference in age distribution may be explained by the extended periods of existence of *T. vaginalis* in individuals, since it is mostly asymptomatic in individuals (Soper, 2004; Fernado *et al*., 2012).

Women who are less educated are more at risk of developing trichomoniasis (Mcllelland *et al*., 2008; Pinheiro de Lemos and Garcia-Zapata, 2010). It may be possible that better education is associated with the early seeking of confirmation of diagnosis and treatment of STDs (Leon *et al*., 2009; Fernado *et al*., 2012). It is possible that less educated women miss out on sex education, which is addressed at later stages of the school curriculum (Leon *et al*., 2009).

Trichomonas vaginalis is primarily transmitted through sexual intercourse, although non-sexual transmission is also a common mode of transmission (Peterson and Dramme, 2010; Crucitti *et al*., 2011). Adu-Sarkodie reported transmission of *T. vaginalis* within a family in Ghana and hypothesized that transmission occurred from the mother to her children through sharing of bathing implements (Adu-Sarkodie, 1995). In a study in Ndola (Zambia) there was a prevalence of 24.7% among virgins and 40% among women who denied any sexual activity (Crucitti *et al*., 2011). The sharing of bed linen, toilets, bath tubs, towels and water have been cited as routes of the non-sexual transmission of *T. vaginalis* (Crucitti *et al*., 2011). Crucitti *et al.* (2011) cited a common traditional practice (elongation of labia majora in young girls by older infected women) as a cause of *T. vaginalis* transmission (Crucitti *et al*., 2011). Vaginal examination by traditional healers has been found to be responsible for the spread of *T. vaginalis* in Gambia (Peterson and Dramme, 2010).

2.6 Pathogenesis and virulence factors of *T. vaginalis*

The host-parasite of *T. vaginalis* interaction is complex and the wide variety of medical symptoms cannot be attributed to a distinct pathogenic mechanism (Sood and Kapil, 2008). As an extracellular parasite, *T. vaginalis* colonizes the mucosal surfaces of the human genitourinary tract without direct invasion (Goodman *et al*., 2011). Infection by *T. vaginalis* involves adhering to the squamous epithelium cells of the genital tract resulting in a multifocal infection (Han *et al*., 2012). Infection usually does not involve the invasion of the parasite into the tissue (Han *et al*., 2012). *Trichomonas vaginalis* has been reported in the urinary tract, fallopian tubes, pelvis and can cause pneumonia, bronchitis and oral lesions (Soper, 2004; Usanga *et al*., 2010). In men, the (external) genitalia, prostate and epididymitis can be infected (Smith and Ramos, 2010). In infected women, the parasite may be found in the vagina, cervix and the bladder, as well as Bartholin's Skene and periurethral glands (Cudmore *et al*., 2004). Despite its extracellular life style, *T. vaginalis* can effect mucosal damage through an apparent variety of mechanisms some of which are dependent on epithelial cell contact (adherence-based cytotoxicity) and others on the release of soluble factors, such as proteinases (Fichorova, 2009). In many cases *T. vaginalis* infection results in a profuse release of host immune response elements that lead to inflammation and further mucosal damage (Goodman et *al*., 2011).

2.6.1 Adhesion of *T. vaginalis*

Adhesion of the trichomonads to the epithelial cells in the vaginal milieu plays a vital role in the pathogenesis of the parasite (Kucknoor *et al*., 2005). The exterior of the trichomonad cell is a medley of adhesion proteins and carbohydrates, which offer the foundation for ligand-receptor binding in the heavily defended vaginal mucosa (Hirt *et al*., 2007). Following cyto-adherence *T. vaginalis* changes to an amoeba configuration with growing cell-to-cell surface inter-digitations (Harp and Chowdhury, 2011). *Trichomonas vaginalis* has a distinctive cell surface with copius lipophosphoglycan (LPG), a carbolipid molecule that contribute significantly to key pathogenic and immune-regulatory functions (Singh *et al*., 2009). Lipophosphoglycans is a key component as an adherence feature in *T. vaginalis* (Crouch *et al*., 2001; Lubick and Burgess, 2004; Fichorova, 2009; Gomez *et al*., 2010). Research on the molecular basis of adhesion of *T. vaginalis* to human cells have discovered that several extra genes' expression [adhesion proteins (AP), fibronectin (FN)-binding protein, laminin-binding orotein, α-actinin, enolase, phosphoglucomutase and preserved GTP-binding protein (GTP-BP)] are upregulated through adhesion (Crouch *et al*., 2001; Lubick and Burgess, 2004; Fichorova, 2009; Gomez *et al*., 2010). The adhesion of the parasite is mediated by four main adhesion proteins which are: AP65, AP51, AP33 and AP23 that operate in a receptor-ligand manner (Crouch *et al*., 2001; Lubick and Burgess, 2004; Gomez *et al*., 2010).

2.6.2 Hydrolases and cytotoxic molecules of *T. vaginalis*

A variety of hydrolases (20 to 110 kDa), such as the cytoplasmic cysteine proteinases, are present in *T. vaginalis* (Leon-Sicairos *et al*., 2004; Schwebke and Burgess, 2004). These hydrolases have trypsin-like activity, which breaks down proteins, such as laminin and fibronectin of the extracellular matrix (ECM) (Sood and Kapil, 2008). The resulting cell-detaching helps in the release of host cells from tissue and mucosal desquamation (Schwebke and Burgess, 2004). *Trichomonas vaginalis* manufactures a number of cytotoxic molecules and incites cytotoxicity through the destruction of the targetted cell's plasma membrane (Gilbert *et al*., 2000). Some of these cytotoxic molecules exhibit perforin-like action and generate holes in the erythrocyte membrane (Calton *et al*., 2007; Sood and Kapil, 2008; Gomez *et al*., 2010). Erythrocytes form a crucial part of the nutritional requirements of *T. vaginalis*, since fatty acids and iron are deficient in the protozoa (Tiwari *et al*., 2008).

2.6.3 Evasion of immune system by *T. vaginalis*

Trichomonas vaginalis eludes the immune system through complement-mediated destruction, molecular imitation and by covering itself with host plasma proteins (Yadav *et al*., 2005; Fichorova, 2009). *Trichomonas vaginalis* manufactures immuno-suppressive cytokines (IL-10, TGFβ) and causes caspase-mediated apoptosis in T-cells, macrophages and dendritic cells (Fichorova, 2009; Dwivedi and Aich, 2011).

2.7 Immunological response to *T. vaginalis*

Trichomonas vaginalis infection in humans result in a site-specific infection and disseminating antibodies are released in the reproductive tract serum (Cudmore *et al*., 2004; Schwebke and Burgess, 2004; Yadav *et al*., 2005). There is also evidence of lymphocyte priming as detected by antigen-specific proliferation of peripheral blood mononuclear cells (Schwebke and Burgess, 2004). Leukocytes produce a number of biochemical molecules: interleukin-8 (IL-8), leukotrienes reactive nitrogen intermediates, macrophage and inflammatory protein-3α (Crouch *et al*., 2001; Lubick and Burgess, 2004; Gomez *et al*., 2010). Leukocytes stimulate nitric oxide synthase (Inos), site-specific immunoglobulin G (lgG) and A (lgA) and promote transmigration of neutrophils across the endothelium (Crouch *et al*., 2001; Lubick and Burgess, 2004; Gomez *et al*., 2010). The assault of the *T. vaginalis* membranes results in bursting, which lead to the disintegration and discharge of its organelles (Lubick and Burgess, 2004; Gomez *et al*., 2010).

2.8 Clinical presentation of *T. vaginalis*

Trichomonas vaginalis affects both men and women although the clinical presentation differs (Swygard *et al*., 2004). Women who contract trichomoniasis are more likely to have symptoms compared to men who become infected. Approximately 75% to 100% of men are asymptomatic in comparison to approximately 50% to 75% of women who are identified as asymptomatic (Miller and Nyiresy, 2011; Dwivedi *et al*., 2012).

2.8.1 Trichomoniasis in women

Trichomonas vaginalis in women may present as abdominal tenderness, pain, itching and irritation of the genital region (Strous, 2009; Sobel, 2012). In roughly 2% of infected women, punctuate bleeding (focal erosions and proliferations of granulation tissue) can be observed (Johnston and Mabey, 2008). Assessment of the vagina might disclose infection accompanied with noticeable ulceration with or without necrosis (death of tissue), the ulcerated area may be coated with purulent exudates with distinct mucosal oedema (Chan and Winkie, 2002).

Trichomonas vaginalis infection in pregnant women leads to difficulties during the pregnancy, which comprise rupture of membranes, premature labour and low birth weight newborns (Johnston and Mabey, 2008; Dwivedi *et al*., 2012). With pregnancy and childbirth being the foremost reason of death for women internationally, an infection that can further jeopardize the wellbeing of mother and child cannot be overlooked (Cudmore and Garber, 2010). Other complications of *T. vaginalis* include a predispotion to cervical cancer, atypical pelvic inflammatory disease and sterility (Madhivana *et al*., 2009; Bachman *et al*., 2011).

Many explanations have been proposed to explain why women display more symptoms than men (Cudmore and Garber, 2010). The explanations include the fact that the female genital tract, bestows a reducing milieu, which encourages the advancement of infection (Gehring and Efferth, 2009). *Trichomonas vaginalis* leads to a decline in lactobacilli (beneficial bacteria) in the vagina and thus presents a more conducive atmosphere for invasion and growth of the parasite (Gehring and Efferth, 2009). Menstrual blood also raises the pH and function as an iron source to enhance the attachment of *T. vaginalis* (Cudmore *et al*., 2004). *Trichomonas vaginalis* is conveyed to babies by breast feeding; however, the baby's estrogen levels (which diminish in the third to sixth week of an infant's life) averts the development of a neonatal type of the disease (Cudmore and Garber, 2010; Sobel, 2012).

2.8.2 Trichomoniasis in men

Infection in men can present in the prostate, seminal vesicles and epidymitis (Sena *et al*., 2007; Sood and Kapil, 2008; Harp and Chowdhury, 2011). In rare cases, complications of *T. vaginalis* infection in men include weakened sperm mobility and viability, which may cause infertility (Lewis, 2010; Ozdemir *et al*., 2011). The oxidative nature of the male genital fluid is

nonetheless perceived to be inhibitory to particular pathogenic factors of the protozoan (Gehring and Efferth, 2009). The presence of zinc in prostatic fluid acts as a cytotoxic factor against the trichomonads (Cudmore *et al*., 2004). The amount of secretory leukocyte protease inhibitors are notably lower in *T. vaginalis* infected male patients (Sood and Kapil, 2008).

2.8.3 Trichomoniasis and HIV coinfection

Infection with *T. vaginalis* can be a marker for high risk sexual behaviour and frequently occurs concomitantly with other STIs, including gonorrhoea, *Chlamydia* and HIV (Hobbs and Sena, 2007). Co-infection of *T. vaginalis* and HIV may increase the infectiousness of both organisms (Hobbs and Sena, 2007). Studies have shown that people with trichomoniasis have a 1.5 to 5 times greater risk of seroconversion when exposed to HIV and *vice versa* (Cudmore and Garber, 2010; Mavedzenge *et al*., 2010). Co-infection of *T. vaginalis* and HIV has been shown to increase the cervical shedding of the HIV virus in women and to lead to higher urethral loads of the HIV virus and HIV RNA in men (Cudmore and Garber, 2010).

Several mechanisms have been proposed to explain why people who have been infected with trichomoniasis have an increased susceptibility to contract HIV (Sood and Kapil, 2008; Shafir *et al*., 2009). Women with trichomoniasis often have disrupted vaginal flora as the presence of *T. vaginalis* leads to a reduction or elimination of *Lactobacillus* species that causes a rise in the pH of the vagina (Sood and Kapil, 2008; Cudmore and Garber, 2010). An increase in pH may not only promote the growth of *T. vaginalis* but also create a more favourable environment for HIV as well as the bacteria associated with other STIs and bacterial vaginosis (Sood and Kapil, 2008; Cudmore and Garber, 2010). Secreted *T. vaginalis* proteins and cell to cell contact dependent mechanisms cause disruption of vaginal epithelium leading to the development of punctuate haemorrhages (Johnston and Mabey, 2008; Mavedzenge *et al*., 2010). These lesions enable HIV access to underlying sub-mucosal tissue facilitating the virus to penetrate and spread beyond the genital tract (Sood and Kapil, 2008; Cudmore and Garber, 2010).

A *T. vaginalis* infection leads to a local vaginal inflammation response that is characterized by recruitment of neutrophils, macrophages and $CD4^+$ helper T cells (Johnston and Mabey, 2008; Mavedzenge *et al*., 2010). The cells that are released during the inflammatory response are susceptible to infection with HIV (Johnston and Mabey, 2008; Mavedzenge *et al*., 2010). This

inflammatory response leads to the production of tumor necrotising factor-alpha (TNF-α), which activates $CD4^+$ T cells, increasing the susceptibility to HIV infection (Guenther *et al*., 2005; Shafir *et al*., 2009; Lazenby, 2011). The induction of TNF-α has been shown *in vitro* to significantly increase HIV infections, since it increases the replication of the HIV infected cells (Cudmore and Garber, 2010; Mavedzenge *et al*., 2010).

2.9 Diagnosis of *T. vaginalis*

Traditionally, physicians diagnosed trichomoniasis on the basis of the clinical picture; however, diagnosis of *T. vaginalis* cannot be made solely on the basis of clinical presentation for primarily two reasons: (i) Most of the patients with *T. vaginalis* are asymptomatic; the classical "strawberry" cervix is seen in approximately 2% of patients and the frothy discharge is seen only in 12% of women with *T. vaginalis* infection (Sood and Kapil, 2008; Houso *et al*., 2011) and (ii) The clinical symptoms may be synonymous with those of other STDs, which include cervicitis, epididymitis and prostatitis (Houso *et al*., 2011; Patil *et al*., 2012). It has been demonstrated that if these classical features are used alone in the diagnosis of trichomoniasis, 88% of the cases will not be diagnosed and 29% of the cases uninfected patients will be falsely indicated as having an infection (Patil *et al*., 2012). This suggests that clinical manifestations are not reliable diagnostic parameters and laboratory diagnosis is therefore, necessary for early and accurate diagnosis (Garber, 2005). Precise, reliable, rapid and low-priced laboratory diagnostic tests play a key role in the diagnosis of *T. vaginalis*. These diagnostic tests can be classified as old (wet mount), newer (culture) and the latest (rapid antigen and nucleic acid amplification) techniques (Harp and Chowdhury, 2011).

2.9.1 Microscopy detection of *T. vaginalis*

Wet mount microscopy is the most extensive method that is used for the detection of *T. vaginalis* (Bachmann *et al*., 2011). Wet mount microscopy involves the visualization of *T. vaginalis* distinctive characteristic size, morphology and characteristic trichomonad movements (jerky, swaying or tumbling) seen in the physiological saline preparation of genital secretions or urine (Garber, 2005). The sensitivity of microscopy varies from 50% to 66% with a specificity of 100% (Pattullo *et al*., 2009). Wet mount microscopy is a low-cost method and positive results can be obtained quickly, allowing patients to be treated in one clinic visit (Garber, 2005; Huppert *et al*., 2005). However, wet mount microscopy is a subjective test that

requires skill and access to a microscope (Huppert *et al*., 2005). A delay in the transport and evaporation of moisture from the specimen reduces the motility and as a result diagnostic sensitivity (Huppert *et al*., 2005; Bachmann *et al*., 2011). Microscopy should thus be done within two hours of specimen collection (Huppert *et al*., 2005; Bachmann *et al*., 2011).

Staining techniques have been used to enhance the sensitivity of microscopic assessments (Garber, 2005). These staining techniques can detect three trichomonads per ml (Garber, 2005). The most common dyes are: Leishman stain, periodic acid-Shiff stain and Fontana dye (Garber, 2005). The sensitivity of the dyes ranges from 30% to 60% and specificity varies between 10% to 30% (Garber, 2005). *Trichomonas vaginalis* fluoresces with a yellow-green banana-shaped nucleus, while epithelial cells and leuckocytes fluoresce light-green (El Sayed *et al*., 2010). Staining with the Giemsa stain shows the characteristic violet pear-shaped trophozoites (El Sayed *et al*., 2010).

2.9.2 *In vitro* growth of *T. vaginalis*

Culturing of *T. vaginalis* is the current "gold standard" for the diagnosis of *T. vaginalis* (Huppert *et al*., 2011). Selective media that can be used for culturing of *T. vaginalis* include Diamond's, modified Diamond's media, Trichosel and InPouch TV (BioMed Diagnostics, San Jose, Calif, USA) (Hobbs and Sena, 2007). None of the culture media seem to have 100% sensitivity for *T. vaginalis* isolation (Huppert *et al*., 2005). After inoculation with a vaginal swab specimen or urine sediment, cultures are incubated for three to five days at 37^0C at 5% CO_2 atmosphere and examined daily for motile trichomonads (Caliendo *et al*., 2005; Schirm *et al*., 2007). Growth of *T. vaginalis* is usually observed within two to three days after incubation (Caliendo *et al*., 2005; Schirm *et al*., 2007). The InPouch TV (BioMed Diagnostics, San Jose, Calif, USA) is a double pouched soft transparent plastic container containing *T. vaginalis* culture media (Hobbs and Sena, 2007). The InPouch has a sensitivity of 85% to 95% and specificity of 95% to 100% (Hobbs and Sena, 2007). The InPouch culture method has several advantages, which include its simplicity of use, the combination of specimen transport, growth and evaluation and its long shelf-life (Huppert *et al*., 2005). The InPouch method requires on-site incubators, microscopes and 24 h to 120 h for final results (Lazenby, 2011). Availability of culturing techniques is limited due to increased financial costs and some *T. vaginalis* isolates cannot be cultured due to strain variation (Garber, 2005).

2.9.3 Commercially available point of care tests for the detection of *T. vaginalis*

Point of care tests for the diagnosis of trichomoniasis in women are commercially available and provide rapid and sensitive detection methods in a short period of time (Brown *et al*., 2004). Point of care tests allow for instant treatment and psychotherapy of patients (Kurth *et al*., 2004). Rapid tests are essential for use in settings where culture and microscopy are not feasible and in populations, such as adolescents and patients seen in emergency departments, both of whom present difficulties to follow-up (Van der Pol *et al*., 2006).

The rapid antigen-antibody test (OSOM TV, Genzyme Diagnostic, Cambridge, USA) is a lateral flow strip test device that detects *T. vaginalis* membrane proteins within 10 min (Bachmann *et al*., 2011). The OSOM rapid test employs a pair of murine monoclonal antibodies, one immobilized on the surface of the dipstick and the other conjugated to particles and dried onto the dipstick (Miller *et al*., 2003). Its reported sensitivity is 85% to 90% from dead organisms with a specificity of 100%, which is similar to those of culture (Huppert *et al*., 2005; Huppert *et al*., 2007). The XenoStrip-Tv (Xenotope Diagnostics, San Antonio, USA) is an immunochromatographic capillary flow assay and has demonstrated a sensitivity of 78.5% to 90% when performed on vaginal swab samples, but it requires up to 20 min to read (Miller *et al*., 2003).

2.9.4 Nucleic acid amplification tests for *T. vaginalis*

The development of highy sensitive nucleic acid amplification tests (NAATs) has provided highly sensitive and specific assays for the detection of *T. vaginalis* (Hobbs and Sena, 2007). Nucleic acid amplification tests are biochemical techniques that directly detect the genetic material of a pathogen (Hobbs and Sena, 2007). There are multiple NAAT methods that fall in this group including the transcription mediated amplification, PCR and ligase chain reaction (Madico *et al*., 1998; Lawing *et al*., 2000; Mayta *et al*., 2000; Jordan *et al*., 2001; Hobbs and Sena, 2007).

Polymerase chain reaction (PCR) assays are the most common NAAT tests and it involves “in-house”, PCR assays, which are based on a protocol developed in a non commercial laboratory (”home brew”) (Madico *et al*., 1998; Lawing *et al*., 2000; Mayta *et al*., 2000; Hobbs and Sena, 2007). Many laboratories have developed their own in-house PCR assays (Madico *et al*., 1998;

Mayta *et al*., 2000; Jordan *et al*., 2001; Hobbs and Sena, 2007). Access to these "in-house" PCR assays has been restricted basically to research laboratories (Hobbs and Sena, 2007). The *T. vaginalis* PCR targets of different "in house" assays include: the beta tubulin gene (Madico *et al*., 1998; Dwivedi *et al*., 2012), the ferredoxin gene (Riley *et al*., 1992; Jordan *et al*., 2001), 18S ribosomal gene (Mayta *et al*., 2000; Simpon *et al*., 2007) and the highly repeated 2-kb DNA repetitive sequence (Kegne *et al*., 1994).

The performance of various PCR primers assessed in different studies varies, with reported sensitivities ranging from 60% to 100% (Hobbs and Sena, 2007). Crucitti and colleagues in 2003 compared the performance of different targets and found sensitivities that ranged from 65.1% to 92.8% (Table 2.5).

Table 2.5: Comparative results of sensitivity, specificity and test efficiency of five different target regions of *T. vaginalis* (Crucitti *et al*., 2003)

Primer set	Sensitivity (%)	Specificity (%)	Efficiency (%)
Culture	34.9	100	87
DNA repeats	92.8	94.6	95.5
Adhesion protein	65.1	75.2	92.5
DNA repeats	84.3	99.7	96.6
Beta tubulin	83.1	98.5	95.5
18S Ribosomal units	69.9	99.4	93.3

The Affirm BD Affirm VPlll Microbial affirm assay (Becton Dickinson, Franklin Lakes, New Jersey) is a transcription based assay, which is commercially available (Andrea and Chapin, 2011). The Affirm BD VPIII is an RNA probe office-based test that can be used on direct-vaginal specimens (Brown *et al*., 2001; Brown *et al*., 2004). Results can be provided within 45 min (Hobbs and Sena, 2007). The sensitivity of the Affirm BD VPIII was found to be 64% and the reported specificity was high at 99.9% (Andrea and Chapin, 2011).

The Anyplex^{TM} II STI-7 (Seegene, Korea) detection assay is a multiplex real-time assay which enables the detection of the presence of seven organisms in a single reaction (Seegene, 2011). This is the only multiplex-real time PCR test that can detect seven of the most common STDs within three and a half hours; providing for fast, effective and economical screening (Seegene, 2011). The seven major STIs detected are *Trichomonas vaginalis, Chlamydia trachomatis*,

Neisseria gonorrhoeae, *Mycoplasma genitalium, Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum* (Seegene, 2011).

2.9.4.1 Automated nucleic acid tests for detection of *T. vaginalis*

Three of the fully automated commercially available molecular assays for the detection of *Trichomonas vaginalis* are the: APTIMA TV (Andrea and Chapin, 2011; Schwebke *et al*., 2011) and BD Probe Tec^{TM} *Trichomonas vaginalis* Qx amplified DNA (BD Diagnostics, New Jersey, USA). Automated machines reduce human intervention and associated variables (Andrea and Chapin, 2011; BD Diagnostics, 2012). The availability of an automated platform is especially important as clinical and public health communities embrace the need for a test that provides timely and accurate diagnosis of trichomoniasis when screening high-risk populations (Andrea and Chapin, 2011; BD Diagnostics, 2012).

The Aptima *Trichomonas vaginalis* assay (Gen Probe, San Diego, USA) is an *in vitro* commercially available transcription based assay for the detection of *T. vaginalis* results can be provided within 45 min (Hobbs and Sena, 2007). The Aptima *T. vaginalis* assay is a nucleic acid amplification tests (NAAT) that combines the technologies of target capture, transcription mediated amplification (TMA) and the chemiluminescent probe hybridization assay (HPA) that utilizes target capture to detect *T. vaginalis* rRNA (Andrea and Chapin, 2011; Schwebke *et al*., 2011). Detection is performed on an automated Tigris DTS system (Gen Probe, San Diego, USA). In a study conducted in the USA on 781 vaginal specimens, the Aptima was shown to be more sensitive than the Affirm assay (100% versus 63.4%) (Andrea and Chapin, 2011). The Aptima *Trichomonas vaginalis* assay (Gen Probe, San Diego, USA) detected 36.65% (41/781) more positive patients than BD Affirm VPIII (26/781) (Andrea and Chapin, 2011).

The BD Probe TEC^{TM} *Trichomonas vaginalis* Qx amplified DNA assay (BD Diagnostics, New Jersey, USA) is for the direct qualitative detection of *T. vaginalis* DNA in specimens using the BD Viper ^{TM} automated system (BD Diagnostics, 2012). The BD Viper ^{TM} system performs automated DNA extraction, simultaneous amplification and detection, which maximizes laboratory efficiency and the quality of results (Schwebke *et al*., 2011; BD Diagnostics, 2012).

2.10 Treatment of *T. vaginalis*

Since, the 1960s, metronidazole and the other 5-nitroimidazoles (tinidazole, ornidazole and secnidazole) have become the treatment of choice for *T. vaginalis* infections (Upcroft *et al*., 2006b; Gehring and Efferth, 2009, Goldman *et al*., 2009, Wright *et al*., 2010). Metronidazole was manufactured in 1959 from the derivation of the antibiotic, azomycin which was obtained from *Streptomyces eurocidicus* (Kita *et al*., 2007). The effectiveness of the drug is high, with curing rates of approximately 85% to 95% of treated patients and re-infections can be prevented through simultaneous treatment of sexual partners (Frenkl and Potts, 2008).

A number of other nitroimidazoles (tinidazole, secnidazole, secnidazole) can be used for treatment of trichomoniasis and have the same mode of action as metronidazole (Gehring and Efferth, 2009). Tinidazole is a second-generation nitro-imidazole with a plasma elimination half-life twice that of metronidazole (12 h to 14 h versus 6 h to 7 h) (Johnson, 2009). The efficacy of tinidazole equals and has been proven to be superior to that of metronidazole (Gehring and Efferth, 2009). In addition, tinidazole has a longer half-life and lower elimination rate in contrast with metronidazole, which may be the basis for its curative effect at smaller dosages (Harp and Chowdhury, 2011). Other nitroimidazoles like ornidazole and secnidazole are comparable to tinidazole in that these antimicrobials have extended half-lifes and lower elimination rates than metronidazole (Gehring and Efferth, 2009). Unlike metronidazole tinidazole is expensive and thus cannot be used routinely in developing countries like South Africa (Kissinger *et al*., 2008).

Hamycin is an aromatic polyene related to amphotericin B (Dunne *et al.*, 2003). It kills *T. vaginalis* by combining briefly to the ergosterols in the plasma membrane and inducing death (Dunne *et al.,* 2003). Hamycin is currently being used in India for topical treatment of trichomoniasis (Cudmore *et al*., 2004).

2.11 Resistance of *T. vaginalis*

Clinical resistance of *T. vaginalis* is defined as the unsuccessful curing of trichomoniasis after at least two successive courses of metronidazole (Sood and Kapil, 2008). Therapy failure is often a consequence of failure to take the medication as recommended or re-infection from an untreated partner (Cudmore *et al*., 2004; Schwebke and Barrientes, 2006). Insufficient oral

absorption, low serum zinc concentrations and inactivation of metronidazole by the vaginal flora also contribute to therapy failure (Cudmore *et al*., 2004). Worldwide approximately 2.5% to 10.1% of *T. vaginalis* isolates are estimated to be resistant to metronidazole (Schwebke and Barrientes, 2006; Kissinger *et al*., 2008; Krashin *et al*., 2010). Increasing dosages and lengthening the time of treatment are generally suggested in situations where there is antimicrobial resistance; however, increasing the dosages may reach levels that are poisonous to the patient (Cudmore and Garber, 2010).

Metronidazole infiltrates the cell and its organelles through passive diffusion in an inactive prodrug form (Gehring and Efferth, 2009). The prodrug is subsequently activated by reduction of metronidazole with electrons (Wright *et al*., 2010). This result in the formation of a cytotoxic nitro-radical anion, which attaches briefly to the DNA to disrupt the nucleotide strands; causing cell death (Hrdy *et al*., 2005).

In a susceptible isolate of *T. vaginalis*, electrons required for the drug reduction are generated by the key hydrogenosomal enzyme, pyruvate: ferredoxin oxireductase (PFOR) (Rasoloson *et al*., 2002; Cudmore *et al*., 2004). Pyruvate: ferredoxin oxireductase (PFOR) catalyzes oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 (Rasoloson *et al*., 2002; Cudmore *et al*., 2004). Electrons released in the PFOR reaction are accepted by the ferredoxin that is subsequently reoxidized by the enzyme hydrogenase (Rasoloson *et al*., 2002; Cudmore *et al*., 2004).

Resistance of *T. vaginalis* to metronidazole can be classified as aerobic resistance or anaerobic resistance (Cudmore *et al*., 2004). The anaerobic resistance occurs by elimination of pathways responsible for the reductive activation of the drug (Rasoloson *et al*., 2002; Cudmore *et al*., 2004). Anaerobic resistance is driven by the reduction or absence of the enzyme pyruvate ferredoxin oxidoreductase (PFOR) and hydrogenase (Dunne *et al*., 2003, Cudmore *et al*., 2004). Anaerobic resistance unlike aerobic resistance has only been demonstrated in laboratory-developed strains, not in clinical isolates (Rasoloson *et al*., 2002).

The aerobic resistance typically occurs in clinical isolates from treatment-refractory patients, it manifests only if oxygen is present (Rasoloson *et al*., 2002). The resistance results from defective oxygen scavenging and subsequent interference of intracellular oxygen with the drug activation (Rasoloson *et al*., 2002). Oxygen scavenging pathways reduce availability of

oxygen, which is important as an electron acceptor (Rasoloson *et al*., 2002). In aerobic resistance, transciption of the ferredoxin gene is reduced and this leads to a reduction in the transfer of electrons (Cudmore *et al*., 2004).

2.12 Genotyping of *T. vaginalis*

All organisms are subject to mutations as a result of normal cellular operations or interactions with the environment, leading to genetic variation or polymorphism (Liu and Cordes, 2004). In conjuction with selection and genetic drift, there arises genetic variation within and among the individuals, species and higher taxonomic groups (Liu and Cordes, 2004). At the DNA level, types of genetic variation include single nucleotide polymorphism (base substitution, insertions or deletions within a locus) and rearrangement of DNA segments within a locus (Liu and Cordes, 2004).

The availability of techniques to track the genetic variation and to examine the population genetics of different organisms is important for the control and prevention programmes of trichomoniasis (Liu and Cordes, 2004; Meade *et al*., 2009). Multiple approaches to typing *T. vaginalis* have produced differing results and the *T. vaginalis* genome's composition provides a potential explanation for the difficulty in genotyping (Calton *et al*., 2007). The *T. vaginalis* genome is highly repetitive with 65% of the genome consisting of repeated sequences (Calton *et al*., 2007; Meade *et al*., 2009).

2.12.1 Genotyping of *T. vaginalis* using pulsed-field gel electrophoresis

The predominant technique used for strain characterization has been pulsed-field gel electrophoresis (PFGE), which was first developed in the early 1980s as a means to genotype microorganisms by electrophoretic separation of chromosomal DNA by molecular weight (Liu and Cordes, 2004; Kumar *et al*., 2009). This technology has proven to be a powerful tool used in conjuction with restriction endonuclease digestion of the DNA in order to understand evolution within a single clone (Upcroft *et al*., 2006a). Pulsed-field gel electrophoresis is a highly reproducible, discriminatory analytical tool to separate large DNA molecules (Upcroft *et al*., 2006a; Zhou *et al*., 2006).

This analytical tool (PFGE) has been applied extensively to problem areas in microbiology (Zhou *et al*., 2006). However, the use of PFGE for *T. vaginalis* genotyping has been limited

(Upcroft *et al*., 2006a). The presence of *M. hominis* and the *T. vaginalis* virus (TVV) greatly influences the PFGE pattern of *T. vaginalis* isolates (Xiao *et al*., 2008). It is postulated that treatment of *T. vaginalis* cultures with doxycycline removes *M. hominis* effectively, enabling informative datasets to be produced; however, the presence of TVV will continue to influence the PFGE assay (Fang *et al*., 2006; Xiao *et al*., 2008).

2.12.2 Genotyping of *T. vaginalis* using the RAPD assay

The random amplified polymorphic DNA (RAPD) technique involves the amplification of random segments of genomic DNA by polymerase chain reaction (PCR) using small distinct primers of arbitrary sequence (Williams *et al*., 1990). Random amplified polymorphic DNA has the distinct advantage over other molecular methods used for genomic characterization as cloning, sequencing or hybridization are not essential (Fraga *et al*., 2002, Fraga *et al*., 2011). Vanacova and colleagues in 1997 used the RAPD technique in 18 isolates of *T. vaginalis* and RAPD proved to be a powerful technique to analyze the genetic diversity of the protozoa. Based on the results that were obtained fom intra-species analysis of 18 *T. vaginalis* isolates, Vanacova and colleagues proposed an accordance between the genetic diversity and metronidazole resistance (Vanacova *et al*., 1997). The intra-species analysis of 18 *T. vaginalis* strains reported some agreement between the genetic relationships and the resistance to metronidazole (Vanacova *et al*., 1997).

Rojas and colleagues (2004) used the RAPD technique and reported a link between *T. vaginalis* genetic polymorphism and clinical categorization based upon vaginal discharge, itch, dysuria, dyspareunia, cervical, vaginal-vulvar erythema and some other characteristics. Panels of 10 random primers determined the type and extent of intraspecific polymorphism in 140 isolates of *T. vaginalis* (Rojas *et al*., 2004). All the primers detected DNA polymorphisms among the isolates (Rojas *et al*., 2004). Four main groups could be distinguished by RAPD data; these groups coincided with the four different patient categories (asymptomatic and symptomatic: light, moderate and severe infection) (Rojas *et al*., 2004). In another study, Kaul and colleagues (2004), used five random primers (OP1 to OP5) to examine the genetic diversity of *T. vaginalis* isolates from 15 symptomatic and 15 asymptomatic women. Two lineages with the upper and lower branch corresponding to the symptomatic and asymptomatic groups respectively were found by phylogenetic analysis using RAPD distance software (Kaul *et al*., 2004).

The random amplified polymophic DNA assay has a number of advantages, which include that it is cheaper and a high capacity method, since it allows the characterization of a large number of samples at once (Rojas *et al*., 2004; Fraga *et al*., 2011). The RAPD technique offers advantages for the quick genetic characterization of isolates when no information of the sequence of the DNA is available (Rojas *et al*., 2004). A potential drawback in its utilization for *Trichomonas* epidemiology is the lack of reproducibility and the intrinsic inconsistency in a reaction to even minor adjustments in the experimental conditions (Stiles *et al*., 2000). The reliability of PCR assays can be compromised by the position of individual micro-centrifuge tubes inside the PCR thermocycler block used, which interrupt the temperature and ramping parameters (Stiles *et al*., 2000). This lack of reproducibility can be averted by optimization and standardization of PCR conditions (Rojas *et al*., 2004).

2.12.3 Genotyping of *T. vaginalis* using the RFLP assay

Restriction fragment length polymorphism (RFLP) is a type of polymorphism that results from a variation in the DNA sequence caused by DNA rearrangements (insertions, deletions or point mutations) where a single base substitution has created or abolished a recognition site for the restriction endonuclease (Stiles *et al*., 2000; Crucitti *et al*., 2008). The advantage of RFLP are that it has better reproducibility, since it is less sensitive to experimental variation, thus the technique would be useful for evaluating PCR-based typing assays among different laboratories (Stiles *et al*., 2000).

Meade and colleagues (2009) used the RFLP technique to assess *T. vaginalis* isolates (129) for genetic diversity. The target of the RFLP was the cytoplasmic heat-shock protein 70 genes (*hsp*70) (Meade *et al*., 2009). Cytoplasmic heat-shock protein 70 in *T. vaginalis* are a large multilocus gene family present in at least eight nearly identical copies (96% to 97% sequence identity) (Calton *et al*., 2007). A single restriction enzyme, *Eco*RI, was used to digest genomic DNA in the molecular typing of *hsp*70 using RFLP (Meade *et al*., 2009). The diversity among *T. vaginalis* isolates was considerable with 105 unique hsp70 patterns identified in the 129 isolates analyzed (Meade *et al*., 2009). The *hsp*70 patterns were reproducible and stable over six years (Meade *et al*., 2009). The disadvantages of RFLP are that it is more time consuming and has low analytical sensitivity, because it lacks the amplification step (Stiles *et al*., 2000). The higher sensitivity of PCR with the reliability of the RFLP technique can be used for genotyping of *T. vaginalis* isolates.

Crucitti and colleagues (2008) used the PCR-RFLP technique to determine the genetic polymorphism of the actin gene of *T. vaginalis* (Crucitti *et al*., 2008). A nested PCR targeting the actin gene was used (Crucitti *et al*., 2008). The authors managed to distinguish eight distinct *T. vaginalis* types (Crucitti *et al*., 2008). The restriction enzymes that were used were *Hind*II, *Mse*I and *Rsa*I, which yielded two, three and four different digestion patterns, respectively (Crucitti *et al*., 2008).

2.13 Prevention and control programmes for *T vaginalis* infection

The transmission of sexually transmitted diseases can be prevented by refraining from sexual activity or to be in a monogamous relationship with a partner who has been tested and is known to be uninfected (Smith and Ramos, 2010). The proper and correct use of latex condoms each time during sexual intercourse can decrease the chances of transmission of trichomoniaisis (Smith and Ramos, 2010). Sexual partners of patients infected with trichomoniaisis must be treated to prevent re-infection and patients must abstain from sexual intercourse until both the patient and partner have finished therapy and are asymptomatic (Kissinger *et al*., 2008). Screening efforts must be intensified because most people are asymptomatic and thus ignorant of being infected, as a result indviduals continue to be sexually active, spreading the protozoan *T. vaginalis* (Sood and Kapil, 2008; Lazenby, 2011).

Increased hygienic awareness among families must be taught by avoiding sharing of towels, basins for bathing and underpants to prevent the non-sexual transmission of *T. vaginalis* (Adu-Sarkodie, 1995; Crucitti *et al*., 2011). Traditional healers should be educated on hand hygiene to avoid the spread of pathogens between patients (Peterson and Dramme, 2010).

A vaccine against *T. vaginalis* can provide long term protection that could be successful in controlling the spread of trichomoniasis (WHO, 2001; UNAIDS/WHO, 2009). A *T. vaginalis* vaccine would potentially be effective in decreasing the incidence and prevalence of *T. vaginalis* worldwide (WHO, 2001, UNAIDS/WHO, 2009). A *T. vaginalis* vaccine candidate has advanced to the point of first phase human clinical trials (Sood and Kapil, 2008). The vaccine involves heat killed *T. vaginalis* cells from patients with trichomoniasis (SolcoTrichovac or Gynatren) (Sood and Kapil, 2008; Cudmore and Garber, 2010). Further clinical trials need to be done to validate data obtained from the initial clinical tria(Sood and Kapil, 2008; Cudmore and Garber, 2010). The presence of a successful *Trichomonas foetus*

vaccine (whole-cell) called "Trichguard" for bovine trichomoniasis (trichomoniasis in cattle caused by a protozoan *T. foetus*) is hopeful in the search for a vaccine against *T. vaginalis* (Cobo *et al*., 2002).

2.14 Summary

Trichomonas vaginalis is a single-celled parasite that colonizes the human urogenital tract (Benchimol, 2004). Infected men may temporarily have urethral irritation, mild discharge, or slight burning after urination or ejaculation (Schwebke *et al*., 2011). Like other *Parabasalids T. vaginalis* acquires nutrients through cell membrane transport and phagocytosis (Strous, 2009). The undulating membrane assists in the phagocytosis of bacteria, red blood cells and even parts of the vaginal wall (Petrin *et al*., 1998; Schwebke and Burgess, 2004; Strous, 2009). Enzymes break down bacteria and red blood cells and convert them into usable energy through glycolysis (Petrin *et al*., 1998; Schwebke and Burgess, 2004). *Trichomonas vaginalis* reproduces asexually through longitudinal fission (Strous, 2009). Unlike other protists, *T. vaginalis* does not have a cyst as part of the reproduction cycle (Strous, 2009).

The trophozoites live in the urinary or reproductive tracts, until the trophozoites are passed onto the next human host *via* unprotected sexual contact where the cycle is repeated again (Strous, 2009). Sexual transmission is the most common mode of transmission of trichomoniasis; however, there are several reports of non-sexual transmission (Crucitti *et al*., 2011). Symptoms of trichomoniasis typically occur after an incubation period of 4 to 28 days (Petrin *et al*., 1998; Schwebke and Burgess, 2004). In comparison to the general population, persons who are infected with trichomoniasis are twice as likely to develop HIV due to damage of the genital tract walls by the protozoa, which facilitate HIV infection (Mavedzenge *et al*., 2010; Watts *et al*., 2005).

The symptoms of trichomoniasis are not specific enough for diagnosis, thus specific tests need to be done to diagnose the infection (Smith and Ramos, 2010; Lazenby, 2011). Different diagnostic techniques, which include wet mount microscopy, culture, staining, serological and molecular based techniques are used for the detection of *T. vaginalis* (Smith and Ramos, 2010; Lazenby, 2011). Wet mount microscopy is used for routine field diagnosis (Johnston and Mabey, 2008; Lazenby, 2011). However, the specificity and sensitivity of microscopes are low resulting in false negatives (Johnston and Mabey, 2008; Lazenby, 2011). Culture is more

reliable for diagnosis but it requires daily microscopic observations for between two to seven days (Hobbs and Sena, 2007). In addition, small numbers and non-viable parasites can be read as a false negative culture result (Dwivedi *et al*., 2012).

Transmission occurs predominately *via* sexual intercourse (Crucitti *et al*., 2011). The organism is most commonly isolated from vaginal secretions in women and urethral secretions in men (Swygard *et al*., 2004). The risk of acquiring *T. vaginalis* infection is increased with age, drug use, an increase in the number of sexual partners and having unprotected sex (Johnston and Mabey, 2008; Sood and Kapil, 2008). *Trichomonas vaginalis* infection is strongly associated with the presence of other STIs, including gonorrhoea, *Chlamydia* infection increases susceptibility to viruses, such as herpes, Human papillomavirus (HPV) and HIV (Watts *et al*., 2005; Smith and Ramos, 2010).

Oral metronidazole (Flagyl) remains the treatment of choice for trichomoniasis (Cudmore *et al*., 2004). In cases in which the first-line agent (metronidazole) proves to be ineffective, metronidazole may be substituted with other nitromidazoles (Strous, 2009; Sobel, 2012). Tinidazole has better *in vitro* activity and is well tolerated but cannot be used particularly in developing countries, because it is expensive (Strous, 2009; Sobel, 2012).

The purpose of this study was to detect *T. vaginalis* in HIV positive female patients' atttending the anti-retroviral clinic at the Tshwane District Hospital, Pretoria using three different methods. The genetic diversity of *T. vaginalis* isolates was determined using the RAPD and PCR-IGS RFLP assays. The study also determined if the RAPD patterns of *T. vaginalis* isolates and the metronidazole antimicrobial results correlated.

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CHAPTER 3

Prevalence and genetic relatedness of *Trichomonas vaginalis* isolates obtained from HIV positive women attending an Anti-retroviral clinic in Pretoria, South Africa

The editorial style of the Journal of Sexually Transmitted Infections was followed in this Chapter

Abstract

Objectives: To determine the prevalence and genetic relatedness of *T. vaginalis* obtained from HIV positive female participants attending the anti-retroviral clinic at the Tshwane District Hospital in the Pretoria region.

Methods: Self-collected vaginal swabs from 380 HIV positive female participants attending the anti-retroviral clinic were included in the study. *Trichomonas vaginalis* was detected from vaginal swabs using microscopy, modified Diamond's media and a commercial PCR assay kit (*Trichomonas vaginalis* 240/520 IC Sacace Biotechnology, Italy) targeting the DNA repeat units of *T. vaginalis.* Ten *T. vaginalis* isolates were subjected to 10 primers (TV1, TV2, TV3, TV4, TV5, TV6, TV7, TV8, TV9 and TV10) in initial random amplified polymorphic DNA (RAPD) assays. The five primers (TV1, TV2, TV3, TV5 and TV 6) with the best discriminatory ability were then used for further analysis. The intergenic spacer region-polymerase chain reaction restriction fragment length polymorphism (IGS-PCR RFLP) assay was performed using five restriction enzymes, namely *Alu*I*, Hinf*I*, Rsa*I*, Sau*3AI and *Tsp*509.

Results: Eight percent (30/380) of the female participants tested positive for *T. vaginalis* using wet mount microscopy. The growth of *T. vaginalis* in modified Diamond's media was observed in 24% (92/380), while 31% (118/380) of the specimens tested positive for *T. vaginalis* using the commercial PCR assay. The RAPD assay's genotyping results showed that the TV2 primer had the highest typing ability with 16 banding types, 15 types were obtained with the TV3 primer, the TV1 primer gave 13 types and the TV6 primer gave 11 types. Five banding types were obtained with the TV5 primer. The IGS-RFLP assay failed to discriminate between the *T. vaginalis* isolates.

Conclusions: A high prevalence 31% (118/380) of *T. vaginalis* infection was observed in the HIV positive female participants. The use of only microscopy for the diagnosis *T. vaginalis* is inadequate and resulted in the missing of most infections. However, the PCR assay which detected 26 more positives than culture (Diamond's media) can be the method of choice for the detection of trichomoniasis. The RAPD assay proved to be a more suitable method to discriminate between the different *T. vaginalis* isolates, while the IGS-PCR RFLP failed to discriminate between the *T. vaginalis* isolates. In future microsatellite analysis could be used to determine the genetic variability of *T. vaginalis* isolates.

Keyword: *Trichomonas vaginalis*, trichomoniasis, wet mount microscopy, random amplified polymorphic DNA (RAPD), intergenic spacer region (IGS), restriction fragment length polymorphism (RFLP)

3.1 Introduction

Sexually transmitted infections (STIs) are not only a cause of increased morbidity in adults but may result in complications and sequelae, such as infertility, ectopic pregnancy, cervical cancer, premature mortality and foetal wastage [1]. Most sexually transmitted diseases caused by bacterial, mycological and protozoal agents are curable using the appropriate antibiotics. However, STIs still continue to be a public health problem in both developing and industrialized countries perpetuated by the human immunodefiency/acquired immunodefiency syndrome (HIV/AIDS) pandemic [1]. The most common STIs are trichomoniasis, chancroid, gonorrhoea, hepatitis B infection, HIV and syphilis [1].

Trichomonas vaginalis is a protozoa pathogen of the human urogenital tract [2]. The typical symptoms of trichomoniasis in women are that of a foul-smelling green-yellowish genital discharge, itching and vaginitis [2]. In men, infection with *T. vaginalis* is mostly asymptomatic but can cause non-gonococcal urethritis and chronic prostatitis [2]. It has been demonstrated that if these classical features are used alone in the diagnosis of trichomoniasis, 88% of these cases will not be diagnosed and 29% of uninfected patients will be falsely diagnosed as having an infection [3]. This suggests that clinical manifestations are not reliable diagnostic parameters and accurate laboratory diagnosis methods are necessary for the detection of *T. vaginalis* [3].

To date the most common clinical method for the diagnosis of *T. vaginalis* remains the microscopic evaluation of motile protozoa from vaginal or cervical specimens and from urethral or prostatic secretions [4]. Culture is the current gold standard for diagnosis of trichomoniasis and overall, culture has a number of advantages including: i) higher specificity and sensitivity than microscopy and ii) detection of as few as three organisms/ml [3-5]. The use of culturing in developing countries is limited by the high costs involved with culturing; also some strains of the protozoa cannot be cultured due to strain variation [4]. Point of care tests that are available for the diagnosis of *T. vaginalis* include the OSOM *Trichomonas* rapid test (Genzyme Diagnostics, Cambridge, Massachusetts, USA), which is an antigen-detection test that uses immunochromatic capillary flow dipstick technology and the Affirm VP III (Becton Dickenson, San Jose, California, USA), which is a nucleic acid probe-hybridization test that tests for *T. vaginalis, Gardnerella vaginalis* and *Candida albicans* [5]. Point of care tests have the advantage of allowing immediate diagnosis and treatment of patients [5].

It is recognized that trichomoniasis may play a critical and under-recognized role in amplifying HIV transmission and may have a major impact on the dynamics of the HIV epidemic [6]. Since South Africa is one of Africa's most developed countries; the influx of migrants from other African countries is one of the main drivers of HIV and *T. vaginalis* transmission [7]. Although South Africa is one of the highly burdened countries with *T. vaginalis* and HIV co-infections, the prevalence of *T. vaginalis* among HIV positive patients have not received much attention.

The association of *T. vaginalis* strains and the variable clinical pictures of trichomoniasis are not well-known, partly due to a lack of consistent and universally acknowledged methods to type and trace distinct isolates [8]. Molecular fingerprinting utilizing intra-specific variation in the microbial genome to identify characteristic subtypes is a valuable epidemiological tool [8]. An ideal molecular marker must be easy and fast, highly polymorphic in nature, must be neutral to environmental and experimental conditions, has high reproducibility and must be able to determine co-dominant inheritance [9]. It is difficult to find a molecular technique that meets all these desired criteria [9]. Two of the most promising molecular typing techniques that have been used to type *T. vaginalis* are: the random amplified polymorphic DNA (RAPD) assay and the intergenic spacer region restriction fragment length polymorphism (IGS-PCR RFLP) assay [8,9].

The purpose of this study was to determine the prevalence of *T. vaginalis* using wet mount microscopy, culture (modified Diamond's media) and a commercial PCR assay. Additionally, this study aimed to characterize *T. vaginalis* isolates in the Pretoria region, South Africa using two molecular typing techniques namely, the RAPD and IGS-PCR RFLP assays.

3.2 Materials and Methods

3.2.1 Study population and clinical specimen collection

Ethical clearance was obtained from the Research Ethics Committee, University of Pretoria, Faculty of Health Sciences at the University of Pretoria (S99/2010). Two self-collected Dracon vaginal swabs (Copan Diagnostics, USA) were collected (from January to June of 2010) from each of the 380 women (consecutive women, with or without complaints) visiting the anti-retroviral clinic at the Tshwane District Hospital, Pretoria who gave informed consent

(Appendix C4). The women were instructed to insert a single swab into the vagina and to rotate the swab in the vagina for 50 s. The specimens were transported on ice to the laboratory where the specimens were processed within 60 min of collection.

3.2.2 Diagnosis of *T. vaginalis* isolates

One of the Dacron swabs (Copan Diagnostics, USA) of each participant was gently agitated in 0.1 ml of saline and immediate microscopic diagnosis of the specimen was done by means of a wet preparation under the 10X and 40 X objectives (Zeiss, Germany). The characteristic trichomonad size, morphology and movements, which can be described as jerky, swaying and tumbling were noted [4,10]. The swab was then inoculated in 2 ml of Diamond's media (Appendix B, section 2.1) and incubated aerobically at 37^0C (Labcon, California, USA) for 24 h. *Trichomonas vaginalis* growth occurs as a granular deposit at the bottom of the bottle. Cultures were examined daily for *T. vaginalis* growth at maximum of 7 days before being reported as negative [10]. In this study an expanded gold standard was used, a specimen was considered positive for *T. vaginalis* if the results were found positive for any one of the methods applied: wet mount microscopy, culture and the commercial PCR assay.

3.2.3 Molecular detection and typing of *T. vaginalis* isolates

Genomic DNA was extracted from all *T. vaginalis* culture positive samples. *Trichomonas vaginalis* was detected using a commercial PCR assay (Sacace Biotechnology, Italy) from all the self-collected vaginal swabs.

3.2.3.1 Total genomic DNA extraction of *T. vaginalis* isolates

Total genomic DNA extraction of all vaginal swabs was performed using the DNA-Sorb-A extraction kit (Sacace Biotechnology, Italy) according to the manufacturer's instructions (Appendix A). The purity and concentration of the extracted DNA was determined by optical density measurements at 260 nm and 280 nm using a Nanodrop spectrophotometer (NanoDrop Technologies Inc, USA). The DNA was stored at -20^0C until needed for analysis (Appendix A, Section 1.2).

3.2.3.2 The detection of *T. vaginalis* using a commercial PCR assay

A commercial PCR amplification assay the *T. vaginalis* 240/520 IC (Sacace Biotechnology, Italy) targeting the DNA repeat units was used in the detection and confirmation of *T. vaginalis* according to the manufacturer's instructions (Appendix A, section 1.3). In brief: the amplification procedure was performed in a Gradient Master Cycler (Eppendorf, Germany). The following programme was used: pre-incubation at 95^0C for 5 min, 42 cycles of denaturation at 95^0C for 1 min, annealing at 65^0C for 1 min, extension at 72^0C for 1 min and a final extension at 72^0C for 1 min. The analysis of the PCR results was based on the presence or absence of specific amplified DNA fragments, namely the 520 bp internal control and the 240 bp *T. vaginalis* bands.

3.2.3.3 Genotyping of *T. vaginalis* using random amplified polymorphic DNA analysis

Samples that were positive by culture (modified Diamond's media) were used for genotyping of *T. vaginalis* isolates using the RAPD assay. Ten of the *T. vaginalis* isolates were subjected to ten different RAPD primers (TV1, TV2, TV3, TV4, TV5, TV6, TV7, TV8, TV9 and TV 10) to determine the type and extent of intra-specific polymorphisms as previously described by Rojas *et al.* [12]. Five (TV1, TV2, TV3, TV5 and TV6) of the ten primers that showed the best discriminatory ability were used for further analysis (Table 3.1).

Table 3.1: Nucleotide sequences of the primers used for the RAPD and RFLP assays [11,12]

Primer name	Nucleotide Sequence 5' ⟶ 3'	PCR product size	Reference
IGSf2.2 IGSr2.2	AATCGCATGTATTAGCACCAG' GTGATGTTGGACCGATACG	2 kb	11
TV1	CGC ACT CGG AGT'	Various band sizes	12
TV2	TCG GCC GCT ATC'		
TV3	CCG TGA CAC GCA'		
TV5	GGG ACA CTC TGG'		
TV6	GGG ACC TAC TGC'		

(All primers were manufactured by TIB-MOLBIOL, Germany)

The TaKaRa Ex Taq^{TM} Master Mix (Takara Biotechnology, Japan) was used according to the manufacturer's instructions (Appendix A section 1.4). The amplification profile used consisted of an initial denaturation step at 94^0C for 5 min, followed by 40 cycles of denaturation at 95^0C

for l min, annealing at 38^0C for 1 min and extension at 72^0C for 1 min. This was followed by a final extension at 72^0C for 1 min. The amplification procedure was performed in a Gradient Master Cycler (Eppendorf, Germany). The gel electrophoresis analysis of the RAPD assay amplification products is described in section 3.2.3.5.

3.2.3.4 Genotyping of *T. vaginalis* using restriction fragment length polymorphism analysis

Samples that were positive by culture (modified Diamond's) were used for genotyping of *T. vaginalis* isolates using the IGS-PCR RFLP assay. Primers IGSf2.2 and IGSr2.2 were designed for the amplification of the 2 kb IGS region of *T. vaginalis*. The IGSf2.2 and IGSr2.2 (Table 3.1) primers were modified from primers originally described by Simoes-Barbosa *et al.* [11]. The IGS primers were checked and optimized using the CLC Main workbench 6.0 software programme (CLC bio, Denmark). Reference sequences for the 18S rRNA (Accession number: U17510) and the 28S rRNA (Accession number: AF202181) were obtained from GenBank (www.ncbi.nlm.nih.gov/nuccore).

The TaKaRa Ex Taq™ Master Mix (Takara Biotechnology, Japan) was used according to the manufacturer's instructions (Appendix A section 1.4). In brief: the amplification profile used consisted of an initial denaturation step at 94^0C for 5 min, followed by 45 cycles of denaturation at 95^0C for l min, annealing at 50^0C for 1 min and extension at 72^0C for 1 min. This was followed by a final extension step at 72^0C for 15 min. After visualization of the amplified products, an aliquot of 15 µl was digested with each of the five restriction enzymes namely: *Alu*I, *Hinf*I, *Rsa*I, *Sau*3AI and *Tsp*509 separately (Fermentas, Thermo Scientific, USA). Gel electrophoresis analysis of the results is described in section 3.2.3.5.

3.2.3.5 Gel electrophoresis analysis of the amplified PCR assay products

The gel electrophoresis analysis of the amplified PCR products was performed using a 2% (m/v) agarose gel (SeaKem, Rockland, USA) at 85 V/cm (Eilte-300 Power supply, Wealtec Corp., Kennesaw, GA) for 2 h in 1 X TAE (pH 8.0 - 8.5) [40 mM Tris Base (Sigma Chemical, USA), 20 mM glacial acid (Merck, Germany), 0.5 M EDTA adjusted with sodium hydroxide pellets to pH 8.0 (Promega, USA) using 0.5 µg/ml ethidium bromide (Promega, Madison,

USA) (Appendix A section 1.5, Appendix A section 1.6). The analysis of each 2 kb IGS-PCR RFLP amplicon of conventional PCR was performed on a 1% (m/v) agarose gel (SeaKem, Rockland, USA). The loading dye that was used is the Fermentas 6X green loading dye solution (Fermentas UAB, Lithiuania). All amplified were visualized using a UV transilluminator (DigiDoc-It imaging system, UVP, Upland) (Section 3.2.3.5). Three different molecular size markers (Fermentas Thermo Scientific, USA) were used as reference markers, namely: i) a 50 bp molecular size marker for conventional PCR products obtained after detection of *T. vaginalis* using a commercial PCR assay, ii) a 1 kb ladder for the RAPD assay and iii) a 100 bp molecular size marker for analysis of each of the restricted samples obtained after the IGS-PCR RFLP.

3.2.3.6 Analysis of DNA banding patterns

The RAPD DNA patterns were analyzed using the GelCompar*II* software programme (Applied Maths, Kortrijk, Belgium). A distance matrix was constructed from the DNA banding patterns using the Dice coefficient. The unweighted pair group method with arithmetic mean (UPGMA) algorithm was used for clustering and construction of the dendrogrammes from the distance matrix.

The RAPD fingerprint profiles were manually analysed. Closely related clusters and sub-clusters showed a high similarity of >70%, which meant that the patterns differed by a single genetic event (point mutation, insertion or deletion of DNA). These typically result in two or three band differences. Isolates were considered possibly related when the sub-clusters and clusters showed a similarity of >50%, which meant that patterns differed by two independent genetic events that resulted in four or six bands [13]. The IGS-PCR RFLP restriction products were analysed by comparing the size of the restricted RFLP products with the 100 bp ladder.

3.3 Results

Self-collected vaginal swabs were obtained from a total of 380 HIV positive women attending the Tshwane District hospital women with a mean age of 33 years (21 years to 60 years) who gave informed consent. Eight percent (30/380) of the specimens were positive by wet mount microscopy. The specificity and sensitivity of wet mount microscopy was found to be 76% and

32%, respectively, when compared to culture (Appendix, Table C2). Twenty-four percent (92/380) of the specimens tested positive using the modified Diamond's culture medium. The median day of growth of *T. vaginalis* was on day three (range day 2 to day 5). Twenty-six out of the ninety-two women who were *T. vaginalis* positive by culture using Diamond's medium reported having trichomoniasis symptoms, such as vaginal discharge, itching and burning. One specimen was positive according to microscopy and the commercial PCR assay; however, it did not grow in the culture media. The specificity and sensitivity of culture was found to be 76% and 78%, respectively (Apendix C2).

Thirty-one percent (118/380) of the swabs were positive according to the commercial PCR assay (*Trichomonas vaginalis* 240/520 IC kit, Sacace Biotechnology, Italy). The internal control band (520 bp) was positive in all the PCR amplification products (Figure 3.1). Culture using the modified Diamond's medium detected 60 more *T. vaginalis* isolates than wet mount microscopy (Figure 3.2). The commercial PCR assay detected 26 more positive specimens compared to culture and 88 more than microscopy. The sensitivity of the commercial PCR assay (*T. vaginalis* 240/520 IC kit Sacace Biotechnology, Italy) was 100%, while the specificity was 91% when compared to culture (Appendix, Table C3).

Ninety-two culture positive *T. vaginalis* isolates were used for the RAPD and IGS-PCR RFLP assays. In the RAPD assay the following was obtained for the different pimers: 16 profiles with TV2, 15 with TV3, 13 with TV1, 11 and five with TV6 and TV5, respectively. The amplified products for the TV1 primer were between 250 bp to 4 000 bp. Bands ranging in size from 400 bp to 4 000 bp were generated using the TV2 primer. Amplification with the TV3 primer ranged from 400 bp to 3 500 bp. The amplified products of the TV5 primer were between 250 bp to 2 500 bp, while the TV6 primer generated bands ranging from 750 bp to 4 000 bp.

The dendrogrammes obtained from the RAPD data grouped the 92 *T. vaginalis* isolates into between six to seventeen clusters with 70% similarity used as the reference value for the definition of a cluster. Primers TV1 generated a dendrogramme with 17 clusters at 70% and 33 outliers, while the TV3 primer resulted in a dendrogramme with 16 clusters at 70% similarity and 31 outliers. The dendrogramme constructed from the TV2 primer's data revealed ten clusters and nine outliers, while the dendrogramme constructed with the TV6 primer's data

revealed 12 clusters and 18 outliers. The TV 5 primer data generated six clusters and 19 outliers (Figure 3).

Amplification of the IGS region generated a 2 kb product. Three to five distinct DNA bands ranging in size from 100 bp to 1 000 bp were detected using the five restriction enzymes. The restriction endonuclease *Sau*3AI generated five distinct restriction products (800 bp, 500 bp, 300 bp, 250 bp and 150 bp), four bands were generated with the three enzymes i) *TSP*5091 (1 000 bp, 500 bp, 350 bp, 150 bp), ii) *Hinf*I (1 000 bp, 500 bp, 350 bp, 350 bp) and iii) *Rsa*I (1 000 bp, 600 bp, 250 bp and 150 bp). Restriction enzyme *Alu*I generated three bands (1 000 bp, 700 bp and 300 bp).

3.4 Discussion

The World Health Organization (WHO) estimates that trichomoniasis causes more than half of all curable sexually transmitted infections worldwide [1]. However, limited emphasis has been placed on the importance of decreasing the prevalence of trichomoniasis infection despite the link to an increased risk of HIV transmission and pregnancy difficulties [11,14].

Self-collected vaginal swabs were used in this study and were found to be practical, rapid and easy to obtain compared to swabs collected by physicians as previously also reported by Dwivedi *et al*. [15]. Wet mount microscopy showed a low sensitivity of 32%, which was lower than the 38% to 82% sensitivity reported in other studies [4,16,17] and it had 76% specificity. The low sensitivity of microscopy reported in this study could be due to specimen handling and the skill of the microscopist, which might have been lacking in this study [16]. A positive wet mount microscopy result is diagnostic while a negative wet mount microscopy result does not exclude trichomoniasis [14]. Culture, which is the gold standard for the diagnostic testing of *T. vaginalis* is not readily available in South Africa and other developing countries, due to the high cost involved with the technique (culture requires on site incubators and media, which are expensive) and the difficulty associated with growth of some *T.vaginalis* strains [14]. Wet mount microscopy has been found to be useful where clinicians and staff have gained experience by performing a large number of tests [18].

Twenty-four percent (92/380) of the specimens were positive according to the modified Diamond's culture medium. The sensitivity of culture in this study was 78%, which was comparable to other studies where the sensitivity varied from 70% to 85% [17]. Although culture had a better sensitivity than wet mount microscopy, it requires a maximum of seven days to be regarded as negative [19]. The seven day turnaround time may allow the patient to transmit the infection before receiving medication [19]. Sood and colleagues [20] showed that culture detected 45% (40/740) more *T.vaginalis* positive specimens than wet mount microscopy, which detected 22/710. In this study, culture detected 67% more positives than wet mount microscopy. Researchers have proposed a stepwise approach for the diagnosis of *T. vaginalis*, where culture should be performed after a negative wet mount microscopy result [17].

In this study 26% (24/92) of women who were *T. vaginalis* positive by culture (Diamond's medium) reported having symptoms of trichomoniasis. This is in agreement with a study that was conducted in Zimbabwe where 75% (295/395) of women denied symptoms on direct questioning [21]. Similar to this study voluntary reporting of symptoms was almost non-existent and this may be due to the social stigma associated with having a sexually transmitted disease in South Africa [21].

One specimen (isolate 34) was detected by wet mount microscopy and the PCR assay and not by culture. This might have been due to the specimen having dried during transportation to the laboratory thus killing the trichomonad [4,12]. Viability has been shown to decrease by up to 20% if culture is delayed by 10 min [22]. This emphasizes the need for the immediate inoculation of vaginal swabs into Diamond's medium after sample collection [22].

In this study 31% (118/380) of specimens were positive according to the commercial PCR assay, which targets the DNA repeat units of *T. vaginalis* (*Trichomonas vaginalis* 240/520 IC kit, Sacace Biotechnology, Italy). The sensitivity of the commercial PCR assay in this study was 100%, which was comparable to the 60% to 92.8% reported in previous studies [11,12,23], while the specificity was 91%, which was lower than the 94% to 97% reported by other authors [11,12,23]. The superior performance of a commercial PCR assay targeting the DNA repeats units was previously reported by Crucitti and colleagues in 2003 during a comparison between the sensitivities and specificities of different primer sets and culture on

self-collected vaginal swabs [23]. Two real-time PCR assays that targeted the DNA repeat units [24] and the beta tubulin [25] have previously been used to test and evaluate the possibility of getting false positive results using the commercial kit *T. vaginalis* 240/520 IC (Sacace Biotechnology, Italy) [26]. The commercial kit *T. vaginalis* 240/520 IC (Sacace Biotechnology, Italy) was found to be 100% sensitive [26]. The DNA repeats were found to sometimes produce false positives [26].

The *T. vaginalis* prevalence of 31% observed in this study is higher than the prevalence recorded among HIV positive women in other Sub-Saharan African countries in similar settings with a heavy burden of HIV [14,27,28]. In Nigeria among HIV positive women, a prevalence of 24.4% (61/250) was observed [14], while in the Congo and the Ivory Coast prevalence rates were 18.6% (40/215) [27] and 27% (260/962) [28], respectively. However, the prevalence rates of *T. vaginalis* infection among HIV infected women in New Orleans, USA of 36% (36/100) was higher than the prevalence observed in this study [29]. In South Africa a prevalence of 32% (46/143) has been reported with Johannesburg having a prevalence of 19% (18/94) while, Cape Town reported a 34% (70/206) *T. vaginalis* prevalence in HIV positive women (30).

Successful epidemiological evaluation of STDs, such as trichomoniasis is accomplished with tools, which identify the causative agent and determine the means of transmission, pathogenicity, virulence and drug resistance [31]. A dependable typing procedure will be useful in trying to explain the diversity of symptoms associated with the world's most prevalent sexually transmitted disease, trichomoniasis [32]. Deoxyribonucleic acid-based techniques like RAPD and PCR-RFLP have the exclusive benefit of evaluating permanent diversity, which is portrayed in the genome of *T. vaginalis* [31].

Random amplified polymorphic DNA technology is a widespread method for determining total genetic variation within a population [12,33,34]. Genetic polymorphism among *T. vaginalis* isolates have been exploited before using the RAPD technique [12,33,34]. However, in South Africa genetic polymorphism in *T. vaginalis* using RAPD technique has not been demonstrated. In this study, the RAPD assay gave better strain variability compared to the IGS-PCR RFLP assay. The simplicity, high capacity and low cost of the RAPD assay facilitated its easy and convenient use in analysis of a large number of samples [33,34]. In this

study the RAPD assay was more genetically discriminatory compared to the IGS-PCR RFLP. This may be due to the RAPD assay having the capability to scan across all regions of the genome, hence, giving the advantage to compare polymorphisms of independent genomic regions instead of focusing on a specific region like the IGS region of *T. vaginalis* [33-36].

The dendrogrammes obtained from the RAPD markers grouped the 92 *T. vaginalis* isolates into between six to seventeen clusters (similarity value of 70% used as the reference value for the definition of a cluster). The outliers in this study ranged between 10% (9/92) in the dendrogramme constructed from the TV5 primer fingerprinting data to 36% (33/92) observed in the dendrogramme constructed from the TV2 primer fingerprinting data.

The RAPD assay results revealed a high genetic diversity among the studied *T. vaginalis* isolates. A high genetic diversity among *T. vaginalis* isolates has been observed previously reported by Conrad *et al*. [34] during a study of genetic diversity of isolates collected from different parts of the world. In addition Meade *et al*.[37] also noted genetic diversity with 105 different RFLP patterns observed with 85 *T. vaginalis* isolates. The large, diverse set of the repeated, mobile genetic elements in the *T. vaginalis* genome may provide an explanation for the high genetic diversity in this study [37,38]. The ability of repeat elements to increase in copy number and transpose throughout the genome may have contributed to a large genetic variability [37,38].

In this study, IGS PCR-RFLP gave uniform banding patterns for each of the five enzymes used namely: *Alu*I, *Hinf*I, *Rsa*I, *Sau*3AI and *Tsp*509. These results proved that the IGS region of *T. vaginalis* may not be a suitable marker for strain discrimination and other regions (beta tubulin and DNA repeats units) need to be investigated, which may be more useful for genetic relatedness analysis. The results obtained in this study are in agreement with a previous study where the PCR-RFLP technique was used for the amplification and restriction of the ribosomal IGS regions with eight restriction enzymes [11]. The observed polymorphism was not statistically significant and the authors recommended further studies on the intergenic spacer region of *T. vaginalis* [11].

Crucitti and colleagues [35] concluded that the PCR-RFLP assay had low genetic strain discrimination when targeting the actin gene and recommended a combination of markers to be

used to enhance the discrimination power of RFLP in *T. vaginalis* [35]. The low discriminatory power of the RFLP markers in this study may have been due to rare occurrences of insertion, deletion and rearrangement events occurring within *T. vaginalis* intergenic spacer region, since it is a highly conserved region [36]. However, these events (insertions rearrangements and deletions) may perhaps be widespread in the genomes of most species [36]. Similarly, in a given *T. vaginalis* genome of 10^9 bp approximately 250 000 restriction sites should exist for any restriction enzyme with a 6 bp recognition sequence that accounts for 0.15% of the entire genome [36]. Base substitutions within these restriction sites are widespread and the chances that any such base substitution would occur within the *T. vaginalis* IGS region is relatively small [36].

All the participants were drawn from Pretoria inner city which is a heavily populated place with migrants from other countries and also from the rural areas of South Africa. As a result the participants represent a broad geographical background and a high genetic diversity [39]. Trichomoniasis is characterised by a major heterogeneity in its clinical manifestations such as pathogenicity, metronidazole sensitivity, sequelae to infection and susceptibility to acquisition of other agent. The genetic diversity among *Trichomonas* isolates demonstrated here suggests that an association between *Trichomonas* genotype and highly variable clinical pathology may exist. Understanding this relationship will be a critical factor in devising strategies to control this disease and its clinical sequelae.

This study showed that traditional methods (microscopy and culture) for the detection of *T. vaginalis* underestimate its prevalence and reflect the inadequacy of relying solely upon these conventional methods. The limitation of this study was that the study population size may not have been an adequate representation of the general South African population. The study was conducted at the Antiretroviral clinic of the Tshwane District Hospital in the Gauteng province, which is one of the nine provinces in the country. These limitations might have affected the adequate interpretation of the public health implications of the findings. Further studies incorporating detailed socio-demographic parameters as well as a larger study population size are proposed. The use of microsatellite analysis have been shown to be useful in genetic typing of *T. vaginalis* [34] futher studies to validate these findings will be useful.

3.5 Conclusion

Diagnosis of *T. vaginalis* using a commercial PCR assay was found to be highly specific and sensitive compared to culture and wet mount microscopy, but its availability and cost effectiveness limit its use in routine diagnostic laboratories. In this study the RAPD assay showed a higher discriminative power than the IGS-PCR RFLP. In addition the RAPD assay was less expensive and required a shorter time to perform compared to the IGS-PCR RFLP assay.

Conflict of Interest: There is no known conflict of interest in the project

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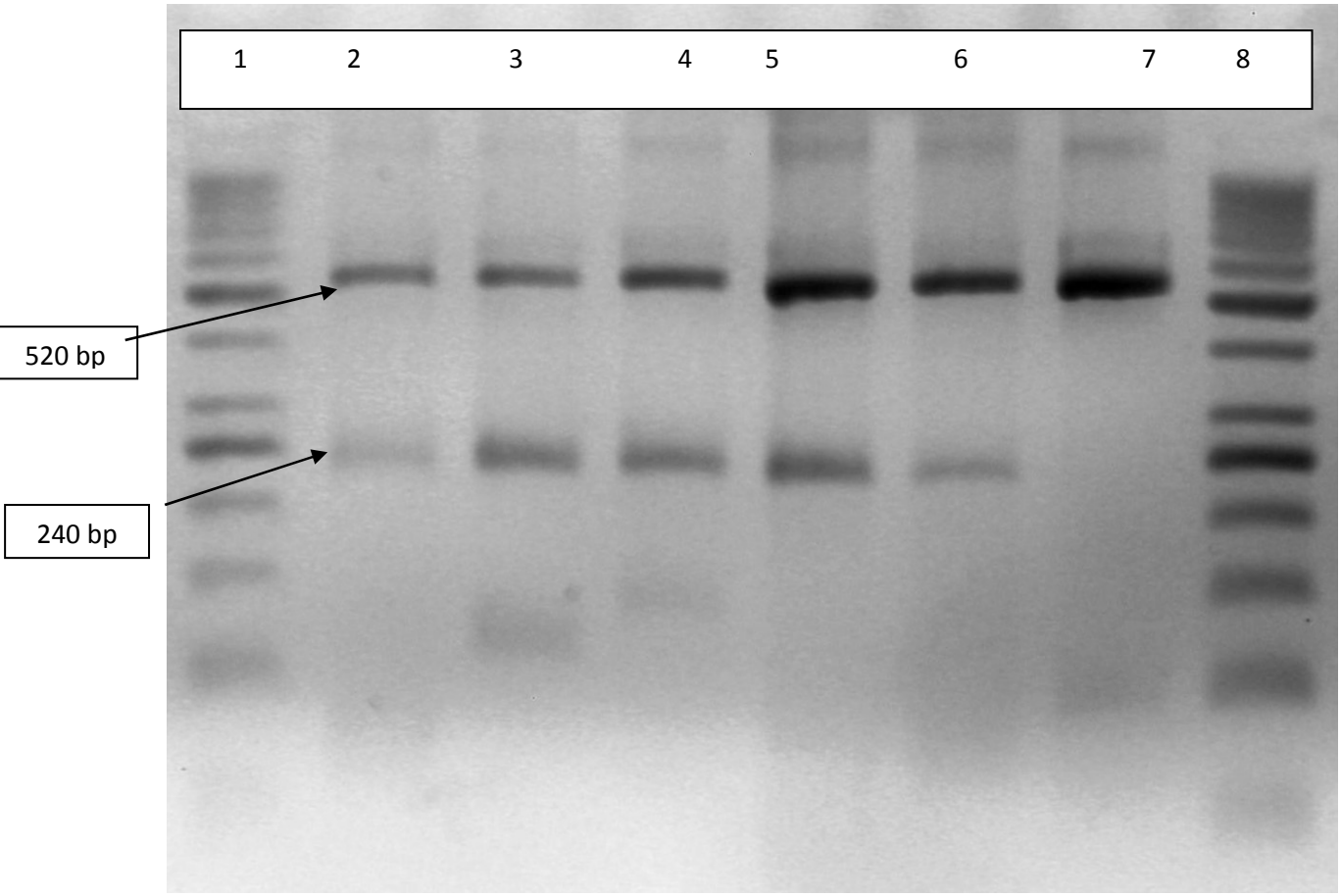

Figure 3.1: **Gel electrophoresis detection of positive *T. vaginalis* specimens. Internal control bands at 240 bp are visible in all the specimens. Lanes 2 to 6 show positive specimens for *T. vaginalis,* while lane 7 shows a negative result.**

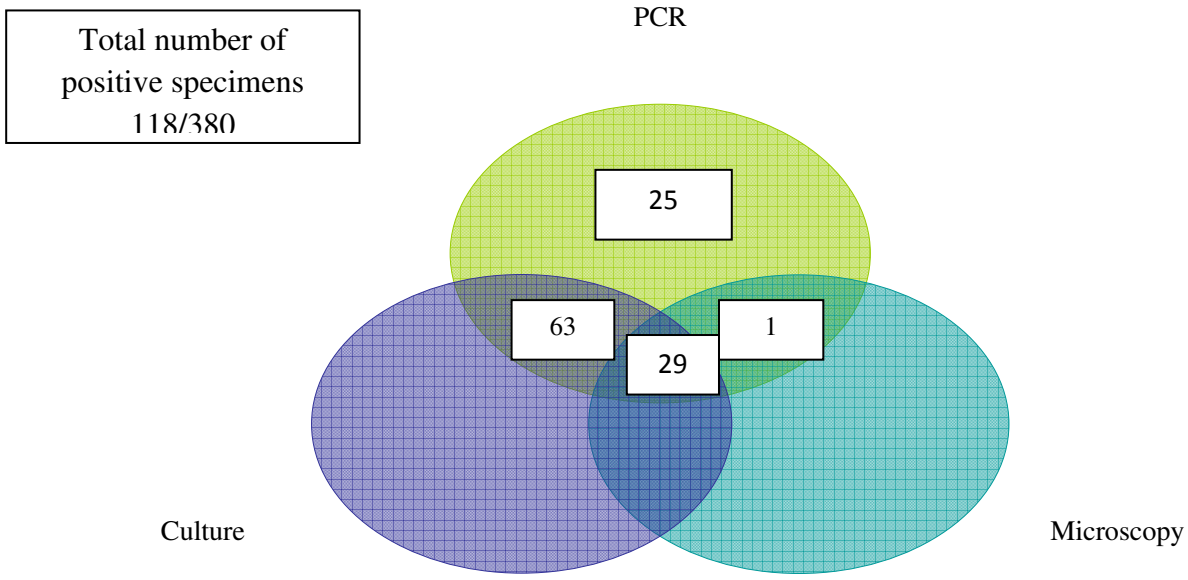

Figure 3.2: **Venn diagram showing the distribution of the positive results between microscopy, culture and PCR. Eight percent (30/380) specimens were positive by microscopy, 24% (92/380) were positive by culture and 31% (118/380) specimens were positive by PCR**

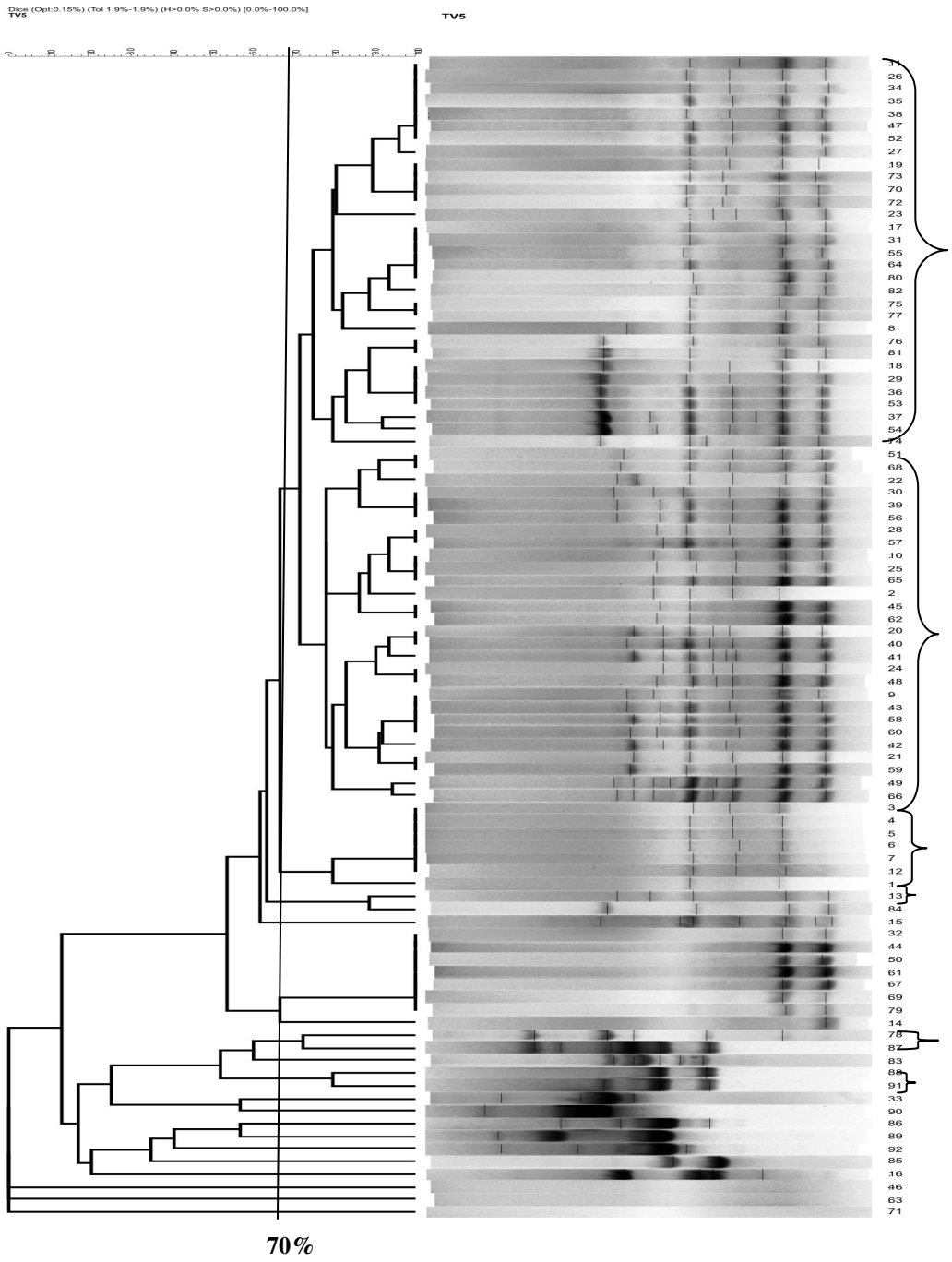

Figure 3.3: **Dendrogramme obtained for the RAPD assay TV5 primer, depicting the genetic relationship of 92 *T. vaginalis* clinical isolates. The reference line represents 70% similarity as a cluster definition**

CHAPTER 4

Susceptibility testing of *Trichomonas vaginalis* isolates from HIV positive women attending an Anti-Retroviral clinic in Pretoria, South Africa

The editorial style of the Journal of Sexually Transmitted Infections was followed in this Chapter

Abstract

Objectives: To determine the *in vitro* prevalence of metronidazole resistance in *T. vaginalis* isolates obtained from women attending the Anti-retroviral clinic at the Tshwane District Hospital, Pretoria.

Materials and Methods: Self-collected vaginal swabs from 30 *T. vaginalis* positive female participants were included in the study. A 24 h and 48 h interval microtiter assay was used to test the antimicrobial drug susceptibility of the isolates under aerobic conditions.

Results: Metronidazole resistance was detected in 6% (2/30) of the *T. vaginalis* isolates. The minimal inhibitory concentration (MIC) was between 0.06 µg/ml and 25 µg/ml. No correlation was observed between metronidazole resistance and the clustering obtained with the RAPD assay.

Conclusion: The prevalence of metronidazole-resistant *T. vaginalis* isolates observed in this pilot study of HIV positive female participants was low. The metronidazole resistant isolates did not cluster together with the RAPD analysis and were genetically diverse.

Keywords: *Trichomonas vaginalis*, metronidazole, antimicrobial drug resistance, minimal inhibitory concentration (MIC).

4.1 Introduction

Trichomonas vaginalis infection in women ranges from an asymptomatic carrier state to acute inflammatory diseases [1]. Infections have been linked to sterility problems, low birth weight and preterm delivery [1,2]. It has been proven that *T. vaginalis* infection predisposes carriers to human immunodefiency virus (HIV) and cervical cancer [1,2].

Antimicrobial drug resistance amongst microorganisms is an increasing problem with relatively few alternatives available [3]. Overuse, abuse and over-the-counter sales of cheap antimicrobial drugs in many parts of the world have already rendered chloroquine, penicillin and methicillin ineffective against *Plasmodium falciparum*, *Streptococcus pneumoniae* and *Staphylococcus aureus* [4].

Nitroimidazoles, in particular metronidazole and tinidazole are used to treat anaerobic protozoa, which include *T. vaginalis*, *Giardia duodenalis* and *Entamoeba histolytica* and even bacterial infections caused by *Helicobacter pylori* [4]. Metronidazole clinical resistance is defined as a failure to cure infection after two consecutive courses of antimicrobial treatment [4]. Treatment failures have traditionally been attributed to re-infection from untreated partners, poor patient compliance and variation in pharmacokinetics [4,5]. It is now postulated that metronidazole resistance may occur for various reasons, including drug resistance, lack of absorption of the medication and lack of transport to the target site [5].

Metronidazole resistance in *T. vaginalis* was reported as early as 1962 and the protozoan has the potential to become resistant to all recommended therapeutics [5]. In a retrospective study conducted at a genitourinary clinic in Birmingham, United Kingdom Das and colleagues postulated that the prevalence of *T. vaginalis,* which is non-responsive to standard doses of metronidazole without any history of re-infection or non-adherence was 0.38% in 1999 to 3.5% in 2002 [6]. Metronidazole is cheap, easy to produce, safe and effective and as a result it is widely prescribed post-operatively and for prophylaxis for a host of diseases, which may contribute to the development of metronidazole resistance [7]. Metronidazole is sold over the counter in many Asian countries, where optimal courses are rarely recommended or taken [7]. Widespread use of metronidazole, which is available without prescription in developing countries, has already led to metronidazole resistance in *H. pylori* [8].

In South Africa (which follows the WHO, 2003 guidelines) a syndromic management (combination therapy for all common causes of the sexually infections (STI) symptoms) have been adapted [9,10]). The syndromic management has the advantage of allowing treatment at the first visit, avoiding problems with follow-up and is also associated with higher cure rates [9,10]. However, the approach might have led to the over-diagnosis and over-treatment of patients and their partners leading to a potential increase in drug resistance in *T. vaginalis* isolates in South Africa. In South Africa information about metronidazole resistant trichomoniasis is scant and sometimes non-existing.

The aim of this study was to determine the metronidazole resistance of *T. vaginalis* isolates obtained from female participants attending the anti-retroviral clinic at the Tshwane District Hospital, Pretoria. The genetic relatedness of metronidazole antimicrobial drug resistant and susceptible *T. vaginalis* isolates was determined using a random amplified polymorphic DNA (RAPD) assay.

4.2 Materials and Methods

4.2.1 Study population and clinical specimen collection

The ethical clearance for this study (S99/2010) was obtained from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria. New vaginal swabs were collected for susceptibility testing. One vaginal swab (Copan Diagnostics, USA) was self-collected by each female participant who gave written informed consent. The female participants were instructed to insert a single swab into the vagina and to rotate the swab for 50 s. A total of 107 specimens were collected until 30 *T. vaginalis* positive specimens were obtained.

4.2.2 Cultivation of *T. vaginalis* isolates

Bijoux bottles containing modified Diamond's medium (Appendix B, Section 2.1) were used for the cultivation of the *T. vaginalis* isolates [11]. The incubator (Labcon, California, USA) was used to bring the media to room temperature $\pm 25^0$C before the inoculation of the clinical specimen. After inoculation, the cultures were incubated (Labcon, California, USA) aerobically at 37^0C for 24 h, with the lid tightly closed. Inoculated cultures were evaluated

after 24 h of incubation (Labcon, California, USA) for the presence of *T. vaginalis* by obtaining a drop of the sediment with the aid of a Pasteur pipette (Becton Dickson, New Jersey, USA) and placing this on a microscopy slide (Lieder, Ludwigsburg, Germany) for wet mount microscopy. A cover slip was placed on the sediment and immediately examined by standard light microscopy (Zeiss, Germany) under the 10X and 40X objectives. Specimens were observed daily for seven days for growth of *T. vaginalis* before being confirmed as negative [12]. The pear shape, size and erratic movements were used to identify *T. vaginalis* [11].

4.2.3 Purification and maintenance of a positive *T. vaginalis* culture

An aliquot of 10 ml sample of actively growing trichomonads was transferred to a 90 ml of prewarmed new culture containing fresh modified Diamond's medium (Appendix B, Section 2.1). Trichomonad isolates were passed in repeated passages of culture to ensure that the isolates were free of contamination. The medium for sub-passage culture was modified Diamond's medium (Appendix B) containing the antibiotics gentamicin (40 µg/ml) (Sigma-Aldrich, St Louis, USA) and ampicillin (1 g/ml) (Sigma-Aldrich, St Louis, USA). The presence of yeast in the specimen was eliminated by the addition of amphotericin B (5 µg/ml) (Sigma-Aldrich, St Louis, USA). The possible presence of bacteria and yeasts was examined by cultivating samples of all *T. vaginalis* cultures on blood agar plates for 24 h in anaerobic atmosphere (5%) CO_2 at 37^0C (HF 212 UV, Shangai Lishen Scientific Equipment, China). Active trichomonads in the logarithmic phase of growth (normally after 48 h incubation) were harvested by centrifugation at 5 000 *x g* (Labnet International, Edison, NJ, USA) for 20 min at room temperature (±25^0C) and washed twice with phosphate buffered saline (PBS) (GIBCO, Life Technologies, USA) [13].

4.2.4 Counting of trichomonads

The number of trichomonads in the pellet was counted in an improved "Neubauer" haemocytometer (Marienfeld, Bad Mergentheim, Germany) (Appendix B, Section 2.4) [11-13]. The cell suspension was adjusted to a concentration of approximately 2 x 10^6 trichomonads per ml (Neubauer haemocytometer) (Marienfeld, Bad Mergentheim, Germany) in antibiotic-free modified Diamond's medium (Appendix B, Section 2.1).

4.2.5 Antimicrobial susceptibility testing

Susceptibility of trichomonads to metronidazole was determined *in vitro* by microdilution and microtitre methods [13]. The analysis was repeated three times per isolate under aerobic conditions. Sterile flat bottom micro-plates (SPL Life Sciences, Korea) with eight rows (A to H) and twelve wells (1 to 12) were used. Three rows (per *T. vaginalis* isolate) were done for the serially decreasing concentrations of metronidazole.

Stock solutions of 2 000 µg/ml and 1 280 µg/ml were filter sterilized (Filter Technology, USA) and stored at 2^0C to 8^0C (Labcon, Johannesburg, South Africa). The working dilutions of metronidazole were made by diluting the stock metronidazole solutions (2 000 µg/ml and 1 280 µg/ml). An aliquot of 5 µl of 2 000 µg/ml stock was diluted with 100 µl of modified Diamond's solution while 7.8 µl of 1 280 µg/ml stock was diluted with 100 µl of modified Diamond's medium to give a 100 µg/ml working solutions. The working solutions were diluted with bromocresol purple indicator (final concentration of bromocresol purple 0.003%) (Appendix B, Section 2.3)

To the first well of the three rows (A to C), 100 µl of the 100 µg/ml from the 2 000 µg/ml stock solution were added and to the next three rows (D to G), 100 µl of the 64 µg/ml from 1 280 µg/ml stock dilution of metronidazole was added. Using a 100 µl to 1 000 µl pipette the wells of three rows (from well 2 to 12) were inoculated with 50 µl of modified Diamond's media containing the indicator bromocresol purple (final concentration of bromocresol purple 0.003%) [14]. Fifty microlitres of the 1:4 dilution of the 2 000 µg/ml stock solution metronidazole was added to well 1 of the rows A, B and C. Fifty microlitres of the 1:4 dilution of the 1 280 µg/ml stock solution metronidazole was added to well 1 of three wells (E, F and G). Serial double dilutions were made as follows: 50 µl from well 1 to 2, 50 µl from well 2 to 3 onwards were performed and finally 50 µl from the 11^{th} well was discarded (Table 4.1). A final metronidazole concentration range from 100 µg/ml to 0.062 µg/ml was obtained. Well 12 was a control well (C) and contained only the trichomonad suspension without any metronidazole. To each of the wells containing the decreasing metronidazole, 150 µl of the 2 X 10^6 trichomonad suspension was added to all the wells of the six rows. At a pH of 6.4 to 6.6 bromocresol purple is a purple coloured solution [14]. With lactate and acetate being the major end products of *T. vaginalis* metabolism, an actively growing culture will produce

enough acid to change the pH of the medium from purple to a deep yellow colour [15]. The plan of a titration plate is as shown in Table 4.1.

Table 4.1: Plan of the microtitre plate showing metronidazole concentrations for the antimicrobial susceptibility testing of *T. vaginalis* isolates

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	50	25	12.5	6.25	3.2	1.6	0.8	0.4	0.2	0.1	C
B	100	50	25	12.5	6.25	3.2	1.6	0.8	0.4	0.2	0.1	C
C	100	50	25	12.5	6.25	3.2	1.6	0.8	0.4	0.2	0.1	C
E	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625	C
F	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625	C
G	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625	C

The plates were covered to prevent contamination and spillage. A damp cloth was included in each sealed bag to prevent the microtitre plate from drying and incubated at 37^0C (Labcon, California, USA) [16,17]. After 24 and 48 h of incubation the micro-titre plate (SPL Life Sciences, Korea) wells were examined with a light microscopy (Carl Zeiss, Germany) under the 10X and 40X objectives. The end-point MIC (minimum inhibitory concentration) of the assays was recorded as the concentration metronidazole at which no motile cells were observed [16,17]. Only MICs were used in this study because the MICs were found to be a reliable and suitable indicator of actual MLC (minimum lethal concentration), which can be used for diagnostic purposes [13].

4.2.6 Molecular characterization of *T. vaginalis* isolates

A commercial kit was used for the DNA extraction of the 30 *T. vaginalis* positive specimens. The genetic relatedness of the culture-positive *T. vaginalis* isolates was determined using the RAPD assay.

4.2.6.1 Extraction of total genomic DNA from *T. vaginalis* isolates

The extraction of culture positive *T. vaginalis* isolates was performed using a DNA-Sorb-A extraction kit (Sacace Biotechnology, Italy) according to the manufacturer's instructions (Appendix A, Section 1.1). The purity and concentration of the extracted DNA was determined by optical density measurements at 260 nm and 280 nm using a Nanodrop

spectrophotometer (NanoDrop Technologies Inc, USA) (Appendix A, Section 1.2). The DNA samples were stored at -20^{0}C until needed.

4.2.6.2 Genotyping using the RAPD analysis of *T. vaginalis* isolates

Five RAPD primers (TV1, TV2, TV3 and TV5, TV6) were used for the RAPD assay analysis [18] (Table 4.2). The primers target arbitrary regions within the *T. vaginalis* genome [18].

Table 4 .2: Nucleotide sequences of the primers used in the RAPD assays [18]

Primer name	Nucleotide Sequence 5 ⟶ 3'	RAPD PCR product size
TV1	CGC ACT CGG AGT	Various band sizes
TV2	TCG GCC GCT ATC	
TV3	CCG TGA CAC GCA	
TV5	GGG ACA CTC TGG	
TV6	GGG ACC TAC TGC	

(All primers were manufactured by TIB-MOLBIOL, Germany)

The TaKaRa Ex Taq^{TM} Master Mix (Takara Biotechnology, Japan) was used according to the manufacturer's instructions. The following amplification cycling profile was used for the RAPD assay: i) an initial denaturation step at 94^{0}C for 5 min; ii) followed by 40 cycles of denaturation at 95^{0}C for l min, annealing at 38^{0}C for 1 min and extension at 72^{0}C for 1 min and iii) final extension step at 72^{0}C for 15 minutes. The amplification procedure was performed in a Gradient Master Cycler (Eppendorf, Germany).

4.2.6.3 Visualization of the amplified PCR products

The PCR amplicons were analysed on a 2.0% (m/v) agarose gel (SeaKem, Upland, USA) containing 0.5 µg/ml ethidium bromide (Promeda, Madison, USA) at 85 V/cm (Eilte-300 Power supply, Wealtec Corp., Kennesaw, GA) for 2 h in 1 X TAE (pH 8.0 - 8.5) [2 M Tris Base (Sigma Chemicals, USA), 20 mM glacial acid (Merck, Germany), 0.5 M EDTA (pH 8.0) (Promega, USA)] (Appendix B, Section 1.5). A 1 kb DNA ladder (Fermentas, Thermo Scientific, USA) was used as the molecular size marker. The amplicons were visualized under UV illumination (TFM-26 Ultra Transilluminator, UVP, Upland, CA). The image was captured using a digital gel documentation system (DigiDoc-It imaging system, UVP, Upland).

4.2.6.4 Analysis of DNA banding patterns

The DNA patterns were analyzed using the GelCompar*II* software programme (Applied Maths, Kortrijk, Belgium). A distance matrix was constructed from the DNA banding patterns using the Dice coefficient. The unweighted pair group method using arithmetic averages (UPGMA) algorithm was used for clustering and construction of the dendrogrammes from the distance matrix.

4.3 Results

A total of 107 specimens were screened for *T. vaginalis* until a total of 30 *T. vaginalis* positive specimens were obtained from HIV positive female participants with a mean age of 30 years (21 years to 69 years old) from February 2012 to March 2012. The improved Neubauer haemocytometer was used to count 2 x 10^6 trichomonads per ml. The concentration of the extracted DNA ranged between 122.4 ng/µl and 499.7 ng/µl using the Nanodrop spectrophotometer sensor (Thermo Scientific, USA).

After 48 h of incubation the colour change was complete, the plates were examined visually and the results were recorded. Control wells (12) (trichomonad were not killed by metronidazole) developed a deep yellow colour, which indicated acid production (Figure 4.1). Microscopic examination, using a microscope in these wells confirmed the presence of numerous active *T. vaginalis* cells.

Microscopic evaluation of wells containing low concentrations of metronidazole (<0.8 µg/ml) indicated a yellow colour and contained active trichomonads. The amount of active trichomonads observed microscopically in these wells was lower than in the control wells (well 12) (Figure 4.1). Wells containing the intermediate concentrations of metronidazole were yellow and microscopic examination of the wells showed a mixture of motile and non-motile trichomonads. Wells containing high metronidazole concentrations (>2 µg/ml) remained purple and no active trichomonads were observed in these wells (Figure 4.2). Figure 4.1 shows the highest MIC of 25 µg/ml, while Figure 4.2 shows the lowest MIC at 0.125 µg/ml. After 48 h of incubation, lysis of the culture occured and no active trichomonads could be seen microscopically.

After 24 h and 48 h of incubation the MIC determined using a microscope ranged between 0.0625 µg/ml to 16 µg/ml and from 0.0625 µg/ml to 25 µg/ml respectively. The MIC determined visually after 48 h was from 0.0625 µg/ml to 25 µg/ml (Table 4.3).

Table 4.3: **Minimum inhibitory concentration (MIC) values determined by absence of characteristic motility after 24 h and 48 h intervals and visual colour changes caused by *T. vaginalis* isolates that were able to survive different metronidazole concentrations**

Lab Number of *T. vaginalis* isolate	MIC determined using a microscope Concentration of metronidazole (µg/ml)		MIC determined by visual colour changes Concentration of metronidazole (µg/ml)
	24 h	**48 h**	**48 h**
1	0.8	0.8	0.5
2	0.8	0.8	0.4
3	2.0	3.2	1.6
4	0.5	0.8	0.125
5	1.6	1.6	1.0
6	2.0	4.0	0.8
7	0.0625	0.1	0.0625
8	0.8	1.0	0.5
9	0.125	0.125	0.1
10	0.5	0.4	0.4
11	0.125	0.2	0.1
12	1.0	1.0	0.8
13	0.125	0.1	0.1
14	0.8	0.8	0.4
15	2.0	4.0	1.6
16	0.5	0.256	0.4
17	0.4	0.4	0.2
18	3.2	3.2	2.0
19	1.0	1.6	0.8
20	12.5	16	12.5
21	0.8	0.8	0.4
22	8.0	12.5	12.5
23	16	25	25
24	6.25	6.25	4.0
25	0.8	1.6	0.4
26	1.6	2.0	1.0
27	1.6	1.6	1.0
28	1.6	2.0	1.6
29	0.0625	0.125	0.0625
30	2.0	2.0	1.0

Fifty percent (15/30) of the trichomonads were inhibited at a metronidazole concentration of 1.0 µg/ml, while 28/30 (93%) were inhibited at a concentration of 12.5 µg/ml (Table 4.4). The minimal inhibitory concentration (MIC) ranged between 0.06 to 25 µg/ml.

Table 4.4: **Percentage and number of *T. vaginalis* isolates inhibited after 48 h aerobic incubation with end points determined visually**

Metronidazole concentration	After 48 h incubation	
	Number of strains	Percentage of strains inhibited
0.125	4	13% (4/30)
0.4	4	27% (8/30)
0.8	5	43% (13/30)
1.0	2	50% (15/30)
1.6	4	63% (19/30)
2.0	3	73% (22/30)
3.2	2	80% (24/30)
6.25	3	90% (27/30)
12.5	1	93% (28/30)
16	1	97% (29/30)
25	1	100% (30/30)

In the RAPD assay the following was obtained for the different primers: 15 profiles for primer TV2, 14 profiles with the TV3 primer, 13 profiles with the TV1 primer, 11 and five profiles with primer TV6 and primer TV5, respectively. *In vitro* metronidazole resistance was observed in isolates 20, 22 and 23 (Figure 4.3). The metronidazole resistant isolates did not cluster together using the RAPD assay.

4.4 Discussion

Metronidazole has been available worldwide since 1960 and is well established as the antimicrobial drug of choice for the treatment of vaginal trichomoniasis [19]. Metronidazole was the first effective anti-trichomonad antimicrobial drug in more than 90% of infected patients [19,20]. The first report of metronidazole treatment failure in women with trichomoniasis occurred within 2 years of the introduction of metronidazole, which may suggest that acquired resistance of the organism is not the only factor in antimicrobial drug resistance [20]. A degree of antimicrobial drug tolerance among trichomonads, insufficient absorption of metronidazole and inadequate transport of the antimicrobial drug to the site of infection may all affect treatment outcome [20].

There have been increasing reports of clinical treatment failure in women with vaginal trichomoniasis [19-22]. Although this may reflect an increase in the number of resistant *T. vaginalis* infections, it may also highlight the growing number of clinicians aware of metronidazole resistance in trichomoniasis [19-22]. In an effort to assess the prevalence of

drug resistance *T.vaginalis* this study investigated the *in vitro* susceptibility of 30 strains of *T. vaginalis* to various concentrations of metronidazole [13].

Trichomonas vaginalis isolates were assessed for metronidazole resistance *in vitro* by determining *in vitro* aerobic MIC of greater than or equal to 8 µg/ml. The method used in this study was multi micro-titre plates with a pH indicator (bromocresol purple) which was added to the modified Diamond's medium. Susceptibility testing of each isolate was performed three times to confirm the reproducibility of the MIC results. The use of micro-titre plates for culturing can achieve considerable savings in time, since the entire content of each well could be readily examined using the 10X and 40X objectives (Zeiss, Germany). The addition of bromocresol purple in the medium showed a noticeable indication of trichomonicidal activity (purple colour), which further reduced the time and work needed to read the MICs in the wells.

The use of the Neubauer haemocytometer standardized inoculums ensured that the same number of trichomonads (2 x 10^6 trichomonads per ml) were used in each microtitre well. The number of 2 x 10^6 trichomonads per ml gave a reliable and reproducible reading after 48 h of incubation. The Neubauer haemocytometer was found to be easy and convenient for counting of trichomonads.

The observed metronidazole resistance concentration of 25 µg/ml reached by three trichomonad isolates in well 20 reflects the ability of the *T. vaginalis* isolates to down-regulate all hydrogenosomal function, through circumventing metronidazole activation and to use alternative metabolic pathways [23]. The less sensitive trichomonads survived and produced delayed logarithmic growth density, which resulted in higher MIC values after 48 h compared to 24 h.

After 24 h and 48 h, the MIC determined using a microscope ranged between 0.0625 µg/ml and 16 µg/ml and from 0.0625 µg/ml to 25 µg/ml respectively. The MIC determined visually after 48 h ranged between 0.0625 µg/ml and 16 µg/ml. Determination of trichomonad viability by direct observation of the colour changes in the plate did not correlate with the motility of the parasite. The possible explanation could be that not enough acid was produced by the 2 x 10^6 number of trichomonads to change colour of the bromocresol indicator. The MIC values were

found to be lower and ranged between 0.125 µg/ml and 2 µg/ml after 24 h as compared to 48 h of incubation. The reason could be the delayed growth of the trichomonads.

Depending on the assay used, aerobic MICs of metronidazole on clinically resistant isolates have been reported to range from 4.25 µg/ml to >200 µg/ml [8,13,23-27]. Clinical antimicrobial resistance and treatment failure has been known to exist with *T. vaginalis* isolates with MICs as low as 4.25 µg/ml [24].

In a study by Conrad *et al*. [28] the *T. vaginalis* isolates obtained worldwide were divided into two groups, type 1 and type 2. It was found that type 1 isolates, which are dominant in Southern Africa, had a median MIC value of 25 µg/ml, while isolates from other parts of the world had a MIC median value of 200 µg/ml [28]. This was found to be in agreement with the results of this study where the MICs ranged between 0.06 µg/ml and 25 µg/ml. In this study, high level resistance was defined as MICs of >50 µg/ml, resistance as MICs ranging between 12 µg/ml and 50 µg/ml and low level resistance was found to be between 4 µg/ml and 12 µg/ml.

The prevalence of resistance in this study was found to be 6% (2/30) in adult women which suggested that there is a low prevalence of metronidazole resistance in HIV positive patients in Pretoria, South Africa. Metronidazole prevalence studies have shown values that range from 1.7% to 10.1% [6,8,19,22]. A high prevalence of metronidazole resistance in *T. vaginalis* isolates 17.4% (14/82) was found in the Goroka region, Papua Guinea where a high level of metronidazole resistance is already a problem in this area [4]. Previous studies have found that the prevalence of metronidazole resistant isolates did not depend on the HIV status of an individual [29]. The low metronidazole resistance among the studied *T. vaginalis* isolates could be explained by i) *T. vaginalis* being a diploid parasite, so changes in a single gene is not sufficient to confer drug resistance; ii) *T. vaginalis* lacks alternative fermentation pathways (few metabolic alternatives to enzyme pyruvate: ferredoxin oxidoreductase (PFOR), which activates metronidazole) [8] and iii) metronidazole is not part of the HIV treatment regimen (which normally includes the antivirals and certain antimicrobials).

The random amplified polymorphic DNA (RAPD) technique is one of the most commonly used molecular techniques for the identification of genetic polymorphisms [30, 31]. In this study, two isolates (isolate 22 and 23) were resistant, one isolate (isolate 20) was moderately

resistant and 27 isolates were found to be sensitive (Figure 4.3). In this study the clustering position of the isolates on the dendrogrammes did not correlate with the *in vitro* metronidazole resistance. Results of this study correlated with the study done by Fraga and collegueas [30], which reported that no statistically significant concordance was found between the RAPD dendrogramme's clustering topology and metronidazole resistance. Similarly, Stiles and colleagues [31] reported no concordance between RFLP subtype and metronidazole resistance.

Correlation between RAPD clustering and metronidazole resistance has been shown in previous studies [25,32,33]. A small sample size of 30 *T. vaginalis* isolates were used in this study may have contributed to statistically insignificant results as compared to 109 *T. vaginalis* isolates sample size used in previous studies where concordance between metronidazole resistance and RAPD clustering was found [25].

As with all studies using RAPD analysis as a typing technique, care must be taken in the analysis of the results due to the arbitrary nature of the PCR priming used in this technique. There is a possibility that amplification from different loci may co-migrate on the gel or that variations in template concentrations may result in sporadic fragments [25,33]. The limitations of the current study were that metronidazole susceptibilty testing of *T. vaginalis* under anaerobic conditions was not performed; however, aerobic metronidazole conditions have been found to be satisfactory in previous studies [13, 34]. In addition, aerobic resistance occurs in *T. vaginalis* isolates, while anaerobic resistance rarely takes place in clinical isolates [13, 34]. The observed metronidazole susceptibility results were not followed-up to determine resistance in patients, only *in vitro* susceptibility testing was performed. The study was limited to female participants attending the ARV clinic in Pretoria, thus findings are not representative of the South African population in general. No reference ATCC strains with known MIC or MLC values were included in this study.

4.5 Conclusion

The MIC read by visual interpretation proved to be easy, fast and did not require high expertise to interpret the results. There was a low prevalence of metronidazole resistance among the *T. vaginalis* isolates obtained from the HIV positive female participants. Although the prevalence of resistance is currently low, reliance on a single class of antimicrobial drug heightens the vulnerability of clinical *T. vaginalis* nitroimidazole resistance to become widespread. Periodic

surveillance of the *T. vaginalis* metronidazole antimicrobial drug susceptibility should be carried out to monitor the possible emergence of resistance. In this study, no correlation was found between metronidazole resistance and RAPD assay clustering. The metronidazole resistant isolates were not genetically related and belonged to different clusters on the RAPD dendrogrammes.

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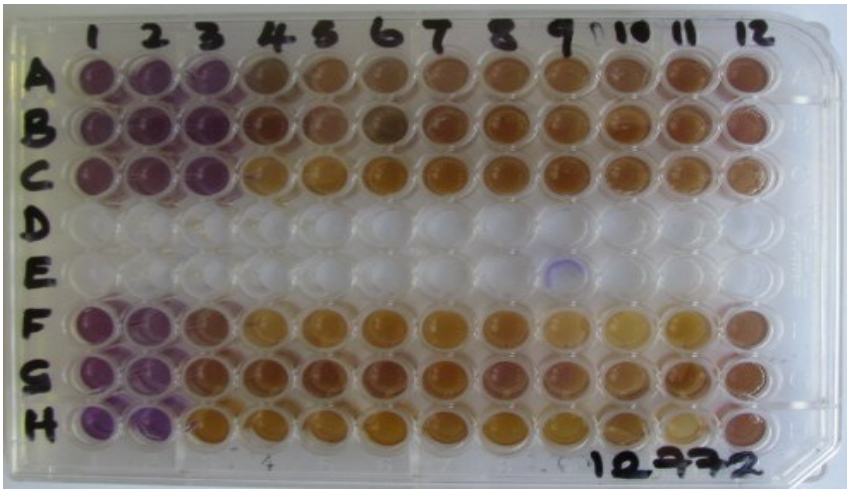

Figure 4.1: Microtitre plate showing the highest MIC (25µg/ml) obtained for a *T. vaginalis* isolate

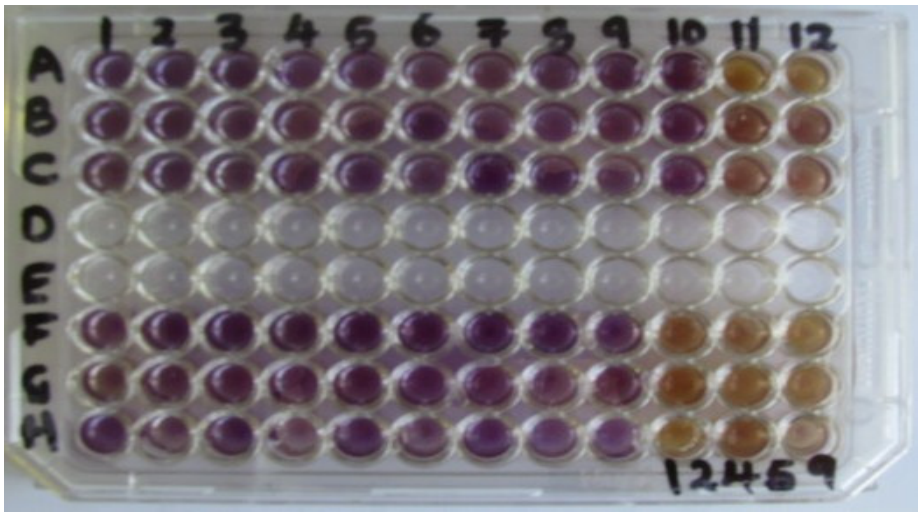

Figure 4.2: Microtitre plate showing the lowest MIC (0.125 µg/ml) obtained for a *T. vaginalis* isolate

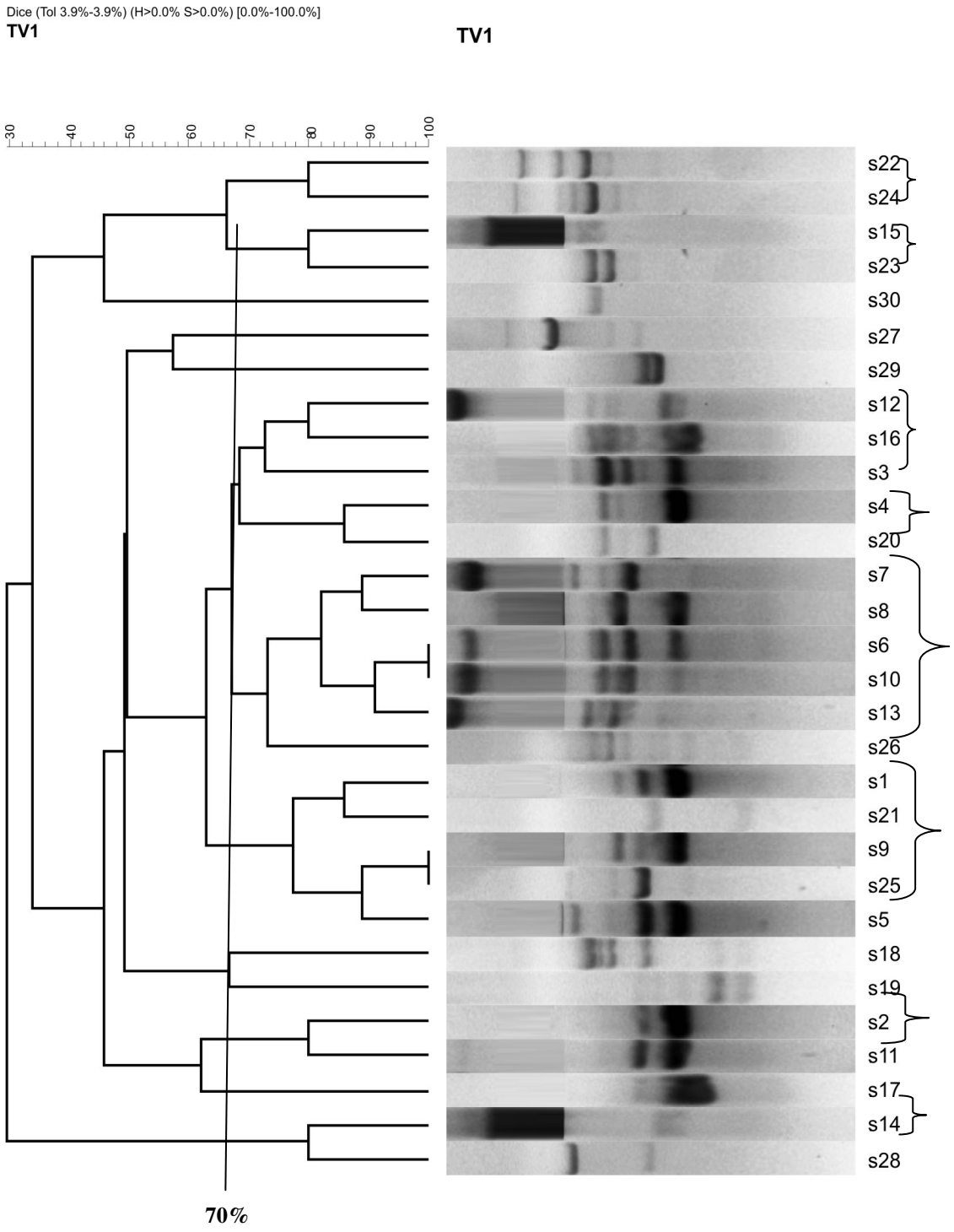

Figure 4.3: Dendrogramme obtained for 30 isolates of *T. vaginalis* using the TV1 primer banding patterns. The dendrogramme was constructed using the UPMGA algorithm from the distrance matrix generated by the Dice coefficient from the banding profiles. Clusters were identified at 70% similarity. Resistant isolates were numbers 20, 22 and 24

CHAPTER 5

CONCLUSION

5.1 Concluding Remarks

Trichomoniasis is a sexually transmitted disease caused by the protozoan *Trichomonas vaginalis* (Mavedzenge *et al*., 2010). Worldwide trichomoniasis is the most common non-viral sexually transmitted disease with approximately 200 million new cases per annum (WHO, 2011). The *T. vaginalis* protozoan is responsible for trichomoniasis and is more common in high-risk behaviour groups, such as prostitutes, drug users and alcoholics (Johnston and Mabey, 2008; Valadkhani *et al*., 2011). Trichomoniasis presents as a broad spectrum of clinical patterns ranging from asymptomatic to severe vaginitis and cervicitis (Rezaeian *et al*., 2009). Infected men may have a report pain in urethra, testicular pain or complicated prostatitis (Schwebke *et al*., 2011).

Trichomoniaisis is widely underdiagnosed due to several factors which include: i) lack of routine testing for *T. vaginalis*; ii) low sensitivity of commonly used diagnostic techniques, such as microscopy and the iii) non-specific clinical symptoms, which are commonly shared by all STIs, such as genital discharge, urethritis and vaginitis (Garber, 2005; Uneke *et al*., 2007; Bachmann *et al*., 2011). Wet mount microscopy remains the most common diagnostic method for the diagnosis of *T. vaginalis* from vaginal or cervical specimens in women and from urethral or prostatic secretions in men (Garber, 2005; Bachmann *et al*., 2011). However, the wet mount microscopy method is used despite of its low sensitivity, since it is rapid and an inexpensive method (Garber, 2005; Bachmann *et al*., 2011).

The purpose of this study was to detect *T. vaginalis* in HIV positive women attending the Anti-Retroviral clinic at the Tshwane District Hospital, Pretoria, South Africa. In this study wet mount microscopy showed that 8% (30/380) of specimens were positive for *T. vaginalis*. Culture showed that 24% (92/380) of the specimens were positive, while the commercial PCR assay detected the most positive *T. vaginalis* specimens 31% (118/380). The current gold standard for the diagnosis of trichomoniasis is culture and it managed to detect three times as many cases of *T. vaginalis* compared to wet mount microscopy, while the commercial PCR assay using a commercial amplification kit 240/520 IC (Sacace Biotechnology, Italy) detected 26 additional positive specimens.

In agreement with Lusk and colleagues in 2010, the conventional diagnostic methods (wet mount microscopy and culture) underestimated the prevalence of *T. vaginalis.* Based on the results in this study it is clear that if a laboratory relies only on microscopy, three quarters of *T. vaginalis* infections will be missed. The use of suboptimal laboratory methods for routine diagnosis could have far-reaching public health implications with regards to *T. vaginalis* transmission dynamics (Lusk *et al.*, 2010). However, should diagnosis be accurately performed by use of quick and sensitive molecular techniques, the treatment of *T. vaginalis* infection is affordable and effective. Infection rates can be minimized by diagnosis and treating; preventing the spread of this protozoan infection (Lazenby, 2011). The active detection and treatment of *T. vaginalis* have the potential to impact on the spread of HIV infection but also the rates of pelvic inflammatory disease, bacterial and viral STIs acquisition, post-operative infection and pregnancy complications.

In resource rich settings, a commercial PCR assay offers the advantage of high sensitivity within a short period of time, whereas in settings with limited resources a combination of wet mount microscopy and culture methods could be used (Patil *et al*., 2012). The commercial PCR assay kit (*Trichomonas vaginalis* 240/520 IC Sacace Biotechnology, USA) for the detection of *T. vaginalis* used in this study proved to be rapid and easy to perform. The commercial PCR assay kit (*Trichomonas vaginalis* 240/520 IC Sacace Biotechnology, USA) contained an internal control, which can be used in the DNA isolation procedure and can serve as an amplification control for each individually processed specimen. The internal control serves to identify possible reaction inhibition. The commercial PCR assay kit (*Trichomonas vaginalis* 240/520 IC Sacace Biotechnology, USA) may not be readily available for use in South Africa and other developing countries because it is expensive. Nucleic acid amplification tests for *T. vaginalis* have improved sensitivity for detecting *T. vaginalis* in infected individuals compared to the existing wet mount microscopy and culture-based methods (Brown *et al*., 2004; Andrea *et al*., 2011).

In South Africa consideration should be given to requiring mandatory testing and reporting of *T. vaginalis* infection, especially in HIV positive patients. This practice has already been adapted in America where the Centres for Disease Control and Prevention (CDC) recommends annual *T. vaginalis* screening of HIV positive women with a repeat screening every three months due to the high rates of recurrence (CDC, 2003; Lazenby, 2011). This approach is

recommended for implementation in South Africa and other developing countries where there are limited resources. To improve the chance of detecting *T. vaginalis* in South Africa a recommendation by Patil *et al*. (2012) where at least two diagnostic techniques namely: wet mount microscopy and culture can be used (Patil *et al*., 2012). The use of highly specific and sensitive nucleic acid amplication tests (NAAT), for the diagnosis of *T. vaginalis* is not practical for routine diagnosis in South Africa.

Despite its importance, very little is known about the genetic relatedness of the causative agent of trichomonasis, particularly in South Africa. Characterization techniques comprising of isoenzyme analysis, antigenic characterization and serotyping with antibodies have been used but these techniques have been restricted by the reliance on protein, polysaccharides or antigen expression, which can be inconsistent (Krieger *et al*., 1985; Proctor *et al*., 1988; Singh, 1997; Stiles *et al*., 2000; Crucitti *et al*., 2008; Conrad *et al*., 2011). Molecular markers have the advantage of showing genetic differences without interference from environmental factors (Stiles *et al*., 2000; Crucitti *et al*., 2008). Two DNA based molecular techniques namely the RAPD assay and IGS-PCR RFLP assay were used to determine the genetic relatedness of *T. vaginalis* isolates in this study.

The RAPD assay is a well verified tool in genetic and taxonomic studies and a suitable method for perceiving total genetic variations and its distribution and among populations (Arya *et al*., 2011). The RAPD assay in this study showed that *T. vaginalis* isolates have diverse genetic variability. An important advantage of the RAPD assay is that it compares polymorphisms in multiple genomic loci instead of polymorphisms in a single locus (Rojas *et al*., 2004). The results of a single locus can be misleading because the phylogeny of one gene can differ from the phylogeny of the species (Hampl *et al*., 2001). The findings in this study suggest that the RAPD assay of *T. vaginalis* fulfilled most of the requirements of an ideal typing method. It was discriminatory, reproducible, simple and inexpensive to perform and thus applicable in many clinical laboratories.

In this study, IGS-PCR RFLP gave uniform bands for each restriction enzyme used and revealed no genetic polymorphism. However, the IGS-PCR RFLP assay was not sensitive to changes in experimental conditions and thus was highly reproducible. The *T. vaginalis* IGS region lack of variability may have led to the low discriminatory ability of IGS-PCR RFLP in

this study. This study proved that the IGS may thus not be a suitable marker for *T. vaginalis* strain discrimination.

Infections with antimicrobial drug resistant strains are one of the major problems in the fight against infectious diseases (Dunne *et al*., 2003). Accurate figures about metronidazole-resistant trichomoniasis cases are limited and sometimes non-existent, especially in Africa (Perez *et al*., 2001). In South Africa, although metronidazole is the antimicrobial drug of choice used to treat *T. vaginalis* and is currently being used in the syndromic manangement of sexually transmitted diseases, this study found a low prevalence of metronidazole resistant isolates 6% (2/30). In this study, at a metronidazole concentration of 25 µg/ml, 100% (30/30) of the *T. vaginalis* isolates had been killed by metronidazole. Hence, this study showed that treatment with metronidazole in HIV positive patients is acceptable and would result in eradication of the trichomoniasis. In rare cases of recurrent trichomoniasis, clinicians should consider sending genital specimens for resistance testing, which may lead to a specific treatment regimen for such patients.

5.2 Future Research

Point of care diagnostic methods have been shown to be useful in developed countries to understand the epidemiology and pathogenesis of *T. vaginalis* (Huppert *et al*., 2005; Harp and Chowdhury, 2011; Lazenby, 2011). The sensitivity and specificity of rapid tests have not been validated in South Africa. Future research in South Africa should focus on point of care diagnostic techniques, such as the rapid antigen test (OSOM TV, Genzyme Diagnostic, Cambridge, MA, USA). Studies to test and validate point of care assays are required before implementation in South African diagnostic laboratories. The addition of point of care tests to the repertoire of techniques available for *T. vaginalis* detection is especially important for facilities in rural areas without access to microscopes or incubators (Huppert *et al*., 2007; Lazenby, 2011). However, the cost of point of care tests may limit the availability in developing countries, like South Africa.

The whole genome sequence of *T. vaginalis* has been published in 2007; however, sequence information alone is unable to explain the complicated and diverse biological characteristics, such as gene regulatory biology, pathogenesis and biochemical kinetics of the protozoan (Calton *et al*., 2007; Huang *et al*., 2008). Proteomics investigations can provide a more direct

assessment of biological processes by monitoring the expressed proteins performing the regulatory, enzymatic and structural functions under different growth and environmental conditions (Huang *et al*., 2008). Proteomics is a key technology in the postgenomic era for investigations of protein synthesis (Huang *et al*., 2008). In addition, proteomics tools are valuable to the research the basic biology of an organism, such as physiology, pathogenesis, potential antimicrobial drug targets and resistance mechanisms (Kaul *et al*., 2004; Meade *et al*., 2009). The proteome reference map of *T. vaginalis* has been established, thus setting the basis for comparative proteomic analysis on the pathogenesis, virulence factors and antimicrobial drug resistance of the *T vaginalis* parasite (Huang *et al*., 2008). Future studies should now focus on using proteomics to investigate why some *T. vaginalis* strains are virulent while others are non-virulent. Comparative proteomics can be used on susceptible and resistant *T. vaginalis* isolates to determine metronidazole resistance markers, which can lead to improved treatment strategies.

Although a number of studies have looked at the prevalence of trichomoniasis in South Africa, the true prevalence and spectrum of *T. vaginalis* is unknown, since only a few studies have assessed the prevalence of *T. vaginalis* among men (Mhlongo *et al*., 2010). Two main reasons have been proposed: i) the majority of men who have trichomoniasis have no knowledge of the infection because they either are asymptomatic or their symptoms are too minor to elicit concern as a result they do not seek treatment (Hobbs and Sena, 2007; Sood and Kapil, 2008) and ii) Diagnosis of *T. vaginalis* in men is often difficult because most commonly used diagnostic tests (wet mount microscopy and culture) often fail to detect *T. vaginalis* in men due to the low protozoa load (Van der Pol, 2007). The wet mount microscopy method requires more than 10^3 trichomonads/ml of live protozoa and culture requires an inoculum of 300 to 500 trichomonads/ml for diagnostic testing (Lee *et al*., 2012). Future research should focus on determining the prevalence of *T. vaginalis* in men and improving the sensitivity of diagnostic tests used to detect the protozoan (Lee *et al*., 2012). The development of new diagnostic tests, in particular the development of the non-invasive urine based nucleic acid amplification tests, will be useful in detecting and finding the true prevalence of *T. vaginalis* infection (Hobbs and Sena, 2007).

High risk sexual behaviours, such as prostitution, drug use and having unproctected sex with multiple partners represent difficult obstacles to overcome in controlling the global burden of

trichomoniasis (Cudmore and Garber, 2010). High risk individuals are likely to be repeatedly re-infected and thus may be responsible for maintaining infection in the community (Cudmore and Garber, 2010). If prevention is based solely on treatment then *T. vaginalis* will continue to be an endemic infection; however, the introduction of a vaccine could provide long-term protection against *T. vaginalis.* Current efforts to design a vaccine, which previously has been left at clinical trial phase one need to be intensified (Cudmore and Garber, 2010).

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APPENDICES

Appendix A

DETAILED MOLECULAR METHODOLOGY

1.1 DNA extraction of *T. vaginalis* isolates

DNA extraction was performed using a DNA-Sorb-A extraction kit (Sacace Biotechnology, Italy) according to the manufacturer's instructions. Briefly: active trichomonads in the logarithmic phase of growth (normally after 48 h incubation) were harvested. An aliquot of 100 µl of the washed cell culture and 300 µl of lysis solution (Sacace Biotechnology, Italy) containing an enzyme that destroys the cells was added to each of the labelled 1.5 ml polypropylene screw-cap tubes (Merck, Germany).

An 10 µl aliquot of internal control (Sacace Biotechnology, Italy) was also added to each screw-cap tube (Merck, Germany). The tubes were vortexed (Labnet International, Edison, NJ, USA) and incubated for 5 min at 65^0C in a dry heat block (Labnet International, Edison, NJ, USA) for 7 to 10 s. An aliquot of 20 µl of Sorbent (Sacace Biotechnology, Italy) was added to each tube and vortexed (Labnet International, Edison, NJ, USA) for 5 s to 7 s. This step was repeated. All the tubes were centrifuged (Labnet International, Edison, NJ, USA) for 30 s at 5 000 *xg* and using a micropipette with a plugged aerosol barrier tip; the supernatant was removed carefully and discarded from each tube without disturbing the pellet.

An aliquot of 500 µl of washing solution (Sacace Biotechnology, Italy) was added to each tube and vortexed (Labnet International, Edison, NJ, USA) vigorously and centrifuged (Labnet International, Edison, NJ, USA) for 30 s at 1 000 *xg*. The supernatant was removed and discarded from each tube. All tubes were incubated at 65^0C in a dry heat block (Labnet International, Edison, NJ, USA) for 5 min and vortexed (Labnet International, Edison, NJ, USA) periodically. The tubes were centrifuged (Labnet International, Edison, NJ, USA) for 1 min at 12 000 *xg*. The supernatant contained DNA ready for amplification. Purity and the concentration of DNA were determined by the optical density measurements at 260 nm and 280 nm using a Nanodrop spectrophotometer (NanoDrop Technologies Inc, USA). The extracted DNA was stored at -20^0C (Whirlpool, USA) until further analysis.

1.2 Determination of the extracted DNA concentration by the Nanodrop spectrophotometer

The concentration of the extracted DNA was determined by pipetting 2 µl of the sample onto the Nanodrop spectrophotometer sensor (Thermo Scientific, USA). The Nanodrop uses the Beer-Lambert equation (C=A/CE*b) to determine the concentration of the nucleic acid (DNA). Where C is the nucleic acid concentration in ng/µl, A is the absorbance in AU, E is the wavelength dependent extinction coefficient in ng-cm/µl and b is the path length in cm. For the determination of DNA concentration, the absorbance peak is at 260 nm and the ratio of 260/280 should be from 1.8– 2.0 to indicate pure and high quality DNA.

1.3 Amplification of *T. vaginalis* DNA

Amplification was performed using the commercial amplification kit *T. vaginalis* 240/520 IC (Sacace Biotechnology, Italy) according to the manufacturer's instructions. Briefly: The required number of tubes containing PCR-mix-1 (containing master mix) was prepared. An aliquot of 10 µl of PCR-mix-2 (containing Taq enzyme) was added into each PCR-mix-1 tube. 10 µl DNA sample was added to appropriate tubes (PCR-mix-1) containing master-mix and Taq enzyme (Sacace Biotechnology, Italy). An aliquot of 10 µl of DNA buffer (Sacace Biotechnology, Italy) and 10 µl of positive control (Sacace Biotechnology, Italy) were added to the negative and positive control tubes respectively. The amplification procedure was performed in the Gradient Master Cycler (Eppendorf, Germany). The following programme was used: pre-incubation at 95^0C for 5 min, 42 cycles of denaturation at 95^0C for 1 min, annealing at 65^0C for 1 min extension at 72^0C for 1 min.

1.4 Random amplified polymorphic DNA analysis (RAPD) and Restriction Fragment Length Polymorphism (RFLP) analysis

The amplification of the RAPD and RFLP assays were done using the TaKaRa Ex Taq™ Master Mix (Takara Biotechnology, Japan). The TaKaRa Ex Taq™ Master Mix (Takara Biotechnology, Japan) was used according to the manufacturer's instructions. Briefly for each individual PCR tube the following were added: i) 5 µl template DNA was added; ii) 5 µl of 10X ExTaq Buffer (Takara Biotechnology, Japan) iii) 1.25 µl primer; iv) 4 µl dNTP mixture; v) 0.25 µl Takara ExTaq™ (Takara Biotechnology, Japan) and vi) 34.5 µl of nuclease free

water (Whitehead Scientific, South Africa) were added to give a total of 50 µl in each PCR tube. The IGS-PCR RFLP amplification programme consisted of an initial denaturation step at 94^0C for 5 min, followed by 45 cycles of i) denaturation at 95^0C for 1 min; iii) annealing at 50^0C for one min and iii) extension at 72^0C for 1 min. This was followed by a final extension step at 72^0C for 15 min. The RAPD amplification programme consisted of an initial denaturation step at 94^0C for 5 min, followed by 40 cycles of denaturation at 95^0C for 1 min, annealing at 38^0C for 1 min and extension at 72^0C for 1 min. The final extension step was at 72^0C for 15 min.

1.5 Preparation of the buffer used for gel electrophoresis of RAPD and RFLP assay products

The 50 X TAE buffer was prepared by adding 242 g of Tris Base (Sigma-Aldrich, St Louis, USA) to deionised water. A volume of 57.1 ml of glacial acid (Merck, Germany) and 100 ml of 0.5 M EDTA (pH 8.0) (Promega, USA) were added to the solution. The 0.5 M EDTA was prepared by dissolving 186.12 g of EDTA (Promega, USA) in a litre of deionised water. The volume of the 50 X TAE was adjusted to a final volume of 1000 ml and stored at room temperature (25ºC) as stock solution until further use. The 1 X TAE working solutions was prepared by adding 10 ml of stock solution into 990 ml of deionised water.

1.6 Preparation of the agarose gel used for gel electrophoresis of PCR products

The 2% (m/v) agarose gel was prepared by adding 2 g of agarose powder (SeaKem, Rockland, USA) in 100 ml of the 1 X TAE working solution. The agarose powder was dissolved in the deionised water by heating in a microwave oven. The mixture was cooled in a Hybridiser HB-1D (Lasec, South Africa) for 30 min. After cooling, 5 µl of ethidium bromide (10 mg/ml) (Promega, Madison, USA) was added. The gel was poured into a casting tray (Bio-RAD, USA) with a comb to form the wells of the gel. The gel was placed into the electrophoresis tank (Bio-RAD, USA) with 1 X TAE buffer after it solidified.

APPENDIX B

DETAILED SUSCEPTIBILITY TESTING METHODOLOGY

2.1 Preparation of modified Diamond's medium

The Diamond's medium was prepared by dissolving 20 g of Trypticase (Merck, Germany), 10 g of Yeast Extract (Merck, Germany), 1 g of L-cysteine HCℓ (Sigma-Aldrich, St Louis, USA) and 1 g of Ascorbic acid (Sigma-Aldrich, St Louis, USA) in 900 ml of distilled water. The pH was adjusted to 6.4 with sodium hydroxide pellets (Merck, Germany) and sterilized by autoclaving at 121^0C for 20 min. After the medium was cooled, the antibiotics and supplements were added namely: 40 µg/ml of gentamicin (Sigma-Aldrich, St Louis, USA), 1 g/ml ampicillin (Sigma-Aldrich, St Louis, USA) and 5 µl/ml of Amphotericin B (Sigma-Aldrich, St Louis, USA) and finally 100 ml of horse serum (Sigma-Aldrich, St Louis, USA).

2.2 Preparation of modified Diamond's medium with bromocresol purple indicator

A bromocresol purple (Merck, Germany) stock solution (0.03%) was made by dissolving 0.003 g bromocresol purple (Merck, Germany) in 100 ml distilled water. The solution was filter sterilized.

2.3 Preparation of *T. vaginalis* cell counting fluid

To prepare a 1% stock solution, 1 g of Gentian violet (Merck, Germany) was dissolved in 100 ml of distilled water.

2.4 Harvesting and counting of *T. vaginalis cells*

A heavy growth of the trichomonads was harvested by obtaining active trichomonads in the logarithmic phase of growth (normally after 48 h incubation). The trichomonad cells were concentrated by centrifuging (Labnet International, Edison, NJ, USA) for 5 min at 5 000 *xg*. A 0.5 ml of Diamond's medium was added to the pellet. A 1/10 dilution of the cell suspension with white cell counting fluid (25 µl of cell suspension + 250 µl counting fluid) (Appendix B: section 2.3) was loaded into an improved Neubauer counting chamber (Marienfeld, Bad Mergentheim, Germany) and 1 mm^2 (middle block counting chamber) was counted. The haemocytometer is a specialised microscope slide on which two grids have been engraved in a

central region that is 0.1 mm lower than the rest of the slide. Each grid comprises 25 large squares, with each containing 16 smaller squares of area 1/400 mm^2. This creates a region of known volume (0.1 mm^3) when a special coverslip is placed over the central region. An aliquot of 10 µl of culture was pipetted under the coverslip and cells counted in the grid squares. Multiplying total number of cells in the entire grid by 10^4 gives the number of cells per ml. The results were recorded as follows 50 cells counted= 5 X 10^4 cells per ml. The entire contents of each well could be readily examined with ±10 fields of view using the 10X objective (Zeiss, Germany). The cell suspension was adjusted to a 2 X 10^6 cells per ml with modified Diamond's medium.

Appendix C

LIST OF PARTICIPANTS AND RESULTS OF THE DIAGNOSTIC ASSAY

Table C1: Formula used for calculating specificity and sensitivity of a diagnostic test

	Positive (+)	Negative (-)	
T. vaginalis (+)	a	b	a + b
T. vaginalis (-)	c	d	c + d
	a+c	b +d	(a + b) + (c + d)

Sensitivity: Probability that the diagnostic test is positive when in facthe person is positive (a/a+b)

Specificity: The probability that the diagnostic test is positive while the person is actually negative (d/c+d)

Table C2: Sensitivity and specificity of microscopy for the detection of *T. vaginalis* isolates against the culture method (N=380)

	Microscopy			
Culture		Positive (+)	Negative (-)	Total
	Positive(+)	29	62	91
	Negative (-)	1	288	289
	Total	30	289	380

Sensitivity: (29/91x100) = 32%

Specificity: (289/380x100) =76%

Table C3: Sensitivity and specificity of PCR for the detection of *T. vaginalis* isolates against the culture method (N=380)

	PCR			
Culture		Positive	Negative	Total
	Positive (+)	92	0	92
	Negative (-)	26	262	288
	Total	92	288	380

Sensitivity: (92/92x100) =100%)

Specificity: (262/288x100) =91%

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
1	06/06/1988	Negative	Negative	Negative	Asymptomatic
2	17/01/1970	Negative	Negative	Negative	Asymptomatic
3	19/02/1968	Negative	Negative	Negative	Asymptomatic
4	21/02/1965	Negative	Negative	Negative	Asymptomatic
5	02/08/1972	Negative	Negative	Positive	Asymptomatic
6	25/07/1950	Negative	Negative	Negative	Symptomatic
7	02/10/1972	Negative	Negative	Negative	Asymptomatic
8	12/11/1968	Negative	Negative	Negative	Asymptomatic
9	26/09/1985	Negative	Negative	Negative	Asymptomatic
10	14/12/1965	Negative	Negative	Positive	Asymptomatic
11	28/02/1971	Negative	Negative	Negative	Asymptomatic
12	08/111/1980	Negative	Negative	Negative	Asymptomatic
13	05/03/1964	Negative	Negative	Negative	Asymptomatic
14	16/03/1976	Negative	Negative	Negative	Asymptomatic
15	04/05/1971	Positive	Positive	Positive	Symptomatic
16	08/06/1975	Negative	Positive	Positive	Asymptomatic
17	31/051962	Positive	Positive	Positive	Asymptomatic
18	11/06/1979	Negative	Negative	Negative	Asymptomatic
19	11/06/1980	Negative	Negative	Negative	Asymptomatic
20	26/03/1979	Negative	Negative	Negative	Symptomatic
21	24/08/1984	Negative	Negative	Negative	Asymptomatic
22	06/08/1977.	Negative	Negative	Negative	Asymptomatic
23	06/12/1974	Negative	Negative	Positive	Asymptomatic
24	12/07/1963	Negative	Negative	Negative	Asymptomatic
25	27/09/1973	Negative	Positive	Positive	Asymptomatic
26	09/01/1975	Negative	Negative	Negative	Asymptomatic
27	09/01/1970	Negative	Negative	Negative	Asymptomatic
28	08/08/1964	Negative	Negative	Negative	Asymptomatic
29	10/10/1975	Negative	Positive	Positive	Asymptomatic
30	16/08/1964	Negative	Negative	Negative	Asymptomatic
31	28/03/1982	Negative	Negative	Negative	Asymptomatic
32	05/02/1963	Negative	Positive	Positive	Asymptomatic
33	28/05/1967	Negative	Negative	Negative	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued……)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
34	29/06/1965	Negative	Negative	Negative	Asymptomatic
35	02/04/1974	Negative	Negative	Negative	Asymptomatic
36	06/06/1966	Negative	Negative	Negative	Asymptomatic
37	19/02/1972	Negative	Negative	Negative	Asymptomatic
38	22/.11/1985	Negative	Negative	Negative	Asymptomatic
39	25/08/1962	Positive	Positive	Positive	Symptomatic
40	04/10/1963	Negative	Negative	Negative	Asymptomatic
41	26/10/1976	Positive	Positive	Negative	Symptomatic
42	07/01/1975	Negative	Negative	Negative	Asymptomatic
43	12/10/1056	Negative	Negative	Negative	Asymptomatic
44	31/05/1076	Negative	Positive	Negative	Symptomatic
45	16/02/1965	Negative	Negative	Negative	Asymptomatic
46	07/02/1967	Negative	Positive	Negative	Asymptomatic
47	30/06/1986	Negative	Negative	Negative	Asymptomatic
48	17/10/1967	Negative	Negative	Negative	Asymptomatic
49	20/08/1964	Negative	Negative	Negative	Asymptomatic
50	07/07/1976	Negative	Positive	Negative	Asymptomatic
51	18/10/1982	Negative	Negative	Negative	Asymptomatic
52	01/02/1952	Negative	Negative	Negative	Asymptomatic
53	12/12/1951	Positive	Positive	Negative	Asymptomatic
54	30/08/1974	Negative	Negative	Negative	Asymptomatic
55	06/06/1981	Negative	Negative	Negative	Asymptomatic
56	22/12/1971	Negative	Negative	Negative	Asymptomatic
57	01/01/1966	Negative	Negative	Negative	Asymptomatic
58	02/08/1957	Positive	Positive	Positive	Symptomatic
59	24/07/1977	Negative	Negative	Negative	Asymptomatic
60	23/07/1956	Negative	Positive	Negative	Asymptomatic
61	26/11/1970	Negative	Negative	Negative	Asymptomatic
62	24/06/1964	Negative	Negative	Negative	Asymptomatic
63	16/12/1960	Negative	Negative	Negative	Asymptomatic
64	06/10/1989	Negative	Negative	Negative	Asymptomatic
65	25/01/1964	Negative	Negative	Negative	Asymptomatic
66	04/04/1970	Negative	Negative	Negative	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued……)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
67	18/12/1969	Negative	Positive	Negative	Asymptomatic
68	05/03/1981	Negative	Negative	Negative	Asymptomatic
69	21/11/1977	Negative	Negative	Negative	Asymptomatic
70	30/06/1952	Negative	Negative	Negative	Asymptomatic
71	11/02/1969	Positive	Positive	Negative	Asymptomatic
72	10/12/1967	Negative	Negative	Negative	Asymptomatic
73	06/06/1969	Negative	Negative	Negative	Asymptomatic
74	02/10/1980	Negative	Negative	Negative	Asymptomatic
75	24/01/1975	Positive	Positive	Positive	Symptomatic
76	04/02/1984	Negative	Negative	Negative	Asymptomatic
77	27/06/1975	Negative	Negative	Negative	Asymptomatic
78	13/11/1963	Negative	Positive	Negative	Symptomatic
79	21/04/1977	Negative	Negative	Negative	Asymptomatic
80	17/09/1975	Negative	Negative	Negative	Symptomatic
81	11/11/1980	Negative	Negative	Negative	Symptomatic
82	23/08/1977	Negative	Negative	Negative	Asymptomatic
83	23/04/1983	Negative	Negative	Negative	Asymptomatic
84	01/10/1962	Negative	Negative	Negative	Symptomatic
85	10/09/1973	Positive	Positive	Positive	Symptomatic
86	27/04/1980	Negative	Negative	Negative	Asymptomatic
87	18/01/1981	Negative	Negative	Negative	Symptomatic
88	09/11/1979	Negative	Negative	Negative	Asymptomatic
89	15/11/1967	Negative	Negative	Negative	Asymptomatic
90	09/11/1977	Negative	Negative	Negative	Asymptomatic
91	23/04/1975	Positive	Positive	Negative	Asymptomatic
92	05/01/1973	Negative	Negative	Negative	Asymptomatic
93	03/03/1965	Negative	Negative	Negative	Asymptomatic
94	14/11/1978	Negative	Negative	Negative	Asymptomatic
95	29/08/1981	Negative	Negative	Negative	Asymptomatic
96	01/09/1968	Negative	Positive	Negative	Asymptomatic
97	15/04/1970	Negative	Negative	Negative	Asymptomatic
98	03/08/1976	Negative	Negative	Negative	Asymptomatic
99	19/08/1973	Positive	Positive	Negative	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued…...)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
100	16/10/1984	Positive	Positive	Positive	Symptomatic
102	26/05/1982	Negative	Negative	Negative	Asymptomatic
102	12/06/1967	Negative	Negative	Negative	Asymptomatic
103	25/03/1979	Positive	Positive	Negative	Asymptomatic
104	11/01/1979	Positive	Positive	Negative	Asymptomatic
105	07/01/1975	Negative	Negative	Negative	Symptomatic
106	01/07/1980	Negative	Negative	Negative	Asymptomatic
107	28/11/1959	Negative	Negative	Negative	Asymptomatic
108	25/02/1982	Positive	Positive	Positive	Asymptomatic
109	12/06/1967	Negative	Negative	Negative	Asymptomatic
110	25/03/1979	Positive	Positive	Negative	Asymptomatic
111	11/01/1979	Positive	Positive	Negative	Asymptomatic
112	07/01/1975	Positive	Positive	Negative	Asymptomatic
113	01/07/1980	Negative	Negative	Negative	Asymptomatic
114	28/11/1959	Negative	Negative	Negative	Asymptomatic
115	22/05/1981	Positive	Positive	Negative	Asymptomatic
116	19/09/1983	Negative	Negative	Negative	Asymptomatic
117	10/01/1984	Negative	Negative	Negative	Symptomatic
118	03/03/1975	Negative	Negative	Negative	Asymptomatic
119	28/07/1969	Negative	Negative	Negative	Symptomatic
120	25/06/1975	Positive	Positive	Negative	Asymptomatic
121	07/10/1957	Negative	Negative	Negative	Asymptomatic
122	15/01/1962	Negative	Negative	Negative	Asymptomatic
123	19/12/1971	Positive	Positive	Positive	Symptomatic
124	11/02/1978	Positive	Positive	Negative	Asymptomatic
125	28/04/1972	Positive	Positive	Negative	Asymptomatic
126	16/06/1971	Positive	Positive	Positive	Symptomatic
127	06/02/1972	Positive	Positive	Negative	Asymptomatic
128	08/08/1963	Negative	Negative	Negative	Asymptomatic
129	07/04/1971	Negative	Negative	Negative	Asymptomatic
130	24/01/1978	Positive	Positive	Negative	Symptomatic
131	11/02/1978	Negative	Negative	Negative	Asymptomatic
132	03/03/1983	Positive	Positive	Negative	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued……)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
133	15/09/1982	Negative	Negative	Negative	Asymptomatic
134	06/05/1973	Negative	Negative	Negative	Asymptomatic
135	04/01/1982	Positive	Positive	Positive	Symptomatic
136	25/09/1972	Negative	Negative	Negative	Negative
137	31/01/1961	Negative	Negative	Negative	Asymptomatic
138	15/05/1965	Positive	Positive	Negative	Asymptomatic
139	14/08/1980	Positive	Positive	Negative	Asymptomatic
140	25/09/1972	Negative	Negative	Negative	Asymptomatic
141	04/09/1985	Positive	Positive	Negative	Asymptomatic
142	15/03/1963	Positive	Positive	Negative	Asymptomatic
143	06/05/1973	Positive	Positive	Negative	Asymptomatic
144	03/03/1983	Positive	Positive	Negative	Asymptomatic
145	28/07/1969	Positive	Positive	Positive	Asymptomatic
146	06/11/1979	Negative	Negative	Negative	Asymptomatic
147	24/01/1978	Positive	Positive	Negative	Symptomatic
148	30/10/1974	Negative	Negative	Negative	Asymptomatic
149	08/01/1976	Negative	Negative	Negative	Asymptomatic
150	10/09/1987	Negative	Negative	Negative	Asymptomatic
151	25/01/1971	Negative	Negative	Negative	Symptomatic
152	15/03/1974	Negative	Positive	Negative	Asymptomatic
153	07/04/1976	Negative	Negative	Negative	Asymptomatic
154	08/11/1975	Negative	Negative	Negative	Symptomatic
155	29/05/1979	Negative	Negative	Negative	Asymptomatic
156	26/12/1987	Negative	Negative	Negative	Asymptomatic
157	03/01/1975	Negative	Negative	Negative	Asymptomatic
158	17/05/1972	Negative	Negative	Negative	Asymptomatic
159	01/01/1969	Negative	Negative	Negative	Asymptomatic
160	07/01/1983	Negative	Negative	Negative	Asymptomatic
161	04/10/1983	Negative	Negative	Negative	Asymptomatic
162	10/12/1972	Positive	Positive	Positive	Asymptomatic
163	25/08/1969	Negative	Negative	Negative	Symptomatic
164	08/06/1987	Negative	Negative	Negative	Asymptomatic
165	31/12/1988	Negative	Negative	Negative	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued……)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
166	10/09/1982	Negative	Positive	Negative	Asymptomatic
167	14/07/1989	Negative	Negative	Negative	Asymptomatic
168	05/08/1975	Negative	Negative	Negative	Asymptomatic
169	08/04/1974	Negative	Negative	Negative	Asymptomatic
170	12/07/1967	Negative	Negative	Negative	Asymptomatic
171	10/04/1963	Positive	Positive	Negative	Symptomatic
172	26/06/1974	Negative	Negative	Negative	Asymptomatic
173	15/03/1969	Negative	Negative	Negative	Asymptomatic
174	01/07/1962	Negative	Negative	Negative	Asymptomatic
175	05/03/1980	Negative	Negative	Negative	Asymptomatic
176	21/05/1974	Positive	Positive	Positive	Asymptomatic
177	06/12/19757	Positive	Positive	Negative	Asymptomatic
178	03/07/1974	Negative	Negative	Negative	Asymptomatic
179	02/02/1961	Negative	Negative	Negative	Asymptomatic
180	21/10/1970	Positive	Positive	Negative	Asymptomatic
181	09/09/1974	Negative	Negative	Negative	Asymptomatic
182	27/03/1978	Negative	Negative	Negative	Asymptomatic
183	09/08/1978	Negative	Negative	Negative	Asymptomatic
184	30/03/1977	Negative	Negative	Negative	Asymptomatic
185	02/09/1963	Positive	Positive	Positive	Symptomatic
186	10/05/1982	Negative	Negative	Negative	Asymptomatic
187	28/02/1978	Negative	Negative	Negative	Asymptomatic
188	27/12/1987	Negative	Negative	Negative	Asymptomatic
189	22/01/1957	Negative	Negative	Negative	Asymptomatic
190	14/01/1975	Negative	Negative	Negative	Asymptomatic
191	08/06/1968	Negative	Positive	Negative	Asymptomatic
192	02/10/1976	Negative	Negative	Negative	Asymptomatic
193	08/01/1976	Negative	Negative	Negative	Asymptomatic
194	12/07/1967	Positive	Positive	Negative	Asymptomatic
195	10/09/1987	Negative	Negative	Negative	Asymptomatic
196	08/11/1975	Negative	Negative	Negative	Asymptomatic
197	15/03/1975	Negative	Negative	Negative	Asymptomatic
198	01/07/1962	Negative	Negative	Negative	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued…...)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
199	26/06/1974	Negative	Negative	Negative	Asymptomatic
200	07/04/1976	Negative	Negative	Negative	Asymptomatic
201	05/03/1980	Negative	Negative	Negative	Asymptomatic
202	18/08/1980	Negative	Negative	Negative	Asymptomatic
203	01/01/1973	Positive	Positive	Positive	Asymptomatic
204	06/12/1975	Negative	Negative	Negative	Asymptomatic
205	21/05/1974	Negative	Negative	Negative	Asymptomatic
206	08/05/1974	Negative	Positive	Negative	Asymptomatic
207	31/12/1980	Negative	Negative	Negative	Asymptomatic
208	02/04/1982	Positive	Positive	Negative	Asymptomatic
209	25/11/1974	Negative	Negative	Negative	Asymptomatic
210	25/01/1971	Negative	Negative	Negative	Asymptomatic
211	10/04/1963	Positive	Positive	Negative	Asymptomatic
212	14/07/1989	Negative	Negative	Negative	Asymptomatic
213	05/08/1975	Negative	Negative	Negative	Asymptomatic
214	30/10/1974	Negative	Negative	Negative	Asymptomatic
215	15/03/1969	Negative	Negative	Negative	Asymptomatic
216	10/12/1972	Positive	Positive	Positive	Symptomatic
217	25/08/1964	Positive	Positive	Negative	Asymptomatic
218	03/12/1988	Negative	Negative	Negative	Asymptomatic
219	08/06/1987	Positive	Positive	Negative	Asymptomatic
220	01/11/1969	Negative	Negative	Negative	Asymptomatic
221	29/05/1974	Negative	Negative	Negative	Asymptomatic
222	17/05/1972	Positive	Positive	Positive	Asymptomatic
223	03/01/1975	Negative	Positive	Negative	Asymptomatic
224	10/04/1983	Negative	Negative	Negative	Asymptomatic
225	26/12/1987	Positive	Positive	Negative	Asymptomatic
226	30/03/1977	Negative	Negative	Negative	Asymptomatic
227	27/03/1978	Negative	Negative	Negative	Symptomatic
228	03/07/1974	Negative	Negative	Negative	Asymptomatic
229	09/09/1974	Negative	Negative	Negative	Asymptomatic
230	09/08/1974	Negative	Positive	Negative	Asymptomatic
231	09/08/1978	Negative	Negative	Negative	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued……)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
232	10/05/1982	Negative	Negative	Negative	Asymptomatic
233	28/02/1978	Negative	Negative	Negative	Asymptomatic
234	27/12/1987	Negative	Negative	Negative	Asymptomatic
235	02/09/1963	Negative	Negative	Negative	Asymptomatic
236	02/02/1961	Negative	Negative	Negative	Symptomatic
237	22/08/1987	Negative	Negative	Negative	Asymptomatic
238	21/10/1970	Negative	Negative	Negative	Asymptomatic
239	10/04/1985	Negative	Negative	Negative	Asymptomatic
240	08/06/1968	Positive	Positive	Positive	Symptomatic
241	22/01/1957	Negative	Negative	Negative	Asymptomatic
242	08/06/1968	Negative	Negative	Negative	Symptomatic
243	14/01/1975	Negative	Positive	Negative	Asymptomatic
244	16/03/1977	Negative	Negative	Negative	Asymptomatic
245	01/01/1961	Positive	Positive	Positive	Asymptomatic
246	11/03/1972	Negative	Negative	Negative	Asymptomatic
247	20/10/1982	Negative	Negative	Negative	Asymptomatic
248	12/09/1972	Positive	Positive	Negative	Symptomatic
249	17/09/1982	Negative	Negative	Negative	Asymptomatic
250	15/06/1981	Negative	Negative	Negative	Symptomatic
251	18/03/1981	Negative	Negative	Negative	Asymptomatic
252	21/05/1984	Negative	Negative	Negative	Asymptomatic
253	30/07/1984	Positive	Positive	Negative	Asymptomatic
254	12/06/1973	Positive	Positive	Positive	Asymptomatic
255	19/06/1976	Positive	Positive	Negative	Asymptomatic
256	11/08/1969	Negative	Negative	Negative	Asymptomatic
257	17/10/1981	Negative	Negative	Negative	Asymptomatic
258	22/03/1976	Negative	Negative	Negative	Asymptomatic
259	15/08/1971	Negative	Negative	Negative	Symptomatic
260	19/11/1972	Positive	Positive	Positive	Symptomatic
261	02/12/1958	Negative	Negative	Negative	Asymptomatic
262	05/10/1981	Negative	Negative	Negative	Asymptomatic
263	11/12/1973	Negative	Negative	Negative	Asymptomatic
264	23/03/1969	Positive	Positive	Positive	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued…...)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
265	20/01/1980	Negative	Negative	Negative	Asymptomatic
266	02/02/1968	Negative	Negative	Negative	Asymptomatic
267	17/11/1962	Negative	Negative	Negative	Asymptomatic
268	31/12/1980	Positive	Positive	Negative	Asymptomatic
269	12/12/1983	Negative	Negative	Negative	Asymptomatic
270	09/01/1974	Negative	Negative	Negative	Symptomatic
271	02/12/1980	Positive	Positive	Positive	Symptomatic
272	03/01/1970	Negative	Negative	Negative	Asymptomatic
273	24/12/1978	Negative	Negative	Negative	Asymptomatic
274	12/06/1981	Negative	Negative	Negative	Asymptomatic
275	19/11/1981	Positive	Positive	Negative	Asymptomatic
276	01/03/1961	Positive	Positive	Negative	Asymptomatic
277	27/09/1975	Positive	Positive	Positive	Symptomatic
278	26/02/1976	Negative	Negative	Negative	Asymptomatic
279	06/11/1976	Negative	Negative	Negative	Asymptomatic
280	05/15/1960	Negative	Positive	Negative	Asymptomatic
281	22/08/1977	Negative	Negative	Negative	Asymptomatic
282	06/02/1971	Negative	Negative	Negative	Asymptomatic
283	11/11/1951	Negative	Negative	Negative	Asymptomatic
284	11/05/1968	Negative	Negative	Negative	Asymptomatic
285	03/11/1981	Positive	Positive	Positive	Symptomatic
286	05/01/1969	Negative	Positive	Negative	Asymptomatic
287	18/09/1961	Negative	Negative	Negative	Asymptomatic
288	31/01/1973	Negative	Negative	Negative	Asymptomatic
289	05/05/1973	Negative	Negative	Negative	Asymptomatic
290	26/01/1981	Negative	Negative	Negative	Asymptomatic
291	25/08/1976	Negative	Negative	Negative	Asymptomatic
292	15/03/1980	Negative	Negative	Negative	Symptomatic
293	10/07/1978	Positive	Positive	Negative	Asymptomatic
294	07/10/1972	Negative	Negative	Negative	Asymptomatic
295	30/09/1983	Positive	Positive	Negative	Asymptomatic
296	23/08/1979	Negative	Negative	Negative	Asymptomatic
297	11/05/1969	Negative	Negative	Negative	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued……)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
298	01/04/1974	Negative	Negative	Negative	Asymptomatic
299	17/10/1989	Negative	Negative	Negative	Asymptomatic
300	27/04/1954	Negative	Negative	Negative	Asymptomatic
301	02/09/1980	Positive	Positive	Positive	Asymptomatic
302	14/11/1978	Positive	Positive	Negative	Asymptomatic
303	09/07/1965	Negative	Negative	Negative	Asymptomati
304	10/10/1976	Negative	Negative	Negative	Asymptomatic
305	06/06/1971	Positive	Positive	Negative	Symptomatic
306	23/07/1957	Negative	Negative	Negative	Asymptomatic
307	21/12/1974	Negative	Negative	Negative	Asymptomatic
308	03/04/1957	Negative	Negative	Negative	Asymptomatic
309	12/11/1984	Negative	Negative	Negative	Asymptomatic
310	02/08/1960	Positive	Positive	Positive	Symptomatic
311	06/07/1973	Negative	Positive	Negative	Asymptomatic
312	12/07/1974	Positive	Negative	Negative	Asymptomatic
313	01/08/1970	Negative	Negative	Negative	Asymptomatic
314	28/01/1963	Positive	Positive	Negative	Asymptomatic
315	16/11/1981	Negative	Positive	Negative	Asymptomatic
316	30/07/1985	Negative	Negative	Negative	Asymptomatic
317	10/11/1968	Negative	Negative	Negative	Asymptomatic
318	06/06/1969	Negative	Negative	Negative	Asymptomatic
319	24/01/1978	Positive	Positive	Negative	Asymptomatic
320	23/06/1979	Negative	Negative	Negative	Asymptomatic
321	03/05/1965	Negative	Negative	Negative	Asymptomatic
322	17/12/1973	Positive	Positive	Negative	Asymptomatic
323	19/10/1962	Negative	Negative	Negative	Asymptomatic
324	09/07/1981	Negative	Negative	Negative	Asymptomatic
325	24/08/1977	Negative	Negative	Negative	Asymptomatic
326	10/09/2010	Negative	Negative	Negative	Asymptomatic
327	24/04/1982	Negative	Negative	Negative	Asymptomatic
328	30/08/1981	Negative	Negative	Negative	Asymptomatic
329	13/06/1970	Negative	Negative	Negative	Symptomatic
330	15/03/1977	Negative	Positive	Negative	Asymptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued….)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
331	15/06/1979	Negative	Negative	Negative	Asymptomatic
332	31/03/1962	Negative	Negative	Negative	Asymptomatic
333	25/01/1966	Positive	Positive	Negative	Symptomatic
334	15/10/1977	Negative	Negative	Negative	Asymptomatic
335	16/08/1953	Positive	Positive	Negative	Asymptomatic
336	03/02/1977	Positive	Positive	Negative	Asymptomatic
337	22/12/1982	Negative	Negative	Negative	Asymptomatic
338	14/12/1954	Positive	Positive	Positive	Symptomatic
339	15/04/1963	Negative	Negative	Negative	Asymptomatic
340	129/02/1984	Negative	Negative	Negative	Asymptomatic
341	05/06/1988	Positive	Positive	Negative	Symptomatic
342	11/05/1983	Positive	Positive	Negative	Asymptomatic
343	28/03/1980	Negative	Negative	Negative	Asymptomatic
344	27/02/1988	Negative	Negative	Negative	Symptomatic
345	02/10/1964	Negative	Negative	Negative	Asymptomatic
346	07/02/1966	Negative	Negative	Negative	Asymptomatic
347	25/08/1989	Positive	Positive	Negative	Asymptomatic
348	21/12/1975	Negative	Negative	Negative	Asymptomatic
349	12/04/1986	Negative	Negative	Negative	Asymptomatic
350	08/06/1978	Negative	Negative	Negative	Asymptomatic
351	22/04/1956	Negative	Negative	Negative	Asymptomatic
352	08/05/1969	Negative	Negative	Negative	Asymptomatic
353	14/01/1967	Positive	Positive	Negative	Symptomatic
354	16/05/1978	Positive	Positive	Negative	Symptomatic
355	10/02/1961	Negative	Negative	Negative	Asymptomatic
356	11/04/1973	Positive	Positive	Negative	Asymptomatic
357	21/11/1981	Negative	Negative	Negative	Asymptomatic
358	11/11/1974	Negative	Positive	Negative	Asymptomatic
359	16/09/1985	Negative	Negative	Negative	Asymptomatic
360	17/09/1981	Negative	Positive	Negative	Asymptomatic
361	19/05/1983	Negative	Negative	Negative	Asymptomatic
362	20/05/1986	Negative	Negative	Negative	Asymptomatic
363	29/06/1983	Negative	Negative	Negative	Symptomatic

Table C4: List of asymptomatic and symptomatic female participants and the results of the diagnostic assays (Continued….)

Specimen number	Date of Birth	Neg/Pos (microscopy)	Neg/Pos (culture)	Neg/Pos (PCR)	AS/S
364	16/08/1953	Positive	Positive	Negative	Asymptomatic
365	03/02/1977	Positive	Positive	Negative	Asymptomatic
366	22/12/1982	Negative	Negative	Negative	Asymptomatic
367	14/12/1954	Positive	Positive	Positive	Symptomatic
368	15/04/1963	Negative	Negative	Negative	Asymptomatic
369	129/02/1984	Negative	Negative	Negative	Asymptomatic
370	05/06/1988	Positive	Positive	Negative	Symptomatic
371	11/05/1983	Positive	Positive	Negative	Asymptomatic
372	28/03/1980	Negative	Negative	Negative	Asymptomatic
373	27/02/1988	Negative	Negative	Negative	Symptomatic
374	02/10/1964	Negative	Negative	Negative	Asymptomatic
375	07/02/1966	Negative	Negative	Negative	Asymptomatic
376	25/08/1989	Positive	Positive	Negative	Asymptomatic
377	21/12/1975	Negative	Negative	Negative	Asymptomatic
378	12/04/1986	Negative	Negative	Negative	Asymptomatic
379	08/06/1978	Negative	Negative	Negative	Asymptomatic
380	22/04/1956	Negative	Negative	Negative	Asymptomatic

A/AS **Asmptomatic/Symptomatic**

Neg/Pos **Negative/Positive specimen**