ANTIMICROBIAL SUSCEPTIBILITY TESTING OF FOUR 5-NITROIMIDAZOLES AGAINST *TRICHOMONAS VAGINALIS*

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PREFACE

The experimental work described in this dissertation was carried in the Infection Prevention and Control Laboratory, Doris Duke Medical Research Institute Building, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, South Africa under the supervision of Dr Bronwyn Joubert.

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

15 March 2016

Andile Mtshali (candidate) Date

15 March 2016

Dr Bronwyn C. Joubert (supervisor) Date

PLAGIARISM DECLARATION

I, **Andile Mtshali** declare as follows:

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ETHICAL APPROVAL

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal (REF: BE220/13).

CONFERENCE PRESENTATIONS

Mtshali AN, Sturm AW, Moodley P and Joubert BC. 2015. Antimicrobial susceptibility testing of four 5-nitroimidazoles against *Trichomonas vaginalis*

- Oral presentation at the College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa (09-10 September 2015)

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ABSTRACT

Background

Trichomonas vaginalis, the causative agent of trichomoniasis, is the most common non-viral sexually transmitted pathogen. It causes vaginal discharge in females and urethritis in males. Trichomoniasis is treated with metronidazole, an antimicrobial agent which belongs to the 5-nitroimidazole family. If left untreated, it causes serious clinical complications including facilitating HIV transmission. South Africa is one of the regions where HIV is rife and the prevalence of *T. vaginalis* is high. No recent studies have evaluated the minimum inhibitory concentrations (MIC) or minimum lethal concentration (MLC) of *T. vaginalis* to the current chemotherapy, metronidazole, or looked for alternatives. In this study we tested the susceptibility *T. vaginalis* isolates to four 5-nitroimidazoles, including metronidazole.

Methodology

Vaginal specimens were collected from women presenting with vaginal discharge syndrome at two different clinics in KwaZulu-Natal, South Africa and cultured in modified Diamonds medium. The MIC and MLC of *T. vaginalis* to four 5-nitroimidazoles was determined in 94 positive clinical isolates using a micro-broth dilution method. Briefly trichomonads were added to Diamonds media containing two-fold dilutions (16 to 0.25 mg/L) of metronidazole, tinidazole, ornidazole or secnidazole and incubated anaerobically at 37°C for 72 hours. The lowest concentration which inhibited trichomonad growth was considered the MIC while the lowest concentration at which no motile trichomonads were detected was considered the MLC. *Propionibacterium acnes* and *Bacteroides fragilis* were used as the resistant and

sensitive controls respectively. The test was repeated for any isolate with MIC > 2 mg/L to confirm results.

Results

From the 617 specimens collected, 106 (17%) were positive for *T. vaginalis*, but only 94 could be cultured and were used in this study. Ten, 2, and 3 isolates had a MIC of > 2 mg/L after 48 hours while 12, 1, and 2 isolates had a MIC > 2 mg/L after 72 hours of incubation for metronidazole, tinidazole and secnidazole respectively. No high MIC was detected for ornidazole. Eighteen, 3, 3 and 2 isolates had a MLC of > 2mg/L after 48 hours while 16, 2, 3 and 2 had a MLC > 2mg/L for metronidazole, tinidazole, secnidazole and ornidazole after 72 hours of incubation. Of the 12 isolates which had a high MIC for metronidazole, 4 also had a high MIC for at least 1 other drug tested but no isolates had a MIC > 2mg/L for all drugs tested. Of the 18 isolates which had a high MLC for metronidazole, 7 also had a high MLC for at least 1 other drug tested but no isolates had a MIC > 2mg/L for all drugs tested. MIC and MLC was usually the same when read after 48 or 72 hours, but some isolates had a lower MIC / MLC after 72 hours, while others had a higher MIC / MLC after 72 hours.

Conclusions

All four of the 5-nitroimidazoles tested have an inhibitory effect on *T. vaginalis* isolates from KwaZulu-Natal. Metronidazole showed the poorest *in vitro* activity and ornidazole showed the best *in vitro* activity against *T. vaginalis*. Isolates which had a MIC or MLC > 2 mg/L for metronidazole had a low MIC or MLC to at least one other drug. Further research is required to correlate *in vitro* findings with clinical outcome.

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ABBREVIATIONS

°C	degrees Celsius
µl	microlitre
µM	micromolar
ATCC	American Type Culture Collection
bp	base pairs
CDC	Centers for Diseases Control and Prevention
Cmax	maximum concentration
CO_2	carbon dioxide
CLSI	Clinical Laboratory Standards Institute
DF	drug-free
DNA	deoxyribonucleic acid
DMSO	dimethyl sulphoxide
e.g	for example
EDTA	Ethylenediaminetetraacetic acid
EUCAST	European Union Committee on Antimicrobial Susceptibility
FDA	Food and Drug Administration
FBS	foetal bovine serum
h	hour

HIV	human immunodeficiency virus
IMI	intramuscular injection
L	litre
mg	milligram
MIC	minimal inhibitory concentration
MIC_{90}	MIC required to inhibit the growth of 90% of organisms
Min	minute
ml	millilitre
MLC	minimal lethal concentration
MLC_{50}	MLC required to kill 50% of organisms
MTZ	metronidazole
NAATs	nucleic acid amplification tests
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NADH:FOR	nicotinamide adenine dinucleotide: ferredoxin oxidoreductase
NICD	National Institute for Communicable Diseases
ORN	ornidazole
PCR	polymerase chain reaction
PFOR	pyruvate:ferredoxin oxidoreductase

RNA	ribonucleic acid
SA	South Africa
SNZ	secnidazole
STD	sexually transmitted disease
STI	sexually transmitted infection
T	temperature
TMA	transcription mediated amplification
TVV	*Trichomonas vaginalis* virus
TZN	tinidazole
UK	United Kingdom
USA	United States of America
WHO	World Health Organisation

CHAPTER 1 – INTRODUCTION

Trichomoniasis, which is caused by the protozoan *Trichomonas vaginalis*, is the most common, non-viral sexually transmitted infection (STI) [1]. According to the World Health Organization (WHO), approximately 276.4 million new cases occur annually worldwide and Sub-Saharan Africa accounts for approximately 42.8 million of these cases [2].

The parasite can infect both the female and the male genital tract [3]. In females it causes vaginal discharge, irritation and discomfort and may lead to adverse pregnancy outcome in untreated pregnant women [3, 4]. In males it can cause urethral discharge but is often asymptomatic [3, 4]. Trichomoniasis is also of great concern because *T. vaginalis* infection is associated with increased risk of acquiring HIV [5].

Trichomoniasis is usually treated with metronidazole. Although there are seven compounds in the 5-nitroimidazole family, only metronidazole and tinidazole are approved for the treatment of trichomoniasis by the US Food and Drug Administration (FDA) [6]. There are no other oral drugs that are known to be effective against *T. vaginalis* other than the 5-nitroimidazoles [6] although there are some research groups working on this [7-9]. For this reason if *T. vaginalis* resistance to the 5-nitroimidazoles were to develop, trichomoniasis caused by the resistant isolates would be untreatable.

T. vaginalis resistance to metronidazole has already been reported in several countries [4, 10-12] and failure of metronidazole to cure trichomoniasis will be cause for concern in a resource limited country like South Africa. The other FDA approved 5-nitroimidazole, tinidazole, is more expensive than metronidazole which is the first choice for treatment. Refractory cases in South Africa are usually treated with increased metronidazole doses, but this leads to more severe side effects [13].

In South Africa, sexually transmitted diseases (STD) such as trichomoniasis are managed syndromically [14-16]. This means that a cocktail of drugs is administered to any patients who present with a particular syndrome without identifying the causative agent or performing susceptibility tests [14-16]. For a system such as this to remain effective, surveillance needs to take place periodically to identify changes in the organisms that are circulating in the population and identify resistance to the drugs used to treat those organisms. No such surveillance has taken place recently in KwaZulu-Natal for *T. vaginalis*, especially with regards to antimicrobial agents.

In this study, we address this problem by collecting fresh isolates of *T. vaginalis* from women presenting with vaginal discharge at two clinics located in KwaZulu-Natal and determining the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of four 5-nitroimidazoles: metronidazole, tinidazole, ornidazole and secnidazole. We used metronidazole because it is the first line treatment for trichomoniasis, tinidazole because it is the only other antimicrobial approved by the FDA for the treatment of trichomoniasis, and ornidazole and secnidazole to assess whether or not trichomonads with a high MIC or MLC to metronizole or tinidazole will also have a high MIC or MLC to other 5-nitroimidazoles.

In order to do this we used a micro-broth dilution test which is the most widely used method for *in vitro* determination of MIC for *T. vaginalis*.

The aim of this study was to:

- Compare the antimicrobial activity of four 5- nitroimidazoles on *T. vaginalis* isolates collected from women with vaginal discharge in KwaZulu Natal.

The objectives of this study were to

- Determine the MIC and MLC of metronidazole, tinidazole, secnidazole and ornidazole on clinical isolates of *T. vaginalis* under anaerobic conditions after 48 and 72 hours of incubation at 37°C
- Determine whether or not isolates which had a high MIC or MLC to metronidazole also had a MIC to other 5-nitroimidazoles

CHAPTER 2 – LITERATURE REVIEW

2.1 *Trichomonas vaginalis*

2.1.1 Global epidemiology of *T. vaginalis*

Trichomonas vaginalis is a flagellated protozoan which causes human trichomoniasis, the most common non-viral sexually transmitted disease (STD) in the world [15, 17-19].According to the World Health Organisation (WHO), over 248 million people are infected with this organism worldwide [20] and it is found in all age, race and socioeconomic groups [19]. There is a lack of data describing *T. vaginalis* incidence and prevalence in the general population despite the high prevalence of sexually transmitted infections (STI's) globally [18]. However the WHO has generated regional and global estimates of *T. vaginalis*. Sub-Saharan Africa is one of the regions most affected by trichomoniasis following South East Asia. Approximately 32 [2] and 42.8 million people [1] were infected with trichomoniasis in Sub-Saharan Africa in the 1990's and 2008 respectively.

2.1.2 Epidemiology in South Africa

In South Africa, STD's are a public health concern due to high prevalence and incidence [14]. There are several factors that are believed to have contributed to this epidemic: the migrant labour system, poor quality of health services and socio-economic and gender inequalities [14, 17]. Currently, there is insufficient data on the epidemiology [18] and magnitude of clinical resistance of important sexually transmitted pathogens in South Africa [14]. Most surveillance studies are conducted in certain regions with different communities

and this may not be representative of the broader population. However, it provides some insight regarding the distribution of organisms present in that region [21].

In a study conducted in a primary health care clinic in Johannesburg in the Gauteng province of South Africa, the occurrence of *T. vaginalis* amongst women presenting with vaginal discharge syndrome was 31.6% in 2013 and 16.1% in 2014 [22]. In males presenting at the same clinic with urethral discharge syndrome, *T. vaginalis* accounted for 4.5% and 3.1% in 2013 and 2014, respectively [22]. In KwaZulu-Natal, *T. vaginalis* was detected in 37% [23] and 20.3% of the high risk HIV negative women with vaginal discharge who presented at primary health care clinics located at KwaMsane and Durban respectively [24]. However, these data may not represent the true prevalence since it was collected from selected population groups that may not be representative of the total population.

In the KZN Provincial Report - Multi-Sectoral Provincial Strategic Plan for HIV and AIDS, STIs and TB 2012-2016 for KwaZulu-Natal, the KwaZulu-Natal Department of Health, reports over 440,000 new cases of trichomoniasis in KwaZulu-Natal in 2010/2011 [25]. Controlling the burden of sexually transmitted infections (STI's) in South Africa has become a priority for the country due to the remarkably high rates of HIV infection [26] and it is one of the tactics for HIV control encouraged by the national Department of Health.

2.1.3 Biology of the organism

2.1.3.1 Structure

T. vaginalis is a flagellated, amitochondrial, micro aerotolerant protozoan which is known to exist in several forms [26, 27]. As shown in figure 1, the organism changes from one form to another in response to different stimuli [28]. The trophozoite and the amoeboid forms are the best characterised [27].

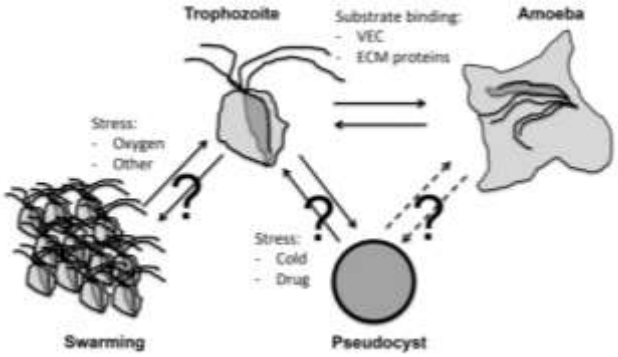


Figure 2.1: Different cellular forms of *T. vaginalis* [30]

T. vaginalis swim freely in the trophozoite form which is considered the infective form. Exposure of trophozoites to cold and other types of stress can induce pseudocyst formation *in vitro* [29, 30] but the importance of this form during infection is unknown [31]. Transformation to the amoeboid form is induced upon attachment to epithelial cells of the urinary or reproductive tract but when they detach they revert back to trophozoites [28, 32]. Tropozoites are able to swarm to produce large cellular aggregates [28]. It is thought that these aggregates allow stronger binding and increased cytotoxicity towards host tissues and protection of the organism from the host's immune responses [33]. It is known to adhere to the squamous epithelium of the genital tract by means of lectin-like adhesins in order to

establish infection [34, 35]. Adherence of the organism to epithelial cells is mediated by adhesin proteins [35, 36] but adherence is also time, temperature and pH dependent [37]. However, some data shows that *T. vaginalis* can also bind to several cell lines (HeLa, CHO and Vero) from different species to the same extent, suggesting that the binding is not only mediated by a specific receptor on the host cells, but rather to the affinity for other structures such as the membrane [34] or surface carbohydrates [38]. *T. vaginalis* adhesins are most abundant on the side opposite the undulating membrane [3, 39]. Each trophozoite contains four anterior flagella plus a single flagellum which is integrated into the undulating membrane. The undulating membrane is supported by non-contractile costa [32, 40]. The cell body is bisected longitudinally by a thin transparent structure called the axostyle which extends at the posterior end [27, 39].

2.1.3.2 Metabolism

T. vaginalis is an obligate parasite and is unable to synthesize many complex essential nutrients such as carbohydrates, iron, vitamins, fatty acids, purines, pyrimidines and lipids [3]. It therefore acquires these molecules from the vaginal secretions [3, 41]. Since these nutrients are essential for *T. vaginalis* growth, they must be incorporated into the culture media for *in vitro* growth [3]. *T. vaginalis* does not have mitochondria [42]. Instead they have hydrogenosomes [27] which are also capable of metabolising carbohydrates [42]. Hydrogenosomes differ from mitochondria in that they lack cytochromes, mitochondrial respiratory chain enzymes and a genome [42-45]. Three other proteins, pyruvate: ferredoxin oxidoreductase (PFOR) (an enzyme absent in mitochondria) [3], ferredoxin and Fe-hydrogenase facilitate the metabolism of pyruvate in *T. vaginalis* [3, 42, 46]. Metabolism in the hydrogenosome resembles that in anaerobic bacteria, however studies on ferredoxin

protein in *T. vaginalis* indicated similarity to the 2Fe-2S ferredoxins present in aerobic bacteria and in mitochondria [3, 47].

2.1.3.3 Replication

T. vaginalis reproduces in the vagina, prostatic secretions and urine and multiplies either via longitudinal binary fission or another form of asexual reproduction in which up to 8 progeny are produced [48]. Replication begins with the doubling of selected locomotor organelles. Attractophores then develop on the sides of the nucleus leading to the formation of poles for division [49]. Chromosomal microtubules are formed from the attractophores and extend towards the nucleus and attach to the centromeres of the chromosomes. The paradesmose is attached to the attractophores and elongate, resulting in the separation of daughter cells. The production of other organelles to complete the cell content occurs in each daughter cell. [49]. Trophozoites in the urinary and reproductive tract live until passed to the next host via unprotected sexual intercourse [50].

2.2 Classification of *T. vaginalis*

T. vaginalis is sub-divided into two types based on the phylogenic sequencing of the full genome, type 1 and type 2 [51, 52]. These types are thought to be associated with the clinical relevance of this organism. Type 1 *T. vaginalis* isolates are usually infected with *Trichomonas vaginalis* virus (TVV), a virus belong to the Totiviriadae family [51, 53] and they usually account for about 50% of all isolates. TVV is not known to replicate in the human host in the absence of *T. vaginalis*. However, the shedding of viral genome, or gene products following *T. vaginalis* lysis by antimicrobial agents can be recognised as foreign by

cells of the human immune system [54]. This leads to the activation of a pro-inflammatory response and phagocytosis of the virions [54]. *T. vaginalis* isolates infected with TVV are usually susceptible to metronidazole [54].

Type 2 isolates are associated with higher resistance to metronidazole. A study by Conrad *et al* 2012 [51] indicated a higher mean minimal lethal concentration (MLC) (224 µg/ml) among type 2 isolates compared to 76.6 µg/ml with type 1 isolates when incubated under aerobic conditions [51]. This study also categorised the global distribution of the two types of *T. vaginalis* isolates in different regions (Eastern USA, Western USA, Mexico, Chile, Italy, Southern Africa and Mozambique, Australia, Paupa New Guinea, and India) [51]. It was found that the two types were equally distributed in most regions with an exception of Mexico where only type 2 isolates were found and Southern Africa where mostly type 1 isolates were found [51].

2.3 Clinical Manifestations and Complications of *T. vaginalis*

Trichomoniasis has a wide variety of clinical presentations in men and women [3]. Severity of infection may be acute, chronic or asymptomatic [3]. In the case of acute infection, the signs include off-white or yellow, frothy discharge and erythema of vulva and cervix and the symptoms include vaginal discharge, itch and dysuria [12, 55]. In chronic infection, major symptoms are usually mild, and vaginal secretions become scanty and mixed with mucus. At this stage, the parasite is highly transmittable [3]. Once the infection is established, it persists longer in females than it does in males. In women, it causes vaginitis and cervicitis [55]. In men it can cause urethritis but is often asymptomatic [56]. These asymptomatic infections are usually untreated and thus contribute to the spread of the disease because “many people unknowingly harbour the parasite and act as carriers spreading the infection in their

community" [12]. It is unclear why some infections are symptomatic and others are asymptomatic, but it has been proposed that it may be due to different virulence properties amongst the isolates [3, 57].

STI's such as trichomoniasis also affect reproductive health. Complications include low birth weight and preterm delivery [58]. Trichomonaisis is also associated with an increase in HIV-1 acquisition [5]. This has been reported in areas with the highest prevalence of HIV-1 especially in developing countries. *T. vaginalis* damages the epithelial cell layer of the genital tract resulting in gaps in the epithelial layer which enables HIV to enter the underlying tissues. Since genital tract infections such as *T. vaginalis* elicit an immune response, when HIV passes through the gaps in the epithelial layer it will come into contact with the target cells for HIV infection [59]. HIV then binds and enters these target cells to establish an infection. Urethral infection with *T. vaginalis* also results in the increased shedding of HIV-1 in the semen [60] and the increased numbers of HIV-infected CD4+ lymphocytes in the genital tract may also facilitate HIV-1 transmission [61].

2.4 Diagnosis of *T. vaginalis* infection

The diagnosis of both ulcerative and non-ulcerative STD's in South Africa, is made based on the signs and symptoms a patient presents with, without further laboratory diagnosis to confirm the aetiology of the disease [14, 16]. Using this approach, a number of asymptomatic cases are often missed leading to further spread of the disease because these cases remain untreated [24]. When laboratory confirmation of *T. vaginalis* infection is required, specimens are obtained from the vagina in females and the urethra in males. Wet mount, culture, antibody tests and other nucleic acid amplification tests (NAATs) are the techniques that are widely used for the diagnosis of *T. vaginalis* [62,63,64].

2.4.1 Wet Mount

Wet mount is the cheapest and most simple method of diagnosis. This is achieved by placing a drop of sterile saline solution with vaginal fluid on a glass slide, covered with a cover slip and visualized using bright field microscopy to identify motile trichomonads [65]. The sample must be kept warm for immediate viewing. This test is performed within 10-20 minutes of sample collection while the trichomonads are still motile. Low sensitivity of this test results from loss of motility after the parasite has been removed from body temperature [3, 66]. Trichomonads are about the size of peripheral blood mononuclear cells (PBMC's), but they can be distinguished from PBMC's in fresh specimens because they are motile or by the beating of flagella which may be visible even if the organism is not swimming [3]. Inappropriate temperature and keeping specimens for too long between collection and assessment may lead to loss of motility and difficulty in differentiating between trichomonas, PBMC's and the nuclei of vaginal epithelial cells present in the sample [65]. The limit for detection using this method is 10^4 trichomonads per millilitre [67]. Males often yield low trichomonad counts, therefore this technique is not recommended for urethral swab specimens collected from males [68]. This test is easy to perform and inexpensive, but has a low sensitivity ranging from 60 to 70% [50].

2.4.2 Culture

Broth culture is the gold standard for diagnosis of *T. vaginalis*. Diamond's medium is widely used for cultivation of this organism but is mainly used for research purposes. Horse, sheep or bovine serum is essential for *T. vaginalis* growth and is the source of lipids, fatty acids, amino acids and other metals in the culture medium [3]. Incubation for up to 7 days is required to confirm the absence of trichomonads, although trichomonads may be detected

earlier. Bacterial and fungal contamination can be problematic when culturing *T. vaginalis* from clinical specimens. Antimicrobial agents including antibacterial and antifungal drugs are therefore used to supplement the medium [4]. However, this is more expensive and time-consuming [3]. Commercially available liquid medium in a clear pouch can also be used for cultivation of *T. vaginalis* and it has been found to be as effective as traditional culture [69]. This method is effective with both clinician-obtained and self-sampled specimens. Specimens collected using swabs can be kept at room temperature for up to 30 minutes before inoculating the pouch [66]. In males, *T. vaginalis* is more difficult to detect by culture than in females. In order to yield better culture results in males, a urethral swab is used with the urine sediment [68]. In males, PCR is the preferred technique for detection [68].

2.4.3 Molecular techniques

Numerous molecular techniques have been developed and validated for the diagnosis of *T. vaginalis* and they've shown to have significantly greater sensitivity than culture and wet mount [70]. These include PCR (Real-time PCR and conventional), NAATs, Research transcription mediated amplification (TMA) [71-73] and BD *T. vaginalis* Q^x (TVQ) amplified DNA assay [74]. Despite their high sensitivity and specificity compared to the traditional method, they are not widely used due to cost and some are not FDA approved [64].

2.5. Syndromic management of sexually transmitted diseases (STD)

In South Africa STDs are poorly managed in both public and private health care facilities [17]. Despite efforts to improve the conditions at clinics, and health care system quality, some problems still exist, mainly due to limited resources in the public health care setting

[17]. These include incorrect treatment, partner notification failure leading to missed opportunities for treatment, and incomplete examination mainly as a result from lack of skills amongst health care workers which leads to failure in diagnosing STD [17]. Other factors that play a role include social stigma, cultural and gender issues (some infected patients feel uncomfortable reporting an STD to the health care worker and do not seek treatment), and treatment compliance [26, 64].

The WHO recommends the treatment of STD's in countries with a high prevalence by syndromic management [14]. This approach aims to reduce the load of STD's by preventing transmission because patients are treated on the same day according to the symptoms with which they present at their first point of contact with the health care system without having to wait for laboratory diagnostic tests [14, 16]. Using this approach, patients benefit by receiving immediate treatment. This approach is especially useful in developing countries such as South Africa where there are limited laboratory facilities especially in the rural areas, laboratory diagnostic tests are expensive, and those for the detection of trichomonads are not very sensitive leading to missed infections which will be left untreated [14, 15]. With the syndromic management approach, all patients presenting with a particular syndrome are treated for the major causative agents for the particular syndrome. Although this approach over treats; the benefits in a high prevalence setting outweigh the disadvantages [3]. Laboratory tests to identify the causative agent are only performed in the case of treatment failure. However, despite the implementation of syndromic management in South Africa, KwaZulu-Natal province reports a high prevalence of STD's including trichomoniasis [16, 21].

2.6 Treatment of vaginal discharge syndrome, including trichomoniasis

Due to the high prevalence in South Africa, STD's are treated based on the WHO syndromic management guidelines [15, 16]. Genital discharge syndrome in KwaZulu Natal is treated with ceftriaxone 250 mg intramuscular injection (IMI), azithromycin 1 g single dose and a 2 g single dose of metronidazole [75]. This is the modified approach adopted by the KwaZulu Natal Province Department of Health from the WHO syndromic management guidelines in 1995 [14, 16]. To prevent reinfection, the sexual partner should also be treated irrespective of the presence of symptoms. However, this is solely dependent on the willingness of the infected person to reveal the infection status to their partners and the willingness of the partner to seek medical care. Metronidazole is usually active against *T. vaginalis,* however resistance screening has not been done in our setting recently. *T. vaginalis* is one of the causative agents of genital discharge syndrome.

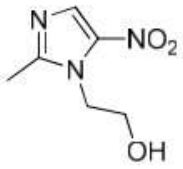
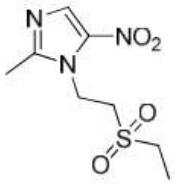
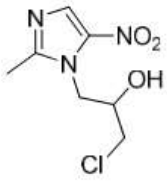
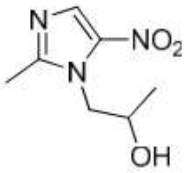
2.7 Nitroimidazoles

The current chemotherapy for trichomonaisis is metronidazole which is a 5-nitroimidazole. Metronidazole, tinidazole, secnidazole and ornidazole are some of the antimicrobials in the nitroimidazole class which have shown antimicrobial activity against trichomoniasis [76]. However, metronidazole and tinidazole are the only antimicrobials that are approved by the FDA for the treatment of trichomoniasis because these drugs have consistently demonstrated the greatest *in vitro* activity against *T. vaginalis* [11, 21]. The antimicrobial activity and pharmacokinetic differences amongst the 5-nitroimidazoles result from chemical substitution at the side chain (Table 2.7).

The mechanism of action for all 5-nitroimidazoles is similar. The drug enters the cell via passive diffusion and it is reduced into a cytotoxic metabolite(s). These metabolites damage

the trichomonad's DNA, and finally the inactive by-products are excreted in urine or faeces of the human host [76].

Table 2.7: A comparison of the properties of four 5-nitroimidazoles

Name	**Metronidazole**	**Tinidazole**	**Ornidazole**	**Secnidazole**
Structure				
Bioavailability*	40 mg/L[77]	40-58 mg/L [76]	14.48 mg/L[78]	37.7-43.6 mg/L [76]
Protein binding	<20% [13]	12% [79]	11-13% [76]	15% [76]
Half life	8.5-8.8 hours [81]	12-14 hours [79]	11-14 hours [76]	17-28.8 hours [76]
Efficacy against *T. vaginalis*	85-95% [12]	86-100% [11]	100% [80]	97% [81]
Relative cost	Cheapest [13]	Expensive [13]	Expensive	expensive
Side effects	Nausea, vomiting, abdominal pain, diarrhoea [82]	Nausea, vomiting, abdominal pain, diarrhoea [82, 83]	Nausea, fatigue, muscle pain and dizziness [81]	Nausea [80]

* 2 hours after a single oral dose of 2 g

2.7.1 Pharmacokinetics of 5-nitroimidazoles in *Trichomonas vaginalis*

There are several factors that influence the choice of antimicrobial agent. These include safety and tolerability, efficacy, cost and compliance [84]. When considering the efficacy of the drug after single dose administration, factors such as half-life, pharmacokinetics and the

peak serum level are considered [85]. The properties of the four 5-nitroimidazoles used in this study are summarised in (Table 2.7). Metronidazole, tinidazole and secnidazole reach a higher level in serum than ornidazole 2 hours after a single 2 g treatment (Table 2.7). Secnidazole has the longest half-life, followed by tinidazole and ornidazole, with metronidazole having the shortest half-life. All four are at least 85% effective against *T. vaginalis* and in a study by Hillstrom *et al* 1977, ornidazole was 100% effective [86]. A reduction in the ornidazole dosage did not affect the cure rate, but patients still reported side effects at the reduced dosage. Metronidazole, the drug of choice for the treatment of trichomoniasis is the cheapest.

2.8 Non-nitroimidazole treatment options for trichomoniasis

There are several non-nitroimidazole drugs which are being tested for their efficacy against *T. vaginalis*. These include intravaginal agents such as acetarsol [87], furazolidone [88], paromycin [87] and pentamycin [88]. These have been used mostly in cases where patients are hypersensitive to metronidazole with limited success [89].

2.9 Drug resistance in *Trichomonas vaginalis*

There are many factors which determine whether an organism is susceptible or resistant to a particular antimicrobial agent. These include the existence of the antimicrobial target in the relevant organism, the amount of antimicrobial that reaches the target site, and the antimicrobial should not be modified or inactivated by the host [90]. In order to understand the antimicrobial resistance mechanisms, one needs to know the target of the particular antimicrobial in the relevant organism [91].

Several mechanisms for drug resistance to anaerobic bacteria and protozoa have been proposed, but they differ among organisms and these are poorly understood [92]. The primary mechanisms include reduced drug activation, inability of the drug to reach the target site, drug detoxification and altered DNA repair [13]. In protozoa, the parasite can change its metabolic pathways and that can affect the activity of the drug by preventing it from reaching its target [99]. Detoxification of the drug will also affect the activity of the drug. Import and export of the drug by the target organism will affect its efficacy by lowering the effective concentration below the threshold required to harm the parasite [46].

T. vaginalis drug resistance to metronidazole has been reported since the early 1960's and is a problem due to the limited alternative drugs available. *T. vaginalis* is considered clinically resistant to metronidazole when the infection is not eradicated after the standard metronidazole treatment regimen. These cases are treated with tinidazole or increased metronidazole dose [13, 61] since there is no effective non-nitroimidazole oral treatment.

Microorganism susceptibility to nitroimidazoles is dependent on the presence of electron generation and transport systems which activate the drug [93]. Metronizadole is the most studied nitroimidazole. Metronidazole enters the host cell via passive diffusion in an inactive form known as a prodrug, the nitro group becomes reduced, thereby activating the drug and rendering it toxic to the parasite [12]. In *T. vaginalis* resistance is thought to occur by one of 3 mechanisms: 1) the lack of PFOR activity, 2) down regulation [94] or loss of ferredoxin [95] or 3) reduced activity of hydrogenase enzymes. Both clinical isolates and laboratory derived strains have demonstrated resistance to nitroimidazoles [4, 11, 61, 81, 86, 94, 96].

2.9.1 Aerobic Resistance

Although *T. vaginalis* is an anaerobic organism, it can survive in environments where small amounts of oxygen are present [97]. Low oxygen concentrations (< 0.25 µM) enable *T.*

vaginalis growth while elevated oxygen concentrations (> 60 µM) are toxic [97]. *T. vaginalis* has protective hydrogenosomal and cytosolic enzymes which rapidly detoxify toxic oxygen radicals keeping them to low levels [97]. However, if the oxygen concentration rises above the detoxification rate it results in cell death [97]. *T. vaginalis* resistance to metronidazole can either be classified as aerobic or anaerobic. Clinical resistance is almost exclusively aerobic. Aerobic resistance occurs when oxygen competes with metronidazole for electrons [98]. Free electrons preferentially bind to oxygen rather than metronidazole [93] and they are rapidly removed from the nitro radical anion resulting in the reformation of the parent compound [93]. Metronidazole therefore remains inactive [46].

2.9.2 Anaerobic Resistance

T. vaginalis resistance to metronidazole in an anaerobic environment is independent of electron scavenging molecules [98]. The mechanism of resistance is poorly understood by is thought to result from an alteration in the metabolic pathways which activate metronidazole. These include lack of PFOR activity [96], reduced activity or down regulation of ferredoxin and lower activities of hydrogenosomal enzymes [46, 98].

2.9.3 Drug resistance testing of *Trichomonas vaginalis*

Macro-broth and micro-broth dilution assays are widely used for protozoal antimicrobial susceptibility testing [99]. Micro-broth dilution assays are preferred since the decreased sample volume reduces cost and increases throughput. Unlike most other organisms, there is no European Committee on Antimicrobial Susceptibility (EUCAST) or Clinical and Laboratory Standards Institute (CLSI) breakpoint for *T. vaginalis*. For this reason different

MIC values have been used to classify *T. vaginalis* isolates as susceptible, intermediate or resistant [4, 10, 11, 61, 100]. Other study assessed the susceptibility of *T. vaginalis* isolates using the anaerobic breakpoint of >15 µg/ml [28]. In 1988 Muller *et al* investigated the correlation between *in vitro* resistance under aerobic and anaerobic conditions and treatment failure [101]. In this study a Minimum Lethal Concentration (MLC) ≥ 3.1 µg/ml was associated with treatment failure. However, the Upcroft group [4] have recommended metronidazole breakpoints under aerobic and anaerobic conditions based on test results obtained from the patients' response to clinical treatment [4]. However, further research is still necessary.

CHAPTER 3 – MATERIALS AND METHODS

3.1 Specimen collection and propagation of clinical isolates

3.1.1 Study participants and specimen collection

Participants were recruited from two public clinics in KwaZulu Natal, South Africa, the Umlazi D Clinic located in Durban, and the Boom Street Clinic located in Pietermaritzburg. A total of 617 specimens were collected from female patients, 18 years or older presenting with vaginal discharge syndrome after obtaining informed consent. Specimens were collected from the vagina using a Dacron swab. The swab was streaked onto a glass slide for another study, and then the same swab was placed in 5 ml of Diamonds media with antibiotics (Appendix A) and transported back to the laboratory at room temperature on the same day.

This study was approved by management at both clinics and the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (REF:BE 220/13).

3.1.2 Propagation of clinical isolates

As soon as the specimens arrived at the laboratory, the tube containing 5 ml Diamonds medium together with the Dacron swab was incubated at 37°C for 48 hours. Each day from day two, a wet mount was prepared by placing one drop of media from each tube on a glass slide and placing a glass coverslip on top. The wet mount was examined with the dark field microscope (Olympus CWHIC, Japan) to look for motile trichomonads. If these were not observed within seven days, the sample was considered negative and the culture was

discarded. If there was growth, 500 µl of the culture was used to inoculate a tube containing fresh Diamond's medium with antibiotics. If there was growth of contaminating organisms in positive samples, it was sub-cultured into Diamonds media containing 100 mg / L tazobactam/piperacillin (Tazobax) (Brimpharm, SA) in order to eliminate contaminating organisms which were resistant to the other antimicrobial agents already present in the Diamonds medium. Positive specimens were sub-cultured every second day until the trophozoites reached the log phase of growth as indicated by the presence of 80-90% motile trophozoites. Positive specimens were then sub-cultured into two 5 ml tubes of drug free Diamonds media. The first tube was used for the susceptibility testing and the other tube for storage.

3.1.3 Quantitation of *T. vaginalis*

The inoculum was standardised using a Neubauer haemocytometer. A drop of culture containing *T. vaginalis* was placed on the haemocytometer and covered with a glass coverslip. The trichomonads were counted using a dark field microscope. The number of organisms per millilitre was calculated using the formula below.

$$\text{Concentration} = \frac{\text{Number of trichomonads} \times 10^4}{\text{Squares counted}}$$

The inoculum was then adjusted in another fresh tube so that each well of the 96 well plate would be inoculated with 3×10^3 trichomonads per well.

3.1.4 Cryopreservation of *Trichomonas vaginalis*

T. vaginalis isolates grown in drug-free Diamonds media were stored at -85°C. To 5.5 ml culture, 2 ml drug-free Diamonds medium, 1.5 ml heat inactivated foetal bovine serum (FBS) (15%) and 1 ml dimethyl sulphoxide (DMSO) (10%) was added to achieve a final volume of 10 ml. Each isolate was stored in triplicate (1 ml per cryovial). The cultures were frozen at -20°C for one hour in a polystyrene rack, followed by -70°C overnight, then transferred to a -85°C freezer for long term storage.

3.1.5 Recovery of *T vaginalis*

Cryovials containing cultures were removed from -85°C and placed at 37°C in a water bath until they thawed. The culture was immediately transferred into 5 ml fresh drug-free Diamonds media and incubated at 37°C. The viability was assessed after 24 hours. If there was sufficient growth, the organism was sub-cultured to remove the excess DMSO. If the trichomonads were evenly dispersed, the tubes were centrifuged at 1500 × g for 5 minutes and the pellet was inoculated into fresh media.

3.2 Bacterial controls

Two bacterial controls were used, one susceptible and one resistant to the drugs used in this study. These are *Bacteroides fragilis* ATCC 25285 and *Propionibacterium acnes* ATCC 11827 respectively. *B. fragilis* is an obligate anaerobe and it is susceptible to both metronidazole and tinidazole at ≤ 4 mg/L [102]. *P. acnes* is an aerotolerant bacterium which is resistant to both metronidazole > 256 mg/L [103] and tinidazole. There were no published

MIC values for secnidazole and ornidazole available. This was determined experimentally three times in triplicate before being used as controls. The MIC for *B. fragilis* was 4 mg/L, while *P. acnes* was completely resistant to both drugs at the highest dilution tested (16 mg/L). Both *B. fragilis* and *P. acnes* were cultured on blood agar plates. Plates were incubated in anaerobic jars at 37°C for 72 hours. Anaerobic conditions were achieved by placing an anaerobic gas pack (Oxoid, England) in a 2.5 L Oxoid anaerobic gas jar together with the culture plates. An indicator strip (Oxoid, England) was also placed into the jar. This was used to confirm whether or not anaerobiosis had been achieved in the anaerobic gas jar. This strip is coated with a redox indicator, resazurin. The colour changes from pink to white under anaerobic conditions and the transition to pink occurs when the environment becomes anaerobic.

3.3 Susceptibility testing

The Minimal Inhibitory Concentration (MIC) and the Minimum Lethal Concentration (MLC) of metronidazole, tinidazole, secnidazole and ornidazole was determined for each clinical isolate. Flat-bottomed 96-well plates were used for the assay. Experiments were conducted in duplicate and 6 isolates or controls were assessed per plate.

Drug stocks were prepared as described in Appendix A. Two hundred microlitres of Diamond's medium containing 32 mg / L drug was added to row A. One hundred microliters of drug-free Diamonds medium was added to rows B to H. Serial 2-fold dilutions of the drug was carried out down the plate (from row A to G) using a multichannel pipette. The resulting drug concentrations from rows A to G was 32, 16, 8, 4, 2, 1, 0.5 and 0.25 mg/L respectively (or 100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0.8 µM). No drug was added to row H which was the drug-free (DF) control. One hundred microlitres of drug free Diamond's medium containing

3×10^3 trichomonads was added to all wells except the susceptible and resistant control wells. The inoculum for the bacterial controls was standardized to a 0.5 McFarland standard (1.5×10^8 cfu / ml). The colonies were suspended in 5 ml sterile distilled water, vortexed and examined visually. The turbidity was adjusted to achieve a 0.5 McFarland standard. The bacterial suspensions were then centrifuged at 2000 × g for 10 minutes. The supernatant was discarded and the pellet was resuspended in an equal amount of Diamonds medium. One hundred microliters of the resulting suspension was added to the relevant wells. The addition of 100 µl inoculum in each well resulted in a further 2-fold dilution of the drug concentration i.e. the 16 to 0.125 mg / L as shown in Figure 3.1. The 96-well plates were placed inside an air-tight 2.5 L Oxoid anaerobic jar containing an anaerobic gas pack and an indicator strip. This was placed inside a 37°C incubator. After 48 hours the plates were removed from the anaerobic container and susceptibility tests were scored visually using an inverted phase contrast microscope (Olympus IXZ-SLP, Japan). The plates were returned to the anaerobic jar together with a new anaerobic gas pack and indicator strip and reincubated at 37°C. The plates were scored again after a further 24 hours then discarded.

The validity of the assay was monitored by processing one susceptible and one resistant bacterial strain per batch, as well as a drug free control to confirm growth of the organism. The experiments were carried out once in duplicate, but when the duplicate wells produced a different score, the experiment was repeated.

	Isolate 1		Isolate 2		Isolate 3		Isolate 4		Resistant control		Susceptible control	
	1	2	3	4	5	6	7	8	9	10	11	12
A	16	16	16	16	16	16	16	16	16	16	16	16
B	8	8	8	8	8	8	8	8	8	8	8	8
C	4	4	4	4	4	4	4	4	4	4	4	4
D	2	2	2	2	2	2	2	2	2	2	2	2
E	1	1	1	1	1	1	1	1	1	1	1	1
F	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
G	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
H	DF	DF	DF	DF	DF	DF	DF	DF	DF	DF	DF	DF

DF, drug free
The resistant control was *Propionibacterium acnes*; the susceptible control was *Bacteroides fragilis*

Figure 3.1: Plate setup for the micro-broth dilution assay indicating the drug concentrations used (mg/L) and the placement of isolates and controls

3.3.1 Interpretation of results

Plates were removed from the incubator and anaerobic jar after 48 hours, and again after a further 24 hours, and visualised using an inverted phase contrast microscope (1XZ-SLP Olympus, Japan. Each well was examined at 100 × magnification (10 × objective lens and 10 × eye piece). A score was assigned to wells which had been inoculated with *T. vaginalis* using the criteria summarised in Table 3.2. The wells containing bacterial controls were

assessed visually for turbidity. Turbid media indicated "growth", whereas clear media indicated "no growth" of the control organism.

Table 3.1: Criteria for scoring *T. vaginalis* growth in the micro-broth dilution assay

Score	Interpretation
0	No motile parasites
1+	Dead or significantly few parasites (1-10)
2+	Several hundred motile parasites
3+	Almost confluent
4+	Confluent

The MIC was defined as the lowest drug concentration which produced a score of 1+. When plates were scored immediately after inoculation of *T. vaginalis* into the wells (Time 0) before the organism had a chance to replicate, a score of 1+ was produced. Therefore a score of 1+ indicates that no replication had taken place i.e. this is the concentration of the drug which inhibits growth. The MLC was reported as the lowest concentration of the drug at which no motile trichomonads were detected i.e. a score of "0" (Table 3.1)

To facilitate comparison of the effect of the different drugs on different isolates, isolates with an MIC or MLC of less than 2 mg/L were classified as has having a low MIC or MLC respectively. Isolates with an MIC or MLC more than 2 mg/L were classified as having a high MIC or MLC respectively.

CHAPTER 4 – RESULTS

4.1 Minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) test results

A total of 617 vaginal specimens were collected. From these 106 (17%) were positive for *Trichomonas vaginalis* but only 94 (15%) could be cultured. Eight isolates could not be cultured due to overgrowth of drug resistant commensal flora and 4 isolates could not be revived from storage (4). Three hundred and sixty two specimens were collected from Boom Street Clinic while 255 were collected from Umlazi D Clinic. Of the 106 *T. vaginalis* positive specimens, 72 (68%) were from Boom Street Clinic and 34 (32%) were from Umlazi D Clinic. The MIC of all 94 isolates was determined for four 5-nitroimidazoles. These were metronidazole, tinidazole, secnidazole and ornidazole. The lowest drug concentration which produced a score of 1+ was taken as the MIC. The MLC was defined as the lowest concentration at which no motile trichomonads were observed (0).

The MICs of the controls *Bacteroides fragilis* and *Propionibacterium acnes* were $\leq$ 4 mg/L and $\geq$ 16 mg/L respectively for all experiments which is in keeping with the published values. The MLC was not determined for the bacteria.

In general the MIC (Table 4.1.) and MLC (Table 4.3) was higher for metronidazole than any of the other 5-nitroimidazoles tested. The MIC for metronidazole ranged from 0.25 – 8 mg/L while the MLC ranged from 0.25 – 16 mg/L. For both tinidazole and secnidazole, the MIC and MLC ranged from 0.25 – 4 mg/L and 0.5 – 4 mg/L respectively. The MICs (0.5 – 2 mg/L) and MLCs (0.5 – 4 mg/L) for ornidazole were lower than for any of the other antimicrobials tested.

Tables 4.2 and 4.4 show the cumulative MICs and MLCs after 48 and 72 hours of incubation. The MIC_{90} was 4 mg/L for metronidazole and 2 mg/L for tinidazole, ornidazole and secnidazole after both 48 and 72 hours of incubation. The MLC_{50} for metronidazole was 2 mg/L and 1 mg/L for tinidazole, ornidazole and secnidazole.

Because the range of MIC and MLC from isolates collected at both clinics are similar. The results are analysed together.

Table 4.1: Minimum inhibitory concentrations (MIC) of 94 *T. vaginalis* isolates from KwaZulu Natal

Antimicrobial	Time point	No. of isolates with MIC (mg/L)						
		0.25	0.5	1	2	4	8	16
Metronidazole	48 h	7	18	32	27	9	1	0
	72 h	10	8	37	27	11	1	0
Tinidazole	48 h	3	27	45	17	2	0	0
	72 h	5	17	46	25	1	0	0
Secnidazole	48 h	3	20	48	22	1	0	0
	72 h	0	17	47	28	2	0	0
Ornidazole	48 h	2	28	54	10	0	0	0
	72 h	3	13	58	20	0	0	0

* MIC was defined as a score of 1+

Table 4.2: Cumulative percentage of *T. vaginalis* isolates (*n* = 94) with each minimum inhibitory concentration (MIC)

Antimicrobial	Time point	Cumulative % MIC (mg/L)						
		0.25	0.5	1	2	4	8	16
Metronidazole	48 h	7	27	61	89	99	100	
	72 h	11	19	59	87	99	100	
Tinidazole	48 h	3	32	80	98	100		
	72 h	5	23	72	99	100		
Secnidazole	48 h	3	24	76	99	100		
	72 h	0	18	68	98	100		
Ornidazole	48 h	2	32	89	100			
	72 h	3	17	79	100			

***** MIC was defined as a score of 1+

Table 4.3: Minimum lethal concentrations (MLC) of 94 *T. vaginalis* isolates from KwaZulu Natal

Antimicrobial	Time point	No. of isolations with MLC (mg/L)						
		0.25	0.5	1	2	4	8	16
Metronidazole	48 h	0	5	40	31	13	3	2
	72 h	3	10	29	35	13	2	2
Tinidazole	48 h	0	8	47	36	3	0	0
	72 h	0	15	44	33	2	0	0
Secnidazole	48 h	0	8	45	37	3	1	0
	72 h	0	6	50	34	3	1	0
Ornidazole	48 h	0	9	56	27	2	0	0
	72 h	0	8	48	36	2	0	0

* MLC was defined as the drug concentration at which no motile parasites were seen

Table 4.4: Cumulative percentage of *T. vaginalis* isolates (*n* = 94) with each minimum lethal concentration (MLC)

Antimicrobial	Time point	Cumulative % MLC* (mg/L)						
		0.25	0.5	1	2	4	8	16
Metronidazole	48 h	0	5	48	81	95	98	100
	72 h	3	14	45	82	96	98	100
Tinidazole	48 h	0	9	59	97	100		
	72 h	0	16	63	98	100		
Secnidazole	48 h	0	9	56	96	99	100	
	72 h	0	6	60	96	99	100	
Ornidazole	48 h	0	10	69	98	100		
	72 h	0	9	60	98	100		

* MLC was defined as the drug concentration at which no motile parasites were seen

4.2 High minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) (> 2 mg/L)

The MIC and MLC values for any isolate which had MIC or MLC > 2 mg/L for any of the 5-nitroimidazoles tested are summarised in Tables 4.5 and 4.6 respectively.

For metronidazole 10 and 12 isolates had a MIC > 2 mg/L after 48 and 72 hours respectively, while 18 and 16 isolates had a MLC > 2 mg/L after 48 and 72 hours respectively. For tinidazole 2 and 1 isolates had a MIC > 2 mg/L after 48 and 72 hours respectively, while 3 and 2 isolates had a MLC > 2 mg/L after 48 and 72 hours respectively. For secnidazole 1 and 2 isolates had a MIC > 2 mg/L after 48 and 72 hours respectively, while 3 isolates had a MLC > 2 mg/L after both 48 and 72 hours. For ornidazole no isolates had a MIC > 2 mg/L after either 48 or 72 hours, while 2 isolates had a MLC > 2 mg/L after both 48 and 72 hours.

Overall there were more isolates with MIC or MLC > 2 mg/L for metronidazole than for any of the other 5-nitroimidazoles tested while ornidazole had the smallest number of isolates with an MIC or MLC > 2 mg/L. None of the isolates had MIC or MLC > 2 mg/L for all four 5-nitroimidazoles tested.

Table 4.5: Minimum inhibitory concentrations (MIC) of all isolates which had an MIC > 2 mg/L for any 5-nitroimidazole tested after either 48 or 72 hours incubation

Isolate number	Metronidazole		Tinidazole		Secnidazole		Ornidazole	
	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
063	4	4	2	2	2	2	2	1
094	8	8	4	4	1	1	1	1
095	4	4	2	2	1	1	1	1
318	4	2	1	2	2	2	1	2
339	4	4	2	2	2	4	2	2
352	4	4	4	2	2	2	1	2
424	4	4	2	2	1	1	1	1
542	4	4	1	2	2	2	1	1
543	2	4	2	2	2	1	0.5	0.5
698	2	2	2	2	4	4	2	2
768	4	4	2	2	2	2	2	1
813	2	4	2	2	2	2	2	2
816	4	4	2	2	2	2	1	2
843	1	4	2	2	2	2	1	1

	MIC < 2 mg/L
	MIC = 2 mg/L
	MIC > 2 mg/L

Table 4.6: Minimum lethal concentrations (MLC) of all isolates which had an MLC > 2 mg/L for any 5-nitroimidazole tested after either 48 or 72 hours incubation

Isolate number	Metronidazole		Tinidazole		Secnidazole		Ornidazole	
	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
062	4	4	2	2	2	2	2	1
063	8	8	2	2	4	4	2	2
094	16	16	4	4	2	2	2	2
095	8	8	2	2	1	1	1	1
312	4	1	2	2	2	2	2	2
318	4	4	2	2	2	2	2	2
339	4	4	2	2	4	4	4	4
347	4	2	2	2	1	1	1	1
352	4	4	4	2	2	2	2	2
542	16	16	2	2	2	2	2	2
543	4	4	2	2	2	1	1	1
655	4	2	1	1	2	2	1	1
698	4	4	2	2	8	8	4	4
768	4	4	2	2	2	2	2	2
802	4	4	2	4	1	2	1	2
813	4	4	2	2	2	2	2	2
816	8	4	4	2	2	2	2	2
843	2	4	2	2	2	2	2	2
870	4	4	2	2	1	1	1	1

	MLC < 2 mg/L
	MLC = 2 mg/L
	MLC > 2 mg/L

4.3. Change in minimum inhibitory concentration (MIC) or minimum lethal concentration (MLC) from 48 to 72 hours

For some isolates there was a shift in MIC or MLC between 48 and 72 hours incubation (Table 4.7). There were more isolates which had a decrease in MIC from 48 to 72 hours for metronidazole, tinidazole and secnidazole (19, 13 and 7 respectively) compared to those which had an increase (10, 3 and 4 respectively). With ornidazole more isolates had an increase (12) in MIC from 48 to 72 hours compared to those which had a decrease (3).

For all of the 5-nitroimidazoles tested, more isolates had an increase in MLC from 48 to 72 hours than those which had a decrease.

Table 4.7: Number of *T. vaginalis* isolates with changes in minimum inhibitory concentration (MIC) or minimum lethal concentration (MLC) from 48 to 72 hours of incubation

	MIC		MLC	
	Increase	**Decrease**	**Increase**	**Decrease**
Metronidazole	10	19	14	6
Tinidazole	3	13	16	6
Secnidazole	4	7	20	5
Ornidazole	12	3	26	3

CHAPTER 5 – DISCUSSION

A micro-broth dilution assay was used to obtain the minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC) of 94 *Trichomonas vaginalis* isolates collected from women presenting with vaginal discharge syndrome at one of two clinics in KwaZulu Natal. These clinics were located at Boom Street in Pietermaritzburg, and Umlazi D in Durban. Specimen collection took place at the Boom Street Clinic from September 2013 to October 2014, and at the Umlazi D Clinic from October 2014 to December 2014. These two clinics were chosen because they are both located within major cities in the province of KwaZulu Natal. We wanted to investigate the MIC and MLC of *T. vaginalis* isolates collected from patients in KwaZulu Natal in order to determine whether or not the isolates collected in our setting are susceptible to the current drug of choice, metronidazole. We also tested three other 5-nitroimidazole compounds in as possible better alternatives for metronidazole. Although *T. vaginalis* does also infect males, specimens were only collected from female patients. *T. vaginalis* accounts for less than 5% of cases of male urethritis [66] and in males *T. vaginalis* infection is frequently asymptomatic. In a heterosexual population we assume that the susceptibility profiles of *T. vaginalis* isolates collected from females are representative of those present in males.

There are several 5-nitroimidazoles. These include metronidazole, tinidazole, secnidazole, ornidazole, benznidazole, carnidazole, and nimorazole. These 5-nitroimidazoles have been reported to have antitrichomonal activities [4, 7, 11, 13, 27, 61, 77, 80, 86, 94, 96, 101, 104, 105] with an exception of benznidazole. We could not find any reports which investigated the activity of benznidazole on *T. vaginalis*. We chose to use metronidazole, tinidazole, secnidazole and ornidazole for this study. Metronidazole was chosen because it is approved by the US Food and Drug Administration (FDA) for the treatment of trichomoniasis and it is

the drug used to treat trichomoniasis in South Africa. For this reason it is included in the syndromic management regimen for the treatment of vaginal discharge syndrome [14, 16]. Tinidazole was chosen because it is the only other drug approved by the FDA for the treatment of trichomoniasis [6] although it is not used in South Africa. Secnidazole and ornidazole were chosen based on their anti-protozoal activity against *T. vaginalis* [80, 86]. Benznidazole, carnidazole, and nimorazole were not available for purchase from a local supplier at the time of conducting the study and were therefore not included.

Currently, there are no FDA approved non-5-nitroimidazole drugs with recognised activity against *T. vaginalis*. The limited chemotherapy options are a cause for concern since resistance has been reported to the currently available 5-nitroimidazoles [4, 11, 12, 27, 61, 94, 96, 98, 101]. Treatment outcome studies have associated clinical failure to 5-nitroimidazoles with resistant *T. vaginalis* isolates [101] or a low bioavailability of the antimicrobial drug at the target site [76]. The absorption of 5-nitroimidazoles differs in different people and this can explain variances in cure rate [76]. In the case of metronidazole, the mean maximal concentration that can be detected in the vagina has been reported as 11.1 µg /ml [106] compared to 40 µg/ml in the serum or plasma 2 hours after oral administration of 2 g [107]. Understanding the pharmacokinetic mechanisms with respect to drug absorption and recovery of the 5-nitroimidazoles will provide additional insights. The difference in aerobic and anaerobic resistance of *T. vaginalis* to 5-nitroimidazoles is thought to result from the interference of oxygen with the mechanism of action of 5-nitroimidazoles [61]. It is therefore, essential that a standardised and reproducible method for resistance testing is available. The micro-broth dilution assay is widely used for the determination of susceptibility testing of *T. vaginalis*. Unlike most other organisms, there is no currently available European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical and Laboratory Standard Institute (CLSI) breakpoints for *T. vaginalis* resistance to 5-nitroimidazoles. Therefore

different researchers use different methods to measure MICs and MLCs [4, 11, 27, 96, 101, 108]. The breakpoint in susceptibility testing is defined as the critical drug concentration that classifies isolates as susceptible, resistant or intermediate [109]. Susceptible isolates are those that are expected to respond to the given antimicrobial dose, while resistant isolates are those that do not respond [109]. Intermediate isolates are those that may or may not respond at the standard dose however they may respond to an increased or longer duration of treatment [109]. Several studies have used different breakpoints to report the susceptibility profile of *T. vaginalis* and correlate high MIC / MLC with isolates from patients with treatment failure using a micro-broth dilution assay [11, 21, 27, 61, 101]. However, these studies focus mainly on metronidazole, the method is not standardised since incubation times varied from 24 to 72 hours, and some expressed drug concentrations as mg/L. These breakpoints are summarized in table 5.1.

Table 5.1: Summary of the published breakpoints of 5-nitroimidazoles against *T. vaginalis* as determined in anaerobic micro-broth dilution assays

Classification	Breakpoint				Incubation time	Reference
	MTZ (mg/L)	TZN (mg/L)	SNZ (mg/L)	ORN (mg/L)		
Susceptible	≤1.6	-	-	-	48 h	[101]
Resistant	≥3.1	-	-	-		
Susceptible	1-9.8	-	-	2	24 and 48 h	[61]
Resistant	39	-	-	8.4		
Susceptible	≤1.1	-	-	-	48 and 72 h	[4]
Intermediate	2.1	-	-	-		
Resistant	≥4.3	-	-	-		
Susceptible	0.51-1.02	-	-	-	48 h	[27]
Resistant	4.25	-	-	-		
Susceptible	≤1.1	-	-	-	72 h	[21]
Intermediate	2.1	-	-	-		
Resistant	≥4.3	-	-	-		

MTZ, metronidazole; TZN, tinidazole; SNZ, secnidazole; ORN, ornidazole

In our study, we determined the MIC of *T. vaginalis* isolates anaerobically against metronidazole, tinidazole, secnidazole and ornidazole. We chose to determine the MIC and MLC under anaerobic conditions since the vagina is an anaerobic environment [105]. We believe that this would provide a more representative estimate of the MIC and MLC than if aerobic conditions were used. The scoring criteria proposed by Upcroft were used to interpret the results [4]. MIC was defined as the lowest drug concentration with a score of 1+. We applied this 1+ as inhibition because it equals the inoculum and therefore correlated with no multiplication of the organism. The MLC was defined as the lowest concentration without any motile parasites. We used the breakpoint used by Upcroft and Upcroft 2001 to classify isolates as high (> 2 mg/L), intermediate (= 2 mg/L) or low (<2 mg/L) MIC [4]. They used

this value (> 2 mg/L) because patients who had failed treatment were infected with isolates with MICs above 2 mg/L [4]. However, this is complicated by the observation that not all patients whose isolates had such a high MIC did fail treatment. The breakpoints reported by Müller *et al* 1988 and Dunn *et al* 2003 [27, 101] are similar to those reported by the Upcroft group (Table 5.1). The breakpoints reported by Meri *et al* (2000) [61] are much higher than the other groups (Table 5.1). This group suggests that a MIC > 39 mg/L indicates resistance compared to 3.1- 4.3 mg/L for the other groups (Table 5.1). We do not classify the isolates as resistant, susceptible or intermediate in this study due to the absence of an agreed upon breakpoint, and since *in vitro* resistance does not always correlate with treatment failure and *vice versa*. We did not use the standardised rules for bacterial MIC because we were not investigating bacterial MIC. Instead we used the bacteria to confirm that our *T. vaginalis* susceptibility tests were reproducible and there were no calculation or dilution errors. The MLC, referred to in bacteriology as the Minimum Bactericidal Concentration (MBC), was not determined for the bacterial controls (*B. fragilis* and *P. acnes*). This would have required an additional subculture procedure.

For trichomonads we used motility as a proxy for viability. This seems not to be correct since some MLC values increased with prolonged incubation. This means that there were still viable organisms in the well that contained the MLC concentration at 48 hours. Since we applied the same assessment with all four drugs, the comparisons are still valid.

Metronidazole had the poorest *in vitro* efficacy with the highest number of MIC > 2 mg/L (10) and MLC > 2 mg/L (18), and the lowest number of MIC < 2 mg/L (57) and MLC < 2 mg/L (45) after 48 hours compared to the other three 5-nitroimidazoles (Table 4.1) However, it is uncertain whether these high MICs indicate *in vivo* resistance. A previous study by Müller *et al* (1988) demonstrated that treatment failure is strongly associated with a high MIC

obtained aerobically, but our work was done anaerobically [101]. Shwebke *et al* [113] indicated that there was no correlation between clinical response and *in vitro* aerobic and anaerobic resistance. Only one isolate which was resistant both aerobically and anaerobically according to the Upcroft and Upcroft 2001 [4] criteria did not respond to treatment. Although the isolates which had a high MIC for metronidazole generally also had a high MIC for tinidazole, secnidazole or ornidazole, there was always at least one drug to which *T. vaginalis* had an MIC < 2 mg/L (Table 4.3).

Ornidazole had the best *in vitro* efficacy with more isolates having an MIC < 2 mg/L after 48 hours (89) and more isolates having an MLC < 2 mg/L (65) than the other three 5-nitroimidazoles (Tables 4.1 and 4.2). These results support the findings of a clinical study [93] which demonstrated that ornidazole had a greater cure rate than tinidazole. A single dose treatment with ornidazole resulted in 100% trichomonad eradication after one week compared to 95% for the same dose of tinidazole.

A significant proportion of isolates (68 %) showed a shift in MIC or MLC with prolonged incubation from 48 to 72 hours. As metronidazole is stable at 37°C [112] it is unlikely that for this compound the observed changes were the result of inactivation of the drug. There are no available data on the stability of the other 5-nitroimidazoles (tinidazole, secnidazole and ornidazole) at 37°C.

Slow killing of trichomonads by drugs could explain the decrease in MIC from 48 to 72 hours of incubation which was observed for 19, 13 and 7 isolates when treated with metronidazole, tinidazole or secnidazole respectively (Table 4.7). However, there were 10, 3 and 4 isolates which demonstrated an increase in MIC for these same 3 drugs under the same conditions. For ornidazole 12 isolates had an increase in MIC from 48 to 72 hours, compared to only 3 which had a decrease. This needs further investigation.

The observation that the MIC or MLC of *T. vaginalis* isolates for a particular antimicrobial agent could either increase or decrease from 48 to 72 hours of incubation although the conditions were the same, suggests that there is strain diversity amongst the isolates circulating in the population.

There was a large number of isolates with an MIC of 2 mg/L (approximately 20-30%) and MLC of 2 mg/L (approximately 30-40%). These MICs could not be classified as high or low using the Upcroft criteria. A difference of one 2-fold dilution falls within the acceptable variation of MIC determination. However, these values were repeatedly the same and therefore should be seen as the true MIC for these isolates. This highlights the need for frequent surveillance to ensure that we continue to treat trichomoniasis with an effective anti-trichomonas drug.

Clinical resistance of *T. vaginalis* to metronidazole treatment has been reported [96]. When this occurs patients are usually treated with longer or higher doses of metronidazole or with tinidazole [61, 108]. In many patients, higher doses result in side effects such as vomiting and dizziness [11, 76]. Clinical trials which correlate treatment outcome, the MIC or MLC of *T. vaginalis* isolates, and the concentration of the drug achieved in the vagina will be useful in determining threshold values for resistance [61]. Reinfection from asymptomatic partners is likely to be common and should be excluded in such studies. This should be done by means of a highly discriminative typing method.

As part of the syndromic management regimen for the treatment of vaginal discharge syndrome, metronidazole is used in combination with other antimicrobials including ceftriaxone and azithromycin [75]. The effect of ceftriaxone, azithromycin and doxycycline on *T. vaginalis* isolates has been tested [111]. Ceftriaxone showed no effect on *T. vaginalis* while doxycycline and azithromycin showed some activity at higher concentrations [111].

The effect of the combination of these antimicrobial agents with metronidazole remains unknown.

Although tinidazole, secnidazole and ornidazole are effective against many isolates with a high MIC or MLC to metronidazole, these drugs are unlikely to offer an immediate solution. These drugs are currently not in use in South Africa. In addition, they have a similar mechanism of action to metronidazole and are therefore likely to share resistance mechanisms. Most organisms with a high MIC or MLC for metronidazole also had a high MIC for one or more (but not all) of the other 5-nitroimidazoles. Since all 5-nitroimidazoles share a similar structure, patients with an allergy to metronidazole may also have an allergy to the other 5-nitroimidazoles [88]. There is a need for a non-5-nitroimidazole alternative for the treatment of trichomoniasis.

The limitations of our study were as follows. Of the 106 positive isolates that were identified, only 94 grew *in vitro*. Drug resistant bacterial and fungal commensal flora from the clinical specimens prevented successful growth of some *T. vaginalis* isolates. However, we were able to optimize the culture media by performing susceptibility tests on the contaminating organisms and incorporating suitable drugs in the culture media. The other limitation of this study was the use of bacteria as the sensitive and resistant controls, but this limitation was minimized by subjecting the bacterial controls to the same conditionsas the *T. vaginalis* isolates, including growth in Diamonds medium.This study was also limited by the fact that inoculated plates for the micro-broth dilution assay which had been incubated anaerobically were removed from the anaerobic jar to be visualised using an inverted phase contrast microscope after 48 hours. Plates were then returned to the anaerobic jar with a fresh anaerobic gas pack and incubated for a further 24 hours. During the time outside the jar, the culture media would have been exposed to oxygen since the plates were not sealed.

CHAPTER 6 – CONCLUSIONS

Our study indicated that *Trichomonas vaginalis* isolates obtained from patients presenting with vaginal discharge syndrome at one of two clinics in KwaZulu Natal have a broad range of minimum inhibitory concentrations (MIC) and minimum lethal concentrations (MLC) to the four 5-nitroimidazoles tested. Metronidazole, which is the current drug of choice in South Africa for the treatment of trichomoniasis had the highest MIC and MLC. Although some isolates which had a high MIC or MLC for metronidazole, also had a high MIC or MLC for other nitroimidazoles, there were no isolates with high MIC or MLC for all four drugs tested. The MIC and MLC of tinidazole, secnidazole and ornidazole were lower, but these drugs are not widely available in South Africa. The high MICs and MLCs are a cause for concern since there is no alternative non-nitroimidazole chemotherapy available for the treatment of trichomoniasis. It is also unknown whether one should use MIC or MLC to measure the efficacy of these drugs. Further research is required to determine which MIC or MLC *in vitro* correlates with clinical failure.

REFERENCES

1. Van Der Pol, B., *Trichomonas vaginalis* infection: the most prevalent nonviral sexually transmitted infection receives the least public health attention. *Clinical Infectious Diseases*, 2007. **44**(1): p. 23-25.

2. WHO, Global prevalence and incidence of selected curable sexually transmitted infections: Overview and estimates, in World Health Organisation 2001.

3. Petrin, D., et al., Clinical and microbiological aspects of *Trichomonas vaginalis.* *Clinical Microbiology Reviews*, 1998. **11**(2): p. 300-317.

4. Upcroft, J.A. and P. Upcroft, Drug susceptibility testing of anaerobic protozoa. *Antimicrobial Agents Chemotherapy*, 2001. **45**(6): p. 1810-1814.

5. McClelland, R.S., et al., Infection with *Trichomonas vaginalis* increases the risk of HIV-1 acquisition. *Journal of Infectious Diseases*, 2007. **195**(5): p. 698-702.

6. Workowski, K.A. and S. Berman, Sexually transmitted diseases treatment guidelines, *2010. MMWR Recommendations and Reports*, 2010. **59**(RR-12): p. 1-110.

7. Roy, R.B., S.M. Laird, and L. Heasman, Treatment of trichomoniasis in the female. A comparison of metronidazole and nimorazole. *British Journal of Venereal Diseases*, 1975. **51**(4): p. 281-284.

8. Chen, M., et al., Acetarsol pessaries in the treatment of metronidazole resistant *Trichomonas vaginalis. International Journal of STD & AIDS*, 1999. **10**(4): p. 277-279.

9. Kranzler, M., et al., Pentamycin shows high efficacy against *Trichomonas vaginalis.* *International Journal of Antimicrobial Agents*, 2015. **45**(4): p. 434-437.

10. Bosserman, E.A., et al., Utility of antimicrobial susceptibility testing in *Trichomonas vaginalis*-infected women with clinical treatment failure. *Sexually Transmitted Diseases*, 2011. **38**(10): p. 983-987.

11. Crowell, A.L., K.A. Sanders-Lewis, and W.E. Secor, In vitro metronidazole and tinidazole activities against metronidazole- resistant Strains of *Trichomonas vaginalis.* *Antimicrobial Agents and Chemotherapy*, 2003. **47**(4): p. 1407-1409.

12. Cudmore, S.L., et al., Treatment of infections caused by metronidazole-resistant *Trichomonas vaginalis. Clinical Microbiology Review*, 2004. **17**(4): p. 783-793, table of contents.

13. Lofmark, S., C. Edlund, and C.E. Nord, Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clinical Infectious Diseases*, 2010. **50 Suppl 1**: p. S16-23.

14. Altini, L. and D. Cootzee, Syndromic management of sexually transmitted infections. *Continuing Medical Education*, 2005. **23**(2). p. 62-66

15. Johnson, L.F., et al., The effect of syndromic management interventions on the prevalence of sexually transmitted infections in South Africa. *Sexual and Reproductive Health*, 2011. **2**(1): p. 13-20.

16. Moodley, P. and A.W. Sturm, Management of vaginal discharge syndrome: how effective is our strategy? *International Journal of Antimicrobial Agents*, 2004. **24 Suppl 1**: p. S4-7.

17. Mayaud, P., Approaches to the control of sexually transmitted infections in developing countries: old problems and modern challenges. *Sexually Transmitted Infections*, 2004. **80**(3): p. 174-182.

18. Poole, D.N. and R.S. McClelland, Global epidemiology of *Trichomonas vaginalis. Sexually Transmitted Infections*, 2013. **89**(6): p. 418-422.

19. Sood, S. and A. Kapil, An update on *Trichomonas vaginalis. Indian Journal of Sexually Transmitted Diseases*, 2008. **29**: p. 7-14.

20. WHO, Global incidence and prevalence of selected curable sexually transmitted infections - 2008 in World Health Organisation2012.

21. Naidoo, S., et al., High prevalence and incidence of sexually transmitted infections among women living in Kwazulu-Natal, South Africa. 2014.

22. Radebe, F., et al., Microbiological surveillance of sexually transmitted infections in Johannesburg, Gauteng Province, 2013-2014, 2014. p. 108-111.

23. Sturm, P.D., et al., Vaginal tampons as specimen collection device for the molecular diagnosis of non-ulcerative sexually transmitted infections in antenatal clinic attendees. *International Journal of STD and AIDS*, 2004. **15**(2): p. 94-98.

24. Mlisana, K., et al., Symptomatic vaginal discharge is a poor predictor of sexually transmitted infections and genital tract inflammation in high-risk women in South Africa. *Journal of Infectious Diseases*, 2012. **206**(1): p. 6-14.

25. KZN Provincial Report - Multi-Sectoral Provincial Strategic Plan for HIV and AIDS, STIs and TB 2012-2016 for KwaZulu-Natal, in *http://www.kznonline.gov.za/hivaids/strategicplans/HIV%20and%20AIDS%204%20web.pdf*2011.

26. Sonko, R., et al., Sexually transmitted infections: An overview of issues on STI management and control in South Africa. South African Health Review 2002, Health Systems Trust. Available: *http://www.hst.org.za/sahr/2002/chapter14.htm*. Accessed 12 April 2015.

27. Dunne, R.L., et al., Drug resistance in the sexually transmitted protozoan *Trichomonas vaginalis. Cell Research*, 2003. **13**(4): p. 239-249.

28. Tasca, T. and G.A. De Carli, Scanning electron microscopy study of *Trichomonas gallinae. Veterinary Parasitology*, 2003. **118**(1–2): p. 37-42.

29. Bogglid, A., C. Sunderman, and B. Esstridge, Localization of post-translationally modified α-tubulin and psedocyst formation in tritrichomonads. *Parasitology Research*, 2002. **88**: p. 468-474.

30. Pereira-Neves, A., K.C. Ribeiro, and M. Benchimol, Pseudocysts in Trichomonads – New Insights. *Protist*, 2003. **154**(3–4): p. 313-329.

31. Afzan, M.Y. and K. Suresh, Pseudocyst forms of *Trichomonas vaginalis* from cervical neoplasia. Parasitology *Research,* 2012. **111**(1): p. 371-381.

32. Arroyo, R., et al., Signalling of *Trichomonas vaginalis* for amoeboid transformation and adhesion synthesis follows cytoadherence. *Molecular Microbiology,* 1993. **7**(2): p. 299-309.

33. Hirt, R.P., *Trichomonas vaginalis* virulence factors: an integrative overview. *Sexually Transmitted Infections*, 2013. **89**(6): p. 439-443.

34. Addis, M.F., P. Rappelli, and P.L. Fiori, Host and tissue specificity of *Trichomonas vaginalis* is not mediated by its known adhesion proteins. Infection and immunity, 2000. **68**(7): p. 4358-4360.

35. Alderete, J.F., et al., Specific parasitism of purified vaginal epithelial cells by *Trichomonas vaginalis. Infection and immunity*, 1988. **56**(10): p. 2558-2562.

36. Alderete, J.F. and G.E. Garza, Identification and properties of *Trichomonas vaginalis* proteins involved in cytadherence. *Infection and immunity*, 1988. **56**(1): p. 28-33.

37. Alderete, J.F. and G.E. Garza, Specific nature of *Trichomonas vaginalis* parasitism of host cell surfaces. *Infection and Immunity*, 1985. **50**(3): p. 701-708.

38. Fichorova, R.N., et al., *Trichomonas vaginalis* lipophosphoglycan triggers a selective upregulation of cytokines by human female reproductive tract epithelial cells. *Infection and Immunity*, 2006. **74**(10): p. 5773-5779.

39. Benchimol, M., Trichomonads under microscopy. *Microscopy and Microanalysis*, 2004. **10**(05): p. 528-550.

40. Harp, D.F. and I. Chowdhury, Trichomoniasis: evaluation to execution. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, 2011. **157**(1): p. 3-9.

41. Heine, P. and J.A. McGregor, *Trichomonas vaginalis:* A reemerging pathogen. *Clinical Obstetrics and Gynecology*, 1993. **36**(1): p. 137-144.

42. Muller, M., The hydrogenosome. *Journal of Genetics and Microbiology*, 1993. **139**(12): p. 2879-2889.

43. Lindmark, D.G., M. Müller, and H. Shio, Hydrogenosomes in *Trichomonas vaginalis.* *The Journal of Parasitology*, 1975. **61**(3): p. 552-554.

44. Schneider, R.E., et al., The *Trichomonas vaginalis* hydrogenosome proteome is highly reduced relative to mitochondria, yet complex compared with mitosomes. *International Journal for Parasitology*, 2011. **41**(0): p. 1421-1434.

45. Shiflett, A. and P.J. Johnson, Mitochondrion-related organelles in parasitic eukaryotes. *Annual Review of Microbiology*, 2010. **64**: p. 409-429.

46. Gehrig, S. and T. Efferth, Development of drug resistance in *Trichomonas vaginalis* and its overcoming natural products. *The Open Bioactive Compounds Journal*, 2009. **2**: p. 21-28.

47. Johnson, P.J., et al., Molecular analysis of the hydrogenosomal ferredoxin of the anaerobic protist *Trichomonas vaginalis. Proceedings of the National Academy of Sciences of the United States of America*, 1990. **87**(16): p. 6097-6101.

48. Yusof, A. and S. Kumar, Ultrastructural changes during asexual multiple reproduction in *Trichomonas vaginalis. Parasitology Research*, 2012. **110**(5): p. 1823-1828.

49. Ribeiro, K.C., L.H. Monteiro-Leal, and M. Benchimol, Contributions of the axostyle and flagella to closed mitosis in the protists *Tritrichomonas foetus* and *Trichomonas vaginalis. Journal of Eukaryotic Microbiology*, 2000. **47**(5): p. 481-492.

50. Schwebke, J.R. and D. Burgess, Trichomoniasis. *Clinical Microbiology Reviews*, 2004. **17**(4): p. 794-803.

51. Conrad, M.D., et al., Extensive genetic diversity, unique population structure and evidence of genetic exchange in the sexually transmitted parasite *Trichomonas vaginalis. PLoS Negleted Tropical Diseases*, 2012. **6**(3): p. 1-11.

52. Fraga, J., et al., Double-stranded RNA viral infection of *Trichomonas vaginalis* and association with clinical presentation. *Acta protozoologica*, 2007. **46**(2): p. 93-98.

53. Weber, B., et al., Double stranded RNA virus in South African *Trichomonas vaginalis* isolates. *Journal of Clinical Pathology*, 2003. **56**(7): p. 542-543.

54. Fichorova, R.N., et al., Endobiont viruses sensed by the human host - beyond conventional antiparasitic therapy. *PLoS One*, 2012. **7**(11): p.1-16.

55. Swygard, H., et al., Trichomoniasis: clinical manifestations, diagnosis and management. *Sexually Transmitted Infections*, 2004. **80**(2): p. 91-95.

56. Hobbs, M.M., et al., Methods for Detection of *Trichomonas vaginalis* in the male partners of infected women: Implications for control of trichomoniasis. Journal of Clinical Microbiology, 2006. **44**(11): p. 3994-3999.

57. Malla, N., Human trichomoniasis due to *Trichomonas vaginalis*–Current perspectives. *Sexually Transmitted Infections*. 2012. p. 107-124

58. Cotch, M.F., et al., *Trichomonas vaginalis* associated with low birth weight and preterm delivery. The vaginal infections and prematurity study group. *Sexually Transmitted Diseases*, 1997. **24**(6): p. 353-560.

59. Iwasaki, A., Antiviral immune responses in the genital tract: Clues for vaccines. *Nature Reviews Immunology*, 2010. **10**(10): p. 699-711.

60. Hobbs, M.M., et al., *Trichomonas vaginalis* as a cause of urethritis in Malawian men. *Sexually Transmitted Diseases*, 1999. **26**(7): p. 381-387.

61. Meri, T., et al., Resistance of *Trichomonas vaginalis* to metronidazole: Report of the first three cases from Finland and oprtimization of in vitro susceptibility testing under various oxygen concentrations. *Journal of Clinical Microbiology*, 2000. **38**(2): p. 763-767.

62. Schwebke, J.R., et al., Molecular testing for *Trichomonas vaginalis* in women: results from a prospective US clinical trial. *Journal of Clinical Microbiology*, 2011. **49**(12): p. 4106-4111.

63. Andrea, S.B. and K.C. Chapin, Comparison of Aptima *Trichomonas vaginalis* transcription-mediated amplification assay and BD Affirm VPIII for detection of *T. vaginalis* in symptomatic women: performance parameters and epidemiological implications. *Journal of Clinical Microbiology*, 2011. **49**(3): p. 866-869.

64. Cudmore, S.L. and G.E. Garber, Prevention or treatment: the benefits of *Trichomonas vaginalis* vaccine. *Journal of Infection and Public Health*, 2010. **3**(2): p. 47-53.

65. Kingston, M.A., D. Bansal, and E.M. Carlin, 'Shelf life' of *Trichomonas vaginalis*. *International Journal Of STD & AIDS*, 2003. **14**(1): p. 28-29.

66. Garber, G.E., The laboratory diagnosis of *Trichomonas vaginalis. Canadian Journal of Infectious Diseases and Medical Microbiology*, 2005. **16**(1): p. 35-38.

67. Thomason, J., et al., Comparison of four methods to detect *Trichomonas vaginalis. Journal of Clinical Microbiology*, 1988. **26**(9): p. 1869-1870.

68. Señea, A.C., et al., A aandomized, double-blind, placebo-controlled trial of combined nevirapine and zidovudine compared with nevirapine alone in the prevention of perinatal transmission of HIV in Zimbabwe. *Clinical Infectious Diseases*, 2007. **44**(1): p. 13-22.

69. Levi, M.H., et al., Comparison of the InPouch TV culture system and Diamond's modified medium for detection of *Trichomonas vaginalis. Journal of Clinical Microbiology*, 1997. **35**(12): p. 3308-3310.

70. Bandea, C.I., et al., Development of PCR assays for detection of *Trichomonas vaginalis* in urine specimens. *Journal of Clinical Microbiology*, 2013. **51**(4): p. 1298-1300.

71. Hardick, A., et al., Comparison between the Gen-Probe transcription-mediated amplification *Trichomonas vaginalis* research assay and Real-Time PCR for *Trichomonas vaginalis* detection using a Roche LightCycler instrument with female self-obtained vaginal swab samples and male urine samples. *Journal of Clinical Microbiology*, 2006. **44**(11): p. 4197-4199.

72. Hollman, D., et al., Screening for *Trichomonas vaginalis* in high-risk adolescent females with a new transcription-mediated nucleic acid amplification test (NAAT): associations with ethnicity, symptoms, and prior and current STIs. *Journal of Pediatric Adolescent Gynecology*, 2010. **23**(5): p. 312-316.

73. Nye, M.B., J.R. Schwebke, and B.A. Body, Comparison of APTIMA Trichomonas vaginalis transcription-mediated amplification to wet mount microscopy, culture, and polymerase chain reaction for diagnosis of trichomoniasis in men and women. *American Journal of Obstetrics and Gynecology*, 2009. **200**(2): p. 188 e1-7.

74. Van Der Pol, B., C.S. Kraft, and J.A. Williams, Use of an adaptation of a commercially available PCR assay aimed at diagnosis of *Chlamydia* and *Gonorrhea* To Detect *Trichomonas vaginalis* in urogenital specimens. *Journal of Clinical Microbiology*, 2006. **44**(2): p. 366-373.

75. DOH, Sexually Transmitted Infections Management Guidelines 2015. *http://www.sahivsoc.org/upload/documents/STIguidelines-1-28-15(LC).pdf*

76. Lamp, K.C., et al., Pharmacokinetics and pharmacodynamics of the nitroimidazole antimicrobials. *Clinical pharmacokinetics*, 1999. **36**(5): p. 353-373.

77. Wood, B.A. and A.M. Monro, Pharmacokinetics of tinidazole and metronidazole in women after single large oral doses. *British Journal of Venereal Diseases*, 1975. **51**(1): p. 51-53.

78. Asane, G.S., et al., Optimization, characterisation and pharmacokinetic studies of mucoadhesive oral multiple unit systems of ornidazole. *Scientia Pharmaceutica*, 2011. **79**(1): p. 181-196.

79. Madhuri, K., M.S. Kumar, and L. Kalyani, A review on tinidazole .*International Journal of Pharmaceutical, Chemical and Biological Sciences*, 2011. 1(1): p. 38-42.

80. Videau, D., et al., Secnidazole: A 5-nitroimidazole derivative with a long half-life. *British Journal of Venereal Diseases*, 1978. **54**: p. 77-80.

81. Sköld, M., H. Gnarpe, and L. Hillström, Ornidazole: a new antiprotozoal compound for treatment of Trichomonas vaginalis infection. *The British Journal of Venereal Diseases*, 1977. **53**(1): p. 44-48.

82. Anjaneyulu, R., S. Gupte, and D. Desai, Single-dose treatment of trichomonal vaginitis: a comparison of tinidazole and metronidazole. *Journal of International Medical Research*, 1973. **1**(2): p. 438-441.

83. Sobel, J.D., Vaginitis. *New England Journal of Medicine*, 1997. **337**(26): p. 1896-1903.

84. Simoens, S., Factors affecting the cost effectiveness of antibiotics. *Chemotherapy Research and Practice*, 2011. **2011**: p. 1-6

85. Levison, M.E. and J.H. Levison, Pharmacokinetics and Pharmacodynamics of Antibacterial Agents. *Infectious disease clinics of North America*, 2009. **23**(4): p. 791-815.

86. Hillstrom, L., L. Petterson, and E. Palsson, Comparison of ornidazole and tinidazole in single-dose treatment of trichomoniasis in women. *British Journal of Venereal Diseases*, 1977. **53**: p. 193-194.

87. Coelho, D.D., Metronidazole resistant trichomoniasis successfully treated with paromomycin. *Genitourinary Medicine*, 1997. **73**(5): p. 397-398.

88. Helms, D.J., et al., Management of *Trichomonas vaginalis* in women with suspected metronidazole hypersensitivity. *American Journal of Obstetrics and Gynecology*, 2008. **198**(4): p.1-7.

89. Secor, W.E., et al., Neglected parasitic infections in the United States: trichomoniasis. *The American journal of tropical medicine and hygiene*, 2014. **90**(5): p. 800-804.

90. Leekha, S., C.L. Terrell, and R.S. Edson, General Principles of Antimicrobial Therapy. *Mayo Clinic Proceedings*, 2011. **86**(2): p. 156-167.

91. Kohanski, M.A., D.J. Dwyer, and J.J. Collins, How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology*, 2010. **8**(6): p. 423-435.

92. Upcroft, P. and J.A. Upcroft, Drug targets and mechanisms of resistance in the anaerobic protozoa. *Clinical Microbiology Reviews*, 2001. **14**(1): p. 150-164.

93. Edwards, D.I. and G.E. Mathison, The mode of Action of Metronidazole Against *Trichomonas vaginalis. Journal of General Microbiology*, 1970. **63**: p. 297-302.

94. Rasoloson, D., et al., Metronidazole-resistant strains of *Trichomonas vaginalis* display increased susceptibility to oxygen. *Parasitology*, 2001. **123**(01). p. 45-56.

95. Quon, D.V.K., C.E. D'Oliveira, and P. Johnson, Reduced transcription of the ferredoxin gene in metronidazole resistant *Trichomonas vaginalis. Journal of Microbiology,* 1992. **89**: p. 4402-4406.

96. Kulda, J., Trichomonas, hydrogenosome and drug resistance. *Internal Journal of Parasitology*, 1999. **29**: p. 199-212.

97. Ellis, J.E., et al., Antioxidant defences in the microerophillic protozoan *Trichomonas vaginalis:* Comparison of metronidazole- resistant and sensitive strains. *Journal of Microbioogyl*, 1994. **140**: p. 2489-2494.

98. Rasoloson, D., et al., Mechanisms of in vitro development of resistance to metronidazole in *Trichomonas vaginalis. Journal of Microbiology*, 2002. **148**: p. 2467-2477.

99. Jorgensen, J.H. and M.J. Ferraro, Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases*, 2009. **49**(11): p. 1749-1755.

100. Wright, J.M., et al., Hydrogenosomes of laboratory-induced metronidazole-resistant *Trichomonas vaginalis* lines are downsized while those from clinically metronidazole-resistant isolates are not. *Journal of Eukaryotic Microbiology*, 2010. **57**(2): p. 171-176.

101. Müller, M., J. Lossick, and T. Gorrell, In vitro susceptibility of *Trichomonas vaginalis* to metronidazole and treatment outcome in vaginal trichomoniasis. *Sexually transmitted diseases*, 1988. **15**(1): p. 17-24.

102. Nagy, E. and E. Urbán, Antimicrobial susceptibility of *Bacteroides fragilis* group isolates in Europe: 20 years of experience. *Clinical Microbiology and Infection*, 2011. **17**(3): p. 371-379.

103. Sowniya, M., et al., *Ocular Propionibacterium Acnes:* A study on Antibiotic Susceptibility Profiling And Their Epidemiological Pattern. *The Internet Journal of Microbiology*, 2010. **9**(2): p. 1-8.

104. Chaudhuri, P. and A. Drogendijk, A double-blind controlled clinical trial of carnidazole and tinidazole in the treatment of vaginal trichomoniasis. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 1980. **10**(5): p. 325-328.

105. Notowicz, A., E. Stolz, and G.A. de Koning, First experiences with single-dose treatment of vaginal trichomoniasis with carnidazole (R 25831). *British Journal of Venereal Diseases,* 1977. **53**(2): p. 129-131.

106. Larsen, B., et al., Implications of metronidazole pharmacodynamics for therapy of trichomoniasis. *Gynecologic and obstetric investigation,* 1986. **21**(1): p. 12-18.

107. Nix, D.E., R. Tyrrell, and M. Müller, Pharmacodynamics of metronidazole determined by a time-kill assay for *Trichomonas vaginalis. Antimicrobial Agents and Chemotherapy*, 1995. **39**(8): p. 1848-1852.

108. Narcisi, E.M. and W.E. Secor, In vitro effect of tinidazole and furazolidone on metronidazole-resistant *Trichomonas vaginalis. Antimicrobial Agents and Chemotherapy*, 1996. **40**(5): p. 1121-1125.

109. MacGowan, A.P. and R. Wise, Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. *Journal of Antimicrobial Chemotherapy,* 2001. **48**(suppl 1): p. 17-28.

110. Larsen, B. and G.R.G. Monif, Understanding the Bacterial Flora of the Female Genital Tract. *Clinical Infectious Diseases,* 2001. **32**(4): p. e69-e77.

111. Naidoo, S., Susceptibility of *Trichomonas vaginalis* to metronidazole and other compunds (Doctorate dissertation). Retrieved from *http:www.researchspace.ukzn.ac.za. accessed on 04 April 2015*, 2014.

112. Erah, P., et al., The stability of amoxycillin, clarithromycin and metronidazole in gastric juice: relevance to the treatment of *Helicobacter pylori infection. Journal of Antimicrobial Chemotherapy*, 1997. **39**(1): p. 5-12.

113. Schwebke, JR., et al., Prevalence of *Trichomonas vaginalis* isolates with resistance to metronidazole and tinidazole. *Antimicrobial Agents and Chemotherapy*, 2006. **50** (12): p. 4209-4210

APPENDICES

APPENDIX A – Antimicrobial Agents

1. Antimicrobial Agents stock preparation

Drug stocks for susceptibility testing experiments (metronidazole, tinidazole, secnidazole and ornidazole) and media supplementation were prepared as per table 1. The powder for each drug was weighed after calculating the percentage purity (HPLC) and dissolved in a required solvent. The stocks were stored as required.

Table A1: Drug stock preparation and storage

Drug	Solvent	HPLC (%)	Amount (g)	Volume (ml)	Conc. (mg/L)	Storage Temp (°C)
Amikacin	Water	100	0.1	10	10	-20
Amphotericin B	DMSO	80	0.0625	10	5	-20
Ciprofloxacin	Acetic acid	98	0.02	10	2	-20
Chloramphenicol	Ethanol	98	1.02	10	100	-20
Metronidazole	Acetic acid	100	0.010	10	32	-20
Ornidazole	Ethanol	98	0.051	5	32	-20
Secnidazole	Water	99.8	0.01	0.998	32	-20
Tazobax	Water	100	4.5	20	100	-4
Tinidazole	Acetic acid	99.7	0.01	0.997	32	-20
Vancomycin	Water	100	0.02	10	2	-20

2. Working solution preparation for susceptibility testing

The working solution was prepared from 10 mg/ml stock of each drug (metronidazole, tinidazole, secnidazole and ornidazole). Briefly, 16 µl of stock (10 mg/ml) was diluted to 5 ml Diamonds media to achieve the required working concentration of 32 mg/L (3.2 mg/L per 1ml medium) final concentration. All antimicrobial agents were manufactured by Sigma-Aldrich, USA.

APPENDIX B – Media Preparation

Diamond's medium was prepared as follows:

20 g BBL™ trypticase peptone (Sigma Aldrich, USA)

10 g yeast extract (Oxoid Ltd, England)

5 g maltose (ACE, SA)

1g L-cysteine hydrochloride (Sigma Aldrich, USA)

0.2 g L-ascobic acid (Sigma Aldrich, USA)

0.8g dipotassium hydrogen phosphate (ACE, SA)

0.5 g potassium dihydrogen phosphate (ACE, SA)

0.5 g agar (Sigma Aldrich, USA)

Dissolve the components in 900 ml sterile deionised water and autoclaved at 121°C for 15 minutes. Allow media to cool to 50°C in the waterbath before the addition of 100 mL heat inactivated horse serum (Biowest) to bring the final volume to 1L.

If the media was to be used for isolation of *T. vaginalis* from clinical specimens the following antimicrobial agents were added to prevent the growth of commensal flora: amphotericin B (5 mg/L), amikacin (4 mg/L), vancomycin (2 mg/L), chloramphenicol (1 mg/L) and ciprofloxacin (2 mg/L). If the media was to be used for susceptibility testing no antimicrobial agent other that the one to be tested was added. Quality control (QC) of the medium was achieved by incubating 5 ml of the media after preparation at 37°C for 24-72 hours. If visible turbidity was visualised in the QC aliquot, all the media in that batch would be discarded; if no turbidity was visualised, the media would be used for culture.

Media was aliquoted in 5 ml aliquots in 15 ml polystyrene tubes (Nest, SA), stored at 4°C and used within 2 weeks.

APPENDIX C – Raw data

Table C1: Minimal Inhibitory Concentrations (MIC) of metronidazole, tinidazole, secnidazole and ornidazole

Isolate number	Metronidazole		Tinidazole		Secnidazole		Ornidazole	
	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
062	2	2	1	2	1	2	1	1
063	4	4	2	2	2	2	2	2
094	8	8	4	4	1	1	1	1
095	4	4	2	2	1	1	1	1
097	0.5	1	1	0.5	1	1	1	1
099	0.5	0.5	0.5	0.5	1	1	1	1
129	0.25	0.5	0.5	0.5	0.5	1	0.5	1
139	0.5	1	0.5	1	0.5	0.5	0.5	1
164	1	1	1	1	1	1	0.5	1
170	2	2	1	1	0.5	0.5	0.5	0.5
176	1	1	0.5	1	0.5	0.5	0.5	0.25
185	1	1	1	1	1	1	1	1
197	2	1	1	1	0.5	0.5	0.5	0.5
210	0.5	0.5	0.5	0.5	0.25	0.5	0.25	0.25
228	1	1	1	1	1	1	0.5	1
231	2	2	1	1	1	1	1	2
246	1	2	2	2	2	1	1	2
253	1	1	1	1	1	1	1	1
264	2	2	0.5	1	2	2	2	2
291	1	1	1	1	2	2	2	2
296	1	1	0.5	0.5	1	1	1	1
298	1	1	1	2	0.5	0.5	0.5	0.5
304	0.5	1	0.5	0.5	1	1	1	1
312	2	1	2	2	2	2	1	1
318	4	2	1	2	2	2	1	2
326	0.5	0.25	2	2	2	2	1	2
339	4	4	2	2	2	4	2	2
347	1	1	1	1	0.5	0.5	0.5	0.5

349	2	2	1	1	1	2	1	2
352	4	4	4	2	2	2	1	2
357	2	2	0.5	1	1	1	1	1
364	0.25	0.25	0.5	0.5	1	2	1	2
368	0.25	0.25	0.25	0.25	1	2	0.5	1
371	0.25	0.25	0.5	0.5	0.5	1	0.5	0.5
378	0.5	0.5	0.5	0.5	1	1	0.5	1
394	0.25	0.25	0.5	0.25	1	1	1	1
401	0.5	1	0.5	1	2	2	1	2
406	0.25	0.25	0.25	0.5	1	1	1	1
411	0.25	0.25	0.5	0.5	2	2	1	1
413	2	2	1	1	0.5	1	1	1
424	4	4	2	2	1	1	1	1
443	2	2	2	2	1	1	0.5	1
449	0.5	0.5	0.5	0.5	1	2	1	1
457	2	2	0.5	1	0.5	0.5	0.5	0.5
481	0.5	0.25	0.5	0.25	1	1	0.5	1
485	0.5	0.25	0.5	0.25	0.5	1	1	1
486	1	1	1	1	1	1	0.5	1
493	0.5	0.25	0.25	0.25	1	1	1	1
527	0.5	0.5	0.5	0.5	1	0.5	0.5	0.5
532	1	1	1	1	2	2	1	2
542	4	4	1	2	2	2	1	1
543	2	4	2	2	2	1	0.5	0.5
545	1	1	1	0.5	1	2	1	2
559	1	1	1	1	1	1	0.5	1
578	2	2	1	1	0.25	1	0.5	1
579	2	2	1	1	2	2	2	2
586	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5
597	1	1	1	1	1	1	0.5	1
614	1	1	1	1	1	1	1	1
615	0.5	1	1	1	0.25	0.5	0.25	0.25
637	2	2	0.5	0.5	0.5	1	1	1
642	1	1	1	1	1	1	0.5	1
643	2	2	1	1	1	1	1	1
647	1	1	1	1	1	2	1	1
651	1	1	1	1	2	2	2	2
652	1	1	1	1	1	1	1	1
655	2	2	0.5	1	1	1	1	1
663	2	2	1	1	1	1	1	1

664	1	1	1	1	1	2	1	1
671	1	1	1	1	0.5	0.5	0.5	0.5
687	1	1	1	1	0.5	1	1	1
688	1	0.5	1	1	1	1	1	1
696	1	1	1	1	1	1	1	1
698	2	2	2	2	4	4	2	2
702	2	2	1	2	1	1	1	1
703	1	1	0.5	1	0.5	0.5	0.5	0.5
716	2	2	1	1	0.5	1	1	1
727	2	2	1	1	2	2	1	1
743	1	1	1	1	0.5	0.5	1	1
748	2	2	1	1	1	1	1	1
768	4	4	2	2	2	2	2	1
774	1	1	1	1	1	1	1	1
777	1	1	1	1	1	1	0.5	0.5
802	2	2	1	2	1	2	1	1
813	2	4	2	2	2	2	2	2
816	4	4	2	2	2	2	1	2
843	1	4	2	2	2	2	1	1
845	2	2	2	2	0.5	0.5	0.5	1
851	1	2	2	2	2	2	2	2
870	2	2	2	2	1	1	1	1
906	0.5	1	1	1	1	0.5	1	1
933	1	2	0.5	2	1	1	1	1
955	0.5	0.5	0.5	1	0.5	0.5	1	0.5
961	0.5	1	0.5	0.5	1	1	0.5	1

Table C2: Minimal Lethal Concentrations (MLC) of metronidazole, tinidazole, secnidazole and ornidazole

Isolate number	Metronidazole		Tinidazole		Secnidazole		Ornidazole	
	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
062	4	4	2	2	2	2	2	1
063	8	8	2	2	4	4	2	2
094	16	16	4	4	2	2	2	2
095	8	8	2	2	1	1	1	1
097	1	1	0.5	0.5	1	1	1	1
099	1	1	1	0.5	1	1	1	1
129	0.5	0.5	0.5	0.5	1	1	1	1
139	1	2	1	0.5	1	1	1	2
164	1	1	1	1	1	1	1	1
170	2	2	2	2	0.5	0.5	0.5	0.5
176	1	1	1	1	1	1	1	1
185	1	2	1	1	1	1	1	1
197	2	2	1	1	0.5	0.5	0.5	0.5
210	0.5	1	0.5	0.5	0.5	1	0.5	0.5
228	1	1	1	1	1	1	1	1
231	2	2	2	2	2	1	1	2
246	2	2	2	2	2	1	1	2
253	2	2	1	1	1	1	1	2
264	2	2	1	1	2	2	2	2
291	2	2	1	1	2	2	2	2
296	1	1	1	1	2	1	1	2
298	2	2	2	2	1	0.5	0.5	0.5
304	1	2	1	1	2	2	1	1
312	4	1	2	2	2	2	2	2
318	4	4	2	2	2	2	2	2
326	1	0.5	2	2	2	2	2	2
339	4	4	2	2	4	4	4	4
347	4	2	2	2	1	1	1	1
349	2	2	1	1	2	2	2	2
352	4	4	4	2	2	2	2	2
357	2	2	0.5	1	1	1	1	1
364	1	0.5	2	0.5	2	2	2	2
368	1	0.25	1	0.5	2	2	1	2
371	0.5	0.5	1	0.5	1	1	1	1
378	1	1	0.5	1	1	1	1	1

394	0.5	0.25	1	1	1	1	1	1
401	1	2	1	1	2	2	2	2
406	0.5	0.25	0.5	0.5	1	1	1	1
411	1	0.5	2	1	2	2	2	2
413	2	2	1	1	1	1	1	1
424	1	1	1	1	2	2	2	2
443	2	2	2	2	1	1	1	1
449	2	0.5	1	0.5	2	2	1	1
457	2	2	1	1	1	1	1	1
481	1	0.5	1	0.5	1	1	1	1
485	1	0.5	1	0.5	1	1	1	1
486	1	1	1	1	1	1	1	1
493	1	0.5	0.5	0.5	2	1	2	2
527	1	0.5	0.5	0.5	0.5	1	0.5	1
532	1	1	1	1	2	2	2	2
542	16	16	2	2	2	2	2	2
543	4	4	2	2	2	1	1	1
545	1	2	2	0.5	2	2	2	2
559	2	2	2	2	2	2	1	2
578	2	2	2	1	1	1	1	1
579	2	2	2	2	2	2	2	2
586	1	1	1	1	0.5	0.5	0.5	0.5
597	1	1	2	2	2	2	1	2
614	1	1	1	1	1	1	1	1
615	1	1	1	1	1	1	0.5	0.5
637	2	2	1	1	1	1	1	1
642	1	1	1	1	1	1	1	2
643	2	2	1	1	1	1	1	1
647	1	1	1	1	2	2	2	2
651	2	1	1	1	4	4	2	2
652	1	1	1	1	1	1	1	1
655	4	2	1	1	2	2	1	1
663	2	2	1	1	1	1	2	1
664	2	2	2	2	2	2	1	1
671	1	1	1	1	0.5	0.5	0.5	0.5
687	2	1	1	1	1	1	1	1
688	1	1	1	1	1	1	1	1
696	1	1	1	1	2	2	1	1
698	4	4	2	2	8	8	4	4
702	2	2	2	2	1	1	1	2
703	1	1	1	1	0.5	1	1	1
716	2	2	2	2	1	1	1	1
727	2	2	1	1	2	2	1	1
743	1	1	1	1	1	1	1	1

748	2	2	2	2	1	1	1	1
768	4	4	2	2	2	2	2	2
774	1	1	1	1	1	1	1	1
777	2	2	1	1	1	1	1	1
802	4	4	2	4	1	2	1	2
813	4	4	2	2	2	2	2	2
816	8	4	4	2	2	2	2	2
843	2	4	2	2	2	2	2	2
845	2	4	2	2	0.5	0.5	1	1
851	1	2	2	2	2	2	2	2
870	4	4	2	2	1	1	1	1
906	1	2	2	2	1	1	0.5	1
933	2	2	2	2	1	1	1	1
955	1	1	1	1	1	1	1	0.5
961	1	1	1	1	1	2	1	1