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TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

Detection of metronidazole resistance in *Trichomonas vaginalis* using uncultured vaginal swabs

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Abstract

Trichomonas vaginalis (*T. vaginalis*) is the most prevalent sexually transmitted infection (STI) globally. Metronidazole is the drug of choice for treating *T. vaginalis* infections although metronidazole-resistant *T. vaginalis* has been reported in clinical isolates. The purpose of this study was to determine the presence of mutations in *nitroreductase* genes associated with metronidazole resistance in vaginal swabs testing positive for *T. vaginalis.* This study included 385 human immunodeficiency virus (HIV)-positive pregnant women. Vaginal swabs were collected from consenting pregnant women and used for the detection of *T. vaginalis* using the TaqMan assay. From the vaginal swabs, nitroreductase genes *ntr4* and *ntr6* containing mutations associated with metronidazole resistance were amplified using a quantitative polymerase chain reaction (PCR) assay. To validate the PCR assay, *T. vaginalis* cultured isolates with known metronidazole resistance profiles were used as controls in the mutation detection assays. The prevalence of *T. vaginalis* in the study population was 12.2% (47/385). Mutations associated with resistance to metronidazole were detected in more than 40% of the samples tested, i.e. 21/47 (45%) and 24/47 (51%) for *ntr4* and *ntr6*, respectively. A total of 19 samples (40%) carried mutations for both *ntr4* and *ntr6* genes associated with metronidazole resistance. The validation assays showed a positive correlation between phenotypic and genotypic resistance profiles. This study found a high prevalence of mutations associated with metronidazole resistance. This is concerning since metronidazole is currently used in the syndromic management of STIs in South Africa. Molecular-based assays for monitoring metronidazole resistance profiles using *nitroreductase* genes may serve as a feasible method for antimicrobial surveillance studies for *T. vaginalis*.

Keywords *Trichomonas vaginalis* · Pregnancy · Metronidazole resistance · HIV · South Africa

Introduction

Trichomonas vaginalis is the parasitic protozoan causing the sexually transmitted infection (STI) trichomoniasis and is the most common and prevalent non-viral STI worldwide (Kissinger 2015a). The World Health Organization (WHO) estimated 142 million cases of *T. vaginalis* among adults (16–45 years of age) globally in 2016, with sub-Saharan Africa (SSA) having the highest incidence (Organization 2016). The last estimated annual incidence of *T. vaginalis* worldwide exceeds that of chlamydia, gonorrhoea, and syphilis combined (Kissinger 2015b; Organization 2018). However, *T. vaginalis* is currently not a reportable disease and the true estimation of disease prevalence is not currently known (Van Der Pol et al. 2021).

In South Africa, the prevalence of *T. vaginalis* infection among pregnant women ranges between 4.8 and 59.6% (Dessai et al. 2020; Green and Taleghani 2020; Joseph Davey

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et al. 2018, 2019; Mabaso et al. 2020; Medina-Marino et al. 2020; Moodley et al. 2015; Morikawa et al. 2018; Mudau et al. 2018; Nyemba et al. 2021; Peters et al. 2021; Price et al. 2018; Smullin et al. 2020). Asymptomatic *T. vaginalis* infections are well documented; approximately 50 to 60% of infected women do not show clinical signs (Bouchemal et al. 2017; Mudau et al. 2018; Vallely et al. 2017). Among women, common sites of infection include the vagina, urethra, and endocervix (Kissinger 2015a). Furthermore, symptomatic women are more likely to experience clinical manifestations, including green or yellow vaginal discharge, vaginal odour, dysuria, vulvar irritation, itching, lower abdominal pain, strawberry cervix, and inflammation (Bosserman et al. 2011; Kissinger 2015a).

T. vaginalis has been reported be associated with several health complications including pelvic inflammatory disease (PID), poor pregnancy outcomes, cervical cancer, prostatitis, and infertility (Akbari and Matini 2017; Masha et al. 2019; Price et al. 2018; Silver et al. 2014). Poor pregnancy outcomes include premature rupture of membranes, low birthweight, preterm delivery, and neonatal death (Kissinger 2015b; Mudau et al. 2018; von Glehn et al. 2017). Furthermore, *T. vaginalis* can also be transmitted to newborn babies during delivery via the birth canal (Akbari and Matini 2017; Kissinger 2015b). Coinfection of *T. vaginalis* with other STIs has been previously reported (Ginocchio et al. 2012; Joseph Davey et al. 2018). *T. vaginalis* infection has also been shown to increase the risk of acquisition and transmission of human immunodeficiency virus (HIV) by 50% via several mechanisms, including damage to the vaginal epithelial membrane by the protozoa (Joseph Davey et al. 2019; Kissinger 2015b; Masha et al. 2019). *T. vaginalis* have been reported to be associated with several risk factors such as older age, sexual behaviour, intravenous drug use, trading sex for goods, commercial sex work, smoking cigarettes, use of oral contraceptives, socioeconomic status, and phase of menstrual cycle (Kissinger 2015a). In women from Africa, older age, marital status, multiple sex partners, a greater number of lifetime sexual partners, poor hygiene, and socioeconomic status have been shown to be risk factors associated with *T. vaginalis* infection (Joseph Davey et al. 2018; Naidoo et al. 2014).

Resistance to metronidazole in the sexually transmitted parasite *T. vaginalis* is a public health concern (Kissinger et al. 2018; Marques-Silva et al. 2021). The WHO and the US Centers for Disease Control and Prevention (CDC) recommend a single 2-g dose of oral metronidazole or tinidazole as first-line treatment and a 7-day dose of oral metronidazole (400 mg or 500 mg twice daily for 7 days) as second-line treatment for *T. vaginalis* infections (Kissinger 2015b; Workowski 2015). However, since metronidazole is generic and affordable, it is primarily used to treat *T. vaginalis* infections in many settings (das Neves et al. 2020; Marques-Silva et al. 2021). Currently, the management and diagnosis of *T. vaginalis* infection in South Africa are based on WHO guidance, using a syndromic approach (Health and Africa, 2015; Organization, 2016). According to South African guidelines, trichomoniasis is characterised by abnormal vaginal discharge, which is treated with a 2 g stat of metronidazole (which is safe to be used in all trimesters of pregnancy) (Health and Africa, 2015). Moreover, emerging nitroimidazole-resistant trichomoniasis is concerning, since few alternatives to standard therapy exist (Alessio and Nyirjesy 2019; Ghosh et al. 2018).

To date, the exact mechanisms of metronidazole resistance in *T. vaginalis* remain unknown and little is known about its molecular basis (Kissinger 2015a). However, it is assumed that it may be due to several mutations in selected targets (Paulish-Miller et al. 2014).

Several studies have reported various factors that modulate metronidazole resistance in *T. vaginalis* such as downregulation of pyruvate-ferredoxin oxidoreductase (PFOR) enzyme activity in anaerobic resistance (Kulda 1999; Land et al. 2001); transcription of ferredoxin enzyme activity reduction in resistant *T. vaginalis* strains in aerobic resistance (Bradic et al. 2017; Yarlett et al. 1986); decreased expressions of flavin reductase; and the downregulation of *nitroreductase* genes (*ntr4* and *ntr6*) (Bradic et al. 2017; Leitsch et al. 2014). The purpose of this study was to determine the presence of mutations in *nitroreductase* genes associated with metronidazole resistance in *T. vaginalis* without the need to culture.

Methods

Ethical statement

Ethics approval for this study (BREC/00001382/2020) was obtained from the Biomedical Research Ethics Committee (BREC), University of KwaZulu-Natal.

Study setting and population

The study was a cross-sectional study of pregnant women attending the antenatal clinic (ANC) at the King Edward VIII Hospital in Durban, South Africa. At the clinic, women were educated on the consequences of STIs during pregnancy and provided with information on risk reduction for STIs. The clinic attends to 80 to 100 women on a daily basis, and the recruitment for this study took place between October 2020 and April 2021. Women were enrolled in this study if they were HIV positive, 18 years and older, willing to provide written informed consent, vaginal swab samples, and socio-demographic,

behavioural, and clinical data. The swab samples were self-collected, and the women were provided with instructions on proper sample collection. Each enrolled woman provided self-collected vaginal swabs (dry swabs) for detection of vaginal infections. The consenting women had also completed a questionnaire on socio-demographic, behavioural, and clinical factors.

Laboratory procedures

Sample processing

After collection, the dry swabs were placed in 2 ml of phosphate buffered saline (PBS). The solution was vortexed to dislodge the cells from the swabs, and the swab was discarded. The suspension was stored at – 20 °C for further molecular analysis.

DNA extraction

DNA extraction was performed on the vaginal swab suspension using the PureLink Microbiome Kit (ThermoFisher Scientific, USA), according to the manufacturer's instructions. Briefly, 2 ml of the vaginal fluid samples was centrifuged for 30 min at 14,000 × *g*. The supernatant was discarded and 800 µl of S1 lysis buffer was added to the pellet and pipetted up and down to mix the sample. The sample was then transferred to the bead tube and 100 µl of S2 lysis enhancer was added to the bead tube, capped, and vortexed briefly. This was incubated at 95 °C for 10 min, followed by vortexing at a maximum speed for 7 min, and further centrifuged at 14,000 × *g* for 1 min. Thereafter, 500 µl of the supernatant was transferred to a clean microcentrifuge tube, avoiding the bead pellet and any cell debris.

To bind DNA to the column, 900 µl of binding buffer was added and vortexed briefly. Following this, 700 µl of the sample mixture was loaded onto a spin column-tube and centrifuged at 14,000 × *g* for 1 min. The flow through was discarded and the spin column was centrifuged at 14,000 × *g* for 30 s. The spin column was placed in a clean tube and 50 µl of elution buffer was added, and the tube was incubated at room temperature for 1 min. After 1 min, the spin column was centrifuged at 14,000 × *g* for 1 min. The concentration of the extracted DNA was determined using the Nanodrop Spectrophotometer (ThermoFisher Scientific, USA). DNA samples were stored at – 20 °C until further molecular analysis. The molecular assays were conducted at the School of Clinical Medicine Research Laboratory at the University of KwaZulu-Natal.

Detection of *T. vaginalis* from vaginal swab DNA extracts

T. vaginalis was detected using the TaqMan Real-time PCR (sensitivity) assay (ThermoFisher Scientific, USA) using commercially available primers and probes specific for *T. vaginalis* (Pr04646256_S1). The assay targets the alpha tubulin 1 gene of from this pathogen. Each PCR reaction was performed in a final volume of 20 µl comprising 1 µl FAM-labelled probe/primer mix, 5 µl Fast Start 4 × probe master mix (ThermoFisher, Part No. 4444434), 2 µl template DNA, and 12 µl nuclease-free water. PCR amplification was performed on the Quant Studio 5 real-time PCR detection system (ThermoFisher Scientific, USA), in a 96-well microtiter reaction plate. Amplification was performed at 95 °C for 30 s followed by 45 cycles comprising of denaturation at 95 °C for 3 s and annealing at 60 °C for 30 s. Detection of amplified fluorescent products was carried out at the end of the annealing phase. The raw fluorescent data that included the C_T mean values were automatically generated by the Quant Studio 5 Real-time PCR system software.

Molecular detection of genes associated with metronidazole resistance

Genes (*ntr4* and *ntr6*) associated with resistance to metronidazole were amplified by quantitative PCR, using specific primers described by Paulish-Miller et al. (2014) (Table 1) on the extracted vaginal swab DNA. The primer and probe sequences which contain the mutations associated with resistance to metronidazole are listed in Table 1. Each PCR reaction was performed in a final volume of 10 µl comprising 5ul Taqman master mix (ThermoFisher Scientific, USA), 10 µM (0.5 µl each) of forward and reserve primer, 0.5 µl probe FAM (wild type) and VIC (mutant), 1 µl template DNA, and 2 µl nuclease-free water. PCR amplification was performed on the Quant Studio 5 real-time PCR detection system (ThermoFisher Scientific, USA), in a 96-well microtiter reaction plate. The cycling conditions comprised of a UDG activation stage for 2 min at 50 °C, initial denaturation for 2 min at 95 °C, followed by 40 cycles consisting of denaturation for 15 s at 95 °C, annealing 15 s at 60 °C with extension for 1 min at 72 °C. To assess any contamination of the PCR, a negative control was included in all PCR runs. Detection of amplified fluorescent products was carried out at the end of the extension phase. The raw fluorescent data that included the Ct mean values were automatically generated by the Quant Studio 5 Real-time PCR system software. The wild-type probes were labelled with FAM and the mutant probes were label with VIC.

Table 1 Primer sequences and targeted genes associated with metronidazole resistance

Targeted genes	Primer sequences 5′–3′	
	Primers	Probes
ntr4	F 5′-GTCAGAGGCCAA GAAAAGCTTGCC-3′ R 5′-GAGCATCGCAGG TGATAACGTTC-3′	C213G 5′-CCTTCC AGGAATAGG CCACCAAAG TAC-3′*
ntr6	F 5′-CGTTGTTACAAA CAAGGAAAAACT CC-3′ R 5′-GGATGCACGCTC ATTCTTGA-3′	A283T 5′-TGGAAT GCAATAGCA GACACATGT -3′**

*VIC labelled

**FAM labelled

Control assays

Sub-culture of stored *T. vaginalis* clinical isolates

T. vaginalis isolates which were stored at − 80 °C were sub-cultured and metronidazole susceptibility assays were performed on the isolates. Sub-culture was performed by transferring 500 µl of the culture into 5-ml fresh Diamond's TYM medium supplemented with amikacin, amphotericin B, ampicillin, chloramphenicol, ciprofloxacin, and vancomycin at 48-h intervals until non- contaminated axenic cultures were obtained. Once axenic cultures were obtained, metronidazole susceptibility assays and DNA extraction were performed.

Metronidazole susceptibility assay

Metronidazole susceptibility was performed in 96-well flat-bottomed microtiter plates under anaerobic incubation conditions. Twofold serial dilutions of metronidazole were performed in Diamond's TYM medium. The resulting concentrations ranged from 0.25 to 16 µg/ml. *T. vaginalis* cultures were then standardised to an inoculum of 1.5×10^4 trichomonads/well. Each *T. vaginalis* isolate inoculum was then added into each well excluding the ATCC control wells. The *T. vaginalis* ATCC 50,148 strain was used as a control strain and untreated cultures of the respective isolates were used as growth controls. Plates were incubated in air-tight anaerobic jars containing Oxoid™ AnaeroGen™ 2.5-L gas pack (ThermoFisher Scientific, USA) and Oxoid™ Resazurin Anaerobic indicator strip (ThermoFisher Scientific, USA) at 37 °C for 48 h. *T. vaginalis* motility and growth were assessed using the inverted microscope at × 400 magnification.

T. vaginalis growth and motility were scored according to the scoring criteria described by Upcroft (Upcroft and Upcroft 2001). Trophozoite numbers were scored 1 + (0–10 motile parasites; not more than 20% coverage of well surface and significantly less active), 2 + (20 to 50% coverage of the well surface and some trophozoite motility), 3 + (more than 50% coverage of the well surface, almost confluent growth with much motility), and 4 + (confluent growth with full motility) (Upcroft and Upcroft 2001). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of metronidazole in which a score of 1 + was observed after 48 h of incubation. Breakpoints suggested by Upcroft were used (Upcroft and Upcroft 2001). MIC ≤ 1 µg/ml was considered susceptible, MIC = 2 µg/ml was considered intermediate (low-level resistance), and MIC ≥ 4 µg/ml was considered resistant (Upcroft and Upcroft 2001). All experiments were performed in triplicate for each *T. vaginalis* isolate.

DNA extraction from *T. vaginalis* isolates

DNA was extracted from the *T. vaginalis* isolates using the phenol–chloroform method (Shaio et al. 1997). Briefly, *T. vaginalis* cells were washed twice in phosphate-buffered saline (pH 7.4) by centrifugation at 1500 × *g* for 10 min. DNA was extracted from the cell pellets by adding 500 µl of lysis buffer and incubated at 65 °C for 30 min. The lysis buffer was prepared from concentrated stock solutions to obtain final concentrations of 100 µg of proteinase K, 450 mM NaCl, 15 mM sodium citrate, and 0.2% sodium dodecyl sulphate (SDS) per ml. The extracted DNA was then purified twice by adding an equal volume of phenol–chloroform (1:1; vol/vol) and centrifuged at 1500 × *g* for 10 min. DNA was then purified once with chloroform only. DNA extracts were then precipitated with 2 volumes of 95% ethanol (vol/vol) and 0.1 volume of 3 M sodium acetate (pH 5.2). The DNA pellets were then washed with 70% (vol/vol) ethanol, air-dried at room temperature, and dissolved in 50 µl of TE buffer. The concentration and purity of the extracted DNA was measured using the NanoDrop Spectrophotometer (ThermoFisher Scientific, USA).

Detection of nitroreductase genes from *T. vaginalis* cultured isolates

Nitroreductase genes (*ntr4* and *ntr6*) associated with resistance to metronidazole were amplified by quantitative PCR, using specific primers described by Paulish-Miller et al. (2014) (Table 1) on the extracted cultured genomic DNA. Each PCR reaction was performed in a final volume of 10 µl comprising 5ul Taqman master mix (ThermoFisher Scientific, USA), 10 µM (0.5 µl each) of forward and reserve primer, 0.5 µl probe, 1 µl template DNA, and 2 µl nuclease-free water. PCR

amplification was performed on the Quant Studio 5 real-time PCR detection system (ThermoFisher Scientific, USA), in a 96-well microtiter reaction plate. The cycling conditions comprised of a UDG activation stage for 2 min at 50 °C, initial denaturation for 2 min at 95 °C, denaturation for 15 s at 95 °C, annealing 15 s at 60 °C with extension for 1 min at 72 °C. To assess any contamination of the PCR, a negative control was included in all PCR runs. Detection of amplified fluorescent products was carried out at the end of the extension phase. The raw fluorescent data that included the Ct mean values were automatically generated by the Quant Studio 5 Real-time PCR system software. The wild-type probes were labelled with FAM and the mutant probes were label with VIC.

To confirm the identity of the PCR products, conventional PCR was performed on the samples using the ntr4 and ntr6 PCR primers only. The amplification reactions were performed in PCR with a total volume of 25 µl. The reaction contained 12.5 µl DreamTaq master mix (ThermoFisher Scientific, MA, USA), 9.5 µl distilled water, 0.5 µl of each primer (reverse and forward), and 2 µl of template DNA was used. The negative control contained 23 µl of PCR mixture and 2 µl of distilled water. Thereafter, the PCR tubes were placed into the thermal cycler and the following conditions were performed, for gene amplification initial denaturation at 95 °C for 5 min, thereafter 35 cycles: of 95 °C 30 s, annealing 60 °C for 1 min, elongation 72 °C for 1 min, and final elongation at 72 °C for 5 min.

The amplicons were sequenced in one direction using the Sanger approach. The sequencing was performed on an ABI3500XL genetic analyser at Inqaba Biotechnical Industries (Hatfield, Pretoria, South Africa). The ABI sequencing files were edited on CHROMAS (Technelysium, Queensland, Australia).

Data analysis

The statistical data analysis was conducted in a freely available Statistical Computing Environment, R software, version 3.6.3 using the RStudio platform. The population characteristics were described using frequency distribution. All the tests were conducted at 5% level of significance.

Results

Detection of *T. vaginalis* from vaginal swab samples

Of the 385 samples tested in this study, 47 samples were positive for *T. vaginalis*.

The prevalence of *T. vaginalis* among the HIV-positive women was 12.2% (47/385). The Ct values of the amplified samples are shown in supplementary Table 1. According to the Ct values, using a cut-off value of 25 for distinguishing low and high positives (< 25 high positives, > 25 low positives), 16 samples were high positives (Ct: < 25), and 31 samples were low positives (Ct: > 25).

Characteristics of the study women who tested positive for *T. vaginalis*

Educational level was significantly associated with being *T. vaginalis* positive ($p < 0.001$), most women reported attending high school, 85.1% when compared to 14.9% who attended college. Other characteristics significantly associated with testing positive for *T. vaginalis* included employment status, marital status, having a regular sex partner, age of first sex, lifetime sex partners, partner having other partners, condom use, partner having symptoms of STIs, trimester of pregnancy, previously treated for STIs, intravaginal practices, and risk of contracting STIs. Of the women who tested *T. vaginalis* positive, 78.7% of the women were unemployed when compared to 21.3% who reported being employed, $p < 0.001$. A higher proportion of positive women, 91.5% were unmarried when compared to 8.5% who reported being married, $p < 0.001$. Most women (93.6.0%) reported having a regular sex partner, compared to 6.4% who reported not having a regular sex partner, $p < 0.001$. The majority of the positive women had experienced their first sex between the ages of 15 and 20 years of age (63.8%) when compared to 34.0% of the women who had their first sex at older than 21 years of age, and 2.1% of the women had their first sex younger than 15 years of age, $p < 0.001$. The majority of the positive women reported having 2–4 lifetime sex partners (55.3%) when compared to 25.5% of the women who reported having greater than 4 lifetime sex partners, and 19.1% of the women reported having 1 lifetime sex partner, $p = 0.005$. The majority of the positive women reported not knowing if their partners had other partners (57.4%) when compared to 23.4% of the women who reported that their partners did not have other partners, and 19.2% of the women reported that their partners had other partners, $p = 0.002$. In addition, 72.3% of the women had reported not using condoms during sex when compared to 22.7% who used condoms during sex, $p = 0.002$. The majority of women reported that their partners did not have symptoms of STIs, 83.0% when compared to 17.0% who reported that their partners had symptoms of STIs, $p < 0.001$. The majority of the positive women were in their third trimester of pregnancy (73.9%) when compared to 23.9% of the women who were in their second trimester of pregnancy, and 2.2% of the women who were in their first trimester of pregnancy, $p < 0.001$. Most women who tested positive for *T. vaginalis* reported not being previously treated for STIs (66.0%) when compared to 34.0% who reported being previously treated for STIs, $p = 0.029$.

Most women who tested positive for *T. vaginalis* reported not engaging in intravaginal practices such as douching, 97.9% when compared to 2.1% who reported such practices, $p < 0.001$. The majority of positive women reported being at risk of contracting STIs (68.1%) when compared to 31.9% who reported not being at risk of contracting STIs, $p = 0.013$ (Table 2).

Detection of mutations associated with metronidazole resistance in control isolates

The control assays were used as validation assays before proceeding to the detection of mutations associated with resistance in the uncultured swabs. The sequencing of the *ntr4* and *ntr6* PCR amplicons showed a 99% identity with

Table 2 Characteristics of the women who tested positive for infection ($n = 47$)

Variable	Response	Overall ($n = 47$)	p-value
Age	Median (IQR)	30.0 (11.5)	0.184
Educational level	High school	40 (85.1)	< 0.001
	College, university	7 (14.9)	
Employed	No	37 (78.7)	< 0.001
	Yes	10 (21.3)	
Married	No	43 (91.5)	< 0.001
	Yes	4 (8.5)	
Regular sex partner	No	3 (6.4)	< 0.001
	Yes	44 (93.6)	
Partners HIV status	Negative	13 (27.7)	0.144
	Positive	22 (46.8)	
	Don't know	12 (25.5)	
Currently living with your husband/ regular partner	No	27 (57.4)	0.307
	Yes	20 (42.6)	
Age of 1st sex	< 15yrs	1 (2.1)	< 0.001
	15–20yrs	30 (63.8)	
	> 21yrs	16 (34.0)	
Lifetime sex partners	1	9 (19.1)	0.005
	2–4	26 (55.3)	
	> 4	12 (25.5)	
Partner has other partners	No	11 (23.4)	0.002
	Yes	9 (19.2)	
	Don't know	27 (57.4)	
Condom during sex	No	34 (72.3)	0.002
	Yes	13 (27.7)	
Partner circumcised	No	18 (38.3)	0.109
	Yes	29 (61.7)	
Partner has symptoms of STIs	No	39 (83.0)	< 0.001
	Yes	8 (17.0)	
Trimester pregnancy	First	1 (2.2)	< 0.001
	Second	11 (23.9)	
	Third	34 (73.9)	
Previously treated for STIs	No	31 (66.0)	0.029
	Yes	16 (34.0)	
Current symptoms of STIs	No	27 (57.4)	0.307
	Yes	20 (42.6)	
Intravaginal practices	No	46 (97.9)	< 0.001
	Yes	1 (2.1)	
Risk of getting STIs	No	15 (31.9)	0.013
	Yes	32 (68.1)	

T. vaginalis G3 nitroreductase family protein (Sequence ID: XM_001307905).

The susceptibility assays which were performed on the stored isolates revealed the presence of two drug-resistant isolates (Table 3). To confirm the presence of mutations associated with the resistant phenotype, metronidazole susceptible ($n=2$) and resistant isolates ($n=2$) were subjected to the amplification assays. According to the analysis, the presence of mutations in the *ntr4* and *ntr6* genes associated with resistance was detected in the resistant isolates and no amplification was observed in the susceptible isolates. This showed a link between phenotypic and genotypic resistance profiles (Table 3).

Detection of mutations associated metronidazole resistance

The Ct values of the samples testing positive for mutations and wild types associated with metronidazole resistance and metronidazole susceptible respectively are shown in Supplementary Table 2. The *ntr4* gene carrying the C213G mutation associated with metronidazole resistance was shown to be present in 21/47 samples (45%). The *ntr6* gene with the A238T mutation associated with metronidazole resistance was shown to be in 24/47 (51%) of the samples. A total of 19 samples (40%) carried mutations for both *ntr4* and *ntr6* (Table 4).

The *ntr4* gene lacking the C213G mutation (wild type) was shown to be present in 27/47 samples (57%) and the *ntr6* gene lacking the A238T mutation was shown to be in 25/47 (53%) samples. Since the study was aimed at detecting resistance profiles from the primary vaginal swab, certain clinical samples contained both mutant and resistant genotypes and this was expected.

Clinical factors associated with metronidazole resistance mutations compared with no metronidazole resistance mutations in any genes

Table 5 provides an overview of clinical factors associated with samples that had metronidazole resistance mutations in both genes compared to samples that had no metronidazole resistance mutations in any of the genes. Of the women who displayed resistance mutations for both genes, 54% reported not having current symptoms of STIs such as discharge, vaginal odour, vaginal itching, genital sore/ulcers, genital warts, and pain during urination and 46% reported having symptoms of STIs. Of the women who displayed no resistance mutations in any genes, 62% reported not having current symptoms of STIs when compared to 38% who reported having STI symptoms.

Of the women who displayed resistance mutations in both genes, 88% reported that their sexual partners did not have symptoms of STIs such as testicular pain, pain during urination, and discharge from the penis and 12% reported that their sexual partners did have symptoms of STIs. For the women who displayed no resistance mutations in any genes, a higher proportion (76%) reported that their sexual partners did not have symptoms of STIs whilst 24% reported that their sexual partners did have symptoms of STIs.

Of the women with resistance mutations in both genes, 77% reported had not been previously treated for STIs when compared to 23% who reported having been previously treated for STIs. For the women who displayed no resistance mutations in any genes, 52% had not been previously treated for a STI when compared to 48% who were treated in the past.

With respect to gestational age, of the women with resistance mutations in both genes, 4% and 15% were on their 1st and 2nd trimesters of pregnancy and 81% of women were in their 3rd trimesters of pregnancy. For the women who displayed no resistance mutations in any genes, 33% and 67% of the women were in their 2nd and 3rd trimesters of pregnancy.

Discussion

Metronidazole is the first line of treatment for *T. vaginalis* infections globally (Kissinger, 2015a). However, growing metronidazole resistance in *T. vaginalis* infections has emerged as a public health concern with long-term health consequences (Bouchemal et al. 2017; Kissinger 2015a; Marques-Silva et al. 2021). In South Africa, metronidazole is used in the syndromic management for the treatment of vaginal discharge syndrome for both the general population and pregnant women (Health and Africa, 2015). This

Table 3 Cultured *T. vaginalis* isolates used as positive controls for *nitroreductase* genes associated with metronidazole resistance

T. vaginalis isolates	Minimal inhibitory concentration (MIC)	Susceptibility profile	Amplification data	
			nrt4	*nrt6*
NK171	1ug/ml	Susceptible	No amplification	No amplification
NK184	1ug/ml	Susceptible	No amplification	No amplification
NK253	4ug/ml	Resistant	21.479 (Ct value)	24.383 (Ct value)
NK270	4ug/ml	Resistant	21.763 (Ct value)	24.850 (Ct value)

Table 4 Detection of mutations in the *ntr4* and *ntr6* genes for the 47 isolates testing positive for *T. vaginalis*

Sample number	Resistance genotype			
	Mutations in ntr4	*Mutation in ntr6*	No *ntr4 mutation*	No *ntr6 mutation*
BN025	Present	Present	Present	Present
BN026	Absent	Present	Present	Present
BN036	Present	Present	Present	Present
BN046	Present	Present	Present	Present
BN055	Present	Present	Present	Present
BN056	Absent	Absent	Absent	Absent
BN064	Present	Present	Present	Present
BN073	Present	Present	Present	Present
BN075	Absent	Present	Present	Present
BN088	Absent	Absent	Absent	Absent
BN089	Present	Present	Present	Present
BN092	Absent	Absent	Present	Present
BN093	Present	Present	Present	Present
BN094	Present	Present	Present	Present
BN101	Present	Present	Present	Present
BN118	Absent	Absent	Absent	Absent
BN124	Absent	Absent	Absent	Absent
BN125	Present	Present	Present	Present
BN127	Absent	Absent	Absent	Absent
BN139	Absent	Absent	Absent	Absent
BN142	Absent	Absent	Absent	Absent
BN178	Absent	Absent	Absent	Absent
BN185	Absent	Absent	Absent	Absent
BN187	Absent	Absent	Absent	Absent
BN188	Present	Present	Present	Present
BN189	Present	Present	Present	Present
BN190	Absent	Absent	Absent	Absent
BN194	Absent	Absent	Absent	Present
BN198	Present	Present	Present	Present
BN201	Present	Present	Present	Present
BN202	Absent	Absent	Present	Absent
BN205	Absent	Absent	Absent	Absent
BN206	Absent	Absent	Absent	Absent
BN209	Present	Absent	Absent	Absent
BN216	Absent	Absent	Present	Absent
BN219	Present	Present	Present	Present
BN232	Absent	Present	Present	Present
BN244	Absent	Absent	Absent	Absent
BN268	Absent	Present	Absent	Absent
BN269	Present	Absent	Present	Present
BN281	Absent	Absent	Absent	Absent
BN299	Present	Present	Present	Present
BN306	Absent	Absent	Absent	Absent
BN335	Present	Present	Present	Present
BN359	Absent	Absent	Absent	Absent
BN368	Present	Present	Present	Present
BN370	Absent	Present	Present	Absent
Total ($N=47$)	21 (45%)	24 (51%)	27 (57%)	25 (53%)

Table 5 Clinical factors associated with metronidazole resistance mutations

Factors $n=47$ (100%)	Resistance patterns	
	Mutations in any one or both genes $n=26$ (55%)	No mutations in any genes $n=21$ (45%)
Current symptoms of STIs*		
No	54%	62%
Yes	46%	38%
Partner has symptoms of STIs**		
No	88%	76%
Yes	12%	24%
Previous treatment for STIs		
No	77%	52%
Yes	23%	48%
Trimester of pregnancy		
1st	4%	0%
2nd	15%	33%
3rd	81%	67%

*Discharge, odour, itching, sore/ulcers, warts, pain during urination

**Testicular pain, pain during urination, discharge from the penis

is especially concerning for pregnant women since resistant *T. vaginalis* infections can have serious consequences for reproductive health, such as an increased risk of HIV and other STIs, as well as perinatal morbidity. Recent metronidazole resistance research studies conducted in South Africa have focused primarily on culture-based approaches rather than molecular-based methods for detecting metronidazole resistance profiles in *T. vaginalis* (Mabaso and Abbai 2021; Rukasha et al. 2013). Additionally, to our best knowledge, this is the first study in our setting to investigate mutations in *nitroreductase* genes associated with metronidazole resistance using molecular-based methods on uncultured vaginal swabs in HIV-positive pregnant women.

In this study, the prevalence of *T. vaginalis* among the HIV-positive women was 12.2%. This prevalence is in accordance with other studies that have been conducted in South Africa among HIV-positive pregnant women (Joseph Davey et al. 2018, 2019; Nyemba et al. 2021). The prevalence of *T. vaginalis* is diverse and depends on various factors. Herein, the factors that were significantly associated with the prevalent infection included attending high school only, being unemployed, being unmarried, not having a regular sex partner, age of first sex between 15 and 20 years of age, having 2–4 lifetime sex partners, partner having other partners, not using a condom, partner having symptoms of STIs, trimester of pregnancy, being previously treated for STIs, intravaginal practices, and risk of contracting STIs. Similar findings have been reported elsewhere (Joseph Davey et al. 2018; Kissinger 2015a).

Nitroreductase genes have been reported to be associated with metronidazole resistance (Ozcelik et al. 2018; Paulish-Miller et al. 2014). According to Ozcelik et al. (2018), the presence of *ntr4* and *ntr6* was associated with metronidazole resistance in *T. vaginalis* and categorised as low (MLC, 50 to 100 µg/ml), moderate (MLC, 200 µg/ml), or high (MLC, ≥ 400 µg/ml) (Ozcelik et al. 2018). In the current study, MIC ≤ 1 µg/ml was considered susceptible, MIC = 2 µg/ml was considered intermediate (low-level resistance), and MIC ≥ 4 µg/ml was considered resistant. In addition, the current study included a mini-validation assay in which the presence and absence of mutations were linked to susceptibility patterns of metronidazole. For the mini-validation assay, TaqMan primers and probes specific for the SNPs associated with the mutations were used. For the susceptible isolates, TV171 and TV184, there was no amplification observed for the presence of *ntr4* or *ntr6*. However, for the resistant isolates, TV253 and TV270, amplification for both *ntr4* and *ntr6* was observed (Table 3). The validation assays performed in this study showed a positive correlation between phenotypic and genotypic resistance profiles. Therefore, our findings are similar to that of Ozcelik et al. (2018). After having performed the validation assays, the presence of the mutations was determined in the clinical samples. In this study, mutations in *ntr4* associated with resistance to metronidazole were detected in 45% of the samples and mutations in *ntr6* were detected in 51% of the samples. In addition, 40% of the samples carried mutations for both *ntr4* and *ntr6* genes.

A study conducted among women attending gynaecology services in Turkey reported a prevalence of 33% for metronidazole-resistant *T. vaginalis* samples (Ozcelik et al. 2018). That study employed both conventional and molecular approaches to detect metronidazole resistance. A second Turkish study reported a prevalence of 7.5% for metronidazole-resistant *T. vaginalis* (Ertabaklar et al. 2016). Similarly, a study conducted in South Africa in *T. vaginalis* isolates obtained from HIV-positive women reported a low prevalence 6% of *T. vaginalis* metronidazole resistance by culture methods (Rukasha et al. 2013). In addition, Matini et al.(2016) and Ghosh et al. (2018) reported a low prevalence of metronidazole resistance, 2% (1/50) and 8% (8/100) in clinical isolates of *T. vaginalis* respectively (Ghosh et al. 2018; Matini et al. 2016). The prevalence estimates of metronidazole-resistant *T. vaginalis* in the USA ranged from 4.3 to 66% (Bosserman et al. 2011; Kirkcaldy et al. 2012; Krashin et al. 2010; Schwebke and Barrientes 2006). A study conducted in Egypt by Abdel-Magied et al. (2017a, b) among symptomatic and asymptomatic women showed a 8.2% prevalence for metronidazole resistance (Abdel-Magied et al. 2017a). Furthermore, Abdel-Magied et al. (2017a, b) assessed in vitro resistance among 30 Egyptian *T. vaginalis*-positive cases between March 2014 and February 2016 with treatment failure and reported

that all isolates, 100% were resistant to metronidazole (Abdel-Magied et al. 2017b). Another two studies that have been conducted in Egypt reported metronidazole resistance prevalence of 0% and 7.7%, respectively (Hussien et al. 2004; Mohamed et al. 2019). All of the studies described above have investigated metronidazole resistance using culture. There is a lack of studies that have determined metronidazole resistance profiles from the molecular level using non-culture methods. The data presented in the current study now reports novel information.

In the current study, the link between clinical factors and metronidazole resistance mutations was determined. According to the findings, most women who carried metronidazole resistance mutations did not present with symptoms of genital infections at enrollment, i.e. they were asymptomatic and would have not been managed by the syndromic approach. The lack of management may lead to the infected women transmitting the infection to their unborn baby or sex partner. A low proportion of women who carried metronidazole resistance mutations had been previously treated for STIs in the past by syndromic management. The lack of diagnosis before treatment initiation using this approach could have contributed to the observed metronidazole resistance mutations. The high proportion of the women who carried metronidazole resistance mutations were in their last trimester of pregnancy. If these infections persist, they can be passed onto their unborn child and have severe neonatal consequences. This study reinforces the need for laboratory diagnosis in order to identify the causative pathogens and antimicrobial surveillance of the pathogens.

Conclusion

The majority of studies that have been conducted focus on the detection of metronidazole resistance using culture-based methods, which explains a huge difference in the frequency of metronidazole resistance among these studies (Kissinger 2015a; Matini et al. 2016; Rukasha et al. 2013). Moreover, these studies focus mainly on general population of women; however, HIV-positive pregnant women are also at higher risk of being infected with *T. vaginalis* and developing resistance to metronidazole (Matini et al. 2016; Rukasha et al. 2013; Schwebke and Barrientes 2006). Studies have shown that molecular-based methods can be used for the detection of metronidazole resistance profiles (Ozcelik et al. 2018; Paulish-Miller et al. 2014). However, there is a lack of this data from our setting.

The current study found a high level of metronidazole resistance, which is of concern, since metronidazole is currently used in syndromic management for STIs and the syndromic management does not include antimicrobial surveillance. Molecular-based methods are rapid, have high sensitivity and specificity, and they can quickly detect specific gene mutations simultaneously from clinical isolates (Alessio and Nyirjesy 2019; Bruni et al. 2019). Based on the findings from this study, molecular-based assays for monitoring metronidazole resistance profiles using *nitroreductase* genes may serve as a feasible method for antimicrobial surveillance studies for *T. vaginalis*.

Limitations

This study had the following limitations: A small sample size (47) was available for testing despite the small sample size, and most of the samples had gene mutations associated with metronidazole resistance. Furthermore, the study participants were recruited from a single clinic; however, King Edward VIII Hospital is a central tertiary hospital that services the most of Durban's population. The study was also limited to HIV-positive pregnant women only. Future research should include a more general population in order to provide more data on antimicrobial resistance patterns in *T. vaginalis.* The study did not investigate all potential genes associated with metronidazole resistance such as *pyruvate ferredoxin* or *flavin reductase*. This is now being considered as a future research endeavour.

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Author contribution NSA designed and funded parts of the study. BN, NM, and NSA recruited the study population. BN performed all the laboratory testing. KSH and RS provided some of the laboratory reagents. PT performed the statistical analysis. BN and NSA wrote the first draft of the manuscript. All authors approved the final version of the manuscript.

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Data availability Data will be available upon request.

Code availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval The study was approved annually by the Biomedical Research Ethics Committee (BREC), University of KwaZulu-Natal as well as KwaZulu-Natal Department of Health.

Informed consent Written informed consent was obtained from all antenatal women that were enrolled in this study.

Consent of publication Not applicable.

Conflict of interest The authors declare no competing interests.

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