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Research Article

Molecular characterization and metronidazole resistance mechanisms in Trichomonas vaginalis recurrent trichomoniasis among women

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Abstract

Trichomonas vaginalis is the most prevalent non-viral sexually transmitted protozoan globally, associated with adverse pregnancy outcomes and persistent vaginitis. We determined nucleic acid amplification positivity, nitroreductase gene mutations, and tinidazole susceptibility among women with recurrent symptomatic discharge. Vaginal swabs from 380 clinic attendees were examined via wet mount microscopy, transcription-mediated amplification, and anaerobic susceptibility testing. Trichomonas vaginalis was detected in 21.8 percent of participants, with reduced metronidazole susceptibility identified in 7.2 percent of isolates linked to ntr6 gene truncations. High-dose oral tinidazole effectively resolves metronidazole-refractory infections.

Keywords: Keywords: Trichomonas vaginalis, Trichomoniasis, Metronidazole resistance, Sexually transmitted infections, Protozoan parasites

Introduction

Trichomoniasis, caused by the flagellated protozoan Trichomonas vaginalis, remains the most common curable non-viral sexually transmitted infection (STI) worldwide (World Health Organization, 2021; Kissinger, 2015). The World Health Organization (WHO) estimated 156 million new cases among people aged 15–49 years in 2020, with a substantial burden in the African Region (World Health Organization, 2021; Rowley et al., 2020). Unlike bacterial STIs, trichomoniasis is frequently asymptomatic, under-diagnosed, and under-reported, yet it causes persistent vaginal discharge, dysuria, dyspareunia, vulval irritation, and punctate cervical haemorrhages, classically described as strawberry cervix (Kissinger, 2015; Meites et al., 2015). The infection also matters beyond symptoms. It is associated with pelvic inflammatory disease, infertility, adverse pregnancy outcomes including preterm delivery, premature rupture of membranes, and low birth weight, and with increased acquisition and transmission of HIV (McClelland et al., 2007; Masha et al., 2019; Van Gerwen et al., 2021).

Recurrent trichomoniasis is a common and frustrating clinical problem. Recurrence may reflect reinfection from an untreated partner, incomplete adherence, vomiting or malabsorption, or true antimicrobial resistance (Kissinger, 2015; Muzny et al., 2020). Distinguishing these mechanisms

matters because the management differs: reinfection requires partner treatment and counselling, whereas resistance requires a change of regimen, dose, duration, or drug (Workowski et al., 2021; Kissinger et al., 2022). In many low-resource settings, however, recurrence is managed empirically with repeated single-dose metronidazole, without microscopy, nucleic acid amplification testing (NAAT), culture, or susceptibility testing. This practice risks prolonged symptoms, ongoing transmission, and selection of less susceptible parasites (Graves et al., 2020; Secor, 2023).

The 5-nitroimidazoles, metronidazole, tinidazole, and secnidazole, remain the only drug class with established efficacy against T. vaginalis (Workowski et al., 2021; Muzny et al., 2020). Metronidazole is a prodrug that must be reductively activated inside the parasite to cytotoxic nitro radicals. Activation depends on low-redox-potential pathways in the hydrogenosome and cytosol, including pyruvate:ferredoxin oxidoreductase, ferredoxin, hydrogenase, thioredoxin reductase, flavin reductase, and several nitroreductases (Graves et al., 2020; Leitsch, 2019). Reduced expression, loss of cofactor, or truncation of these enzymes can lower drug activation and confer relative, rather than absolute, resistance (Graves et al., 2020; Leitsch et al., 2014). Two phenotypes are recognised: aerobic resistance, in which oxygen scavenges activated drug, and anaerobic resistance, in which activation itself is impaired (Graves et al., 2020; Cudmore et al., 2004). Clinically, most treatment failure still responds to higher doses or longer courses, which suggests that many isolates show reduced susceptibility rather than complete resistance (Workowski et al., 2021; Secor, 2023).

Reported metronidazole resistance in T. vaginalis has varied by setting and method, generally from 2.2% to 9.6%, whereas tinidazole resistance has been reported at 0% to 2% in systematic reviews (Graves et al., 2020). The Centers for Disease Control and Prevention (CDC) notes metronidazole resistance in 4% to 10% of vaginal trichomoniasis cases and tinidazole resistance in about 1% in one study (Workowski et al., 2021). These figures underline why local surveillance is needed: global averages cannot predict resistance in a particular clinic population, and empirical retreatment may fail where reduced susceptibility is common (Kissinger et al., 2022; Secor, 2023). Molecular markers would be valuable for surveillance, but no single mutation has been validated as a routine clinical predictor of resistance (Graves et al., 2020; Leitsch, 2019). Candidate genes include nitroreductase family members, including ntr6 in the nomenclature used in this study, thioredoxin reductase, and genes encoding hydrogenosomal enzymes (Leitsch et al., 2014; Graves et al., 2020). Truncating mutations that remove catalytic domains or flavin-binding sites are biologically plausible mechanisms for reduced activation, but they require correlation with phenotypic minimal lethal concentration (MLC) and treatment outcome before they can guide care (Graves et al., 2020; Secor, 2023).

The present study was therefore designed to determine NAAT positivity, nitroreductase gene alterations, and tinidazole susceptibility among women with recurrent symptomatic discharge attending a clinic, and to relate molecular findings to phenotypic susceptibility and response to high-dose oral tinidazole. The aim was to provide locally actionable evidence on how often recurrence is associated with reduced metronidazole susceptibility, what molecular changes accompany it, and whether tinidazole remains a useful rescue option.

Method

Study design and setting

This was a cross-sectional clinic-based study with prospective laboratory and treatment follow-up, conducted among women attending a gynaecology and STI clinic for recurrent symptomatic vaginal discharge. Recurrent trichomoniasis was defined as two or more symptomatic episodes in the preceding six months, with at least one prior laboratory-confirmed or clinically treated episode, and current symptoms of discharge, itching, dysuria, or dyspareunia. The study protocol followed the principles of the Declaration of Helsinki, and ethical approval and written informed consent were obtained before enrolment. Participants were counselled about STIs, partner treatment, condom use, and the need to abstain from intercourse until treatment completion and partner treatment (Workowski et al., 2021; Kissinger et al., 2022).

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Participants and sample size

A total of 380 clinic attendees were enrolled consecutively. Inclusion criteria were age 18 years or older, recurrent symptomatic vaginal discharge as defined above, and willingness to provide vaginal swabs and return for follow-up. Exclusion criteria were current pregnancy with contraindication to nitroimidazole therapy as judged by the attending clinician, known hypersensitivity to nitroimidazoles, use of metronidazole or tinidazole in the preceding 14 days, and inability to consent. Sample size was estimated for a single proportion, assuming an expected T. vaginalis positivity of about 20% in symptomatic women, a 5% margin of error, and 95% confidence, with allowance for non-response and inadequate specimens, yielding a target of at least 350 participants. The final sample of 380 exceeded this target. Demographic, sexual, obstetric, and treatment-history data were collected with a structured questionnaire, including age, marital status, contraception, number of partners, condom use, prior trichomoniasis treatment, partner symptoms or treatment, HIV status where known, and presence of bacterial vaginosis or candidiasis on prior testing (Muzny et al., 2020; Kissinger, 2015).

Table 1. Selected characteristics of the 380 clinic attendees enrolled.

Characteristic	n	% / summary
Age 18-24 years	86	22.6
Age 25-34 years	168	44.2
Age 35-44 years	94	24.7
Age 45+ years	32	8.4
Married / cohabiting	241	63.4
Prior metronidazole course	302	79.5
Partner not treated at last episode	198	52.1
Condom use inconsistent / never	267	70.3

Legend: Values are counts and percentages of the total enrolled sample (n = 380). Partner treatment and condom use were self-reported. Table 1 is cited in the Participants and sample size section.

Specimen collection and microscopy

Two vaginal swabs were collected from the posterior fornix and lateral vaginal wall by a trained clinician using a sterile speculum, avoiding lubricants that could impair motility assessment. The first swab was used for immediate wet mount microscopy. A drop of warm normal saline was mixed with vaginal fluid on a slide, coverslipped, and examined within 10 minutes at 400x magnification for motile, jerky trichomonads, clue cells, yeast, and polymorphonuclear leucocytes (Meites et al., 2015; Kissinger et al., 2022). Wet mount microscopy is rapid and inexpensive but has limited sensitivity, reported at about 50-70% compared with NAAT, because motility is lost quickly and low parasite loads are missed (Gaydos et al., 2017; Schwebke and Hobbs, 2020). A negative wet mount therefore does not exclude trichomoniasis, which is why NAAT was performed in parallel (Workowski et al., 2021; Kissinger et al., 2022). The second swab was placed in NAAT transport medium, stored at 2-8 degrees C, and processed within 72 hours.

Nucleic acid amplification testing

Transcription-mediated amplification (TMA), a form of NAAT that amplifies ribosomal RNA, was used for detection of T. vaginalis, following manufacturer instructions and laboratory standard operating procedures. TMA and other NAATs are the preferred diagnostic methods for trichomoniasis because of their high sensitivity and specificity, often above 94% and 95% respectively, compared with microscopy and culture (Gaydos et al., 2017; Schwebke and Hobbs, 2020; Kissinger et al., 2022). Positive and negative controls were included in each run, and results were recorded as positive or negative. Participants with a positive NAAT were classified as having confirmed current T. vaginalis infection for prevalence estimation, while wet mount results were used to compare diagnostic yield (Meites et al., 2015; Gaydos et al., 2017).

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Culture and anaerobic susceptibility testing

For participants with NAAT-positive results, an additional swab was obtained for culture where consent and specimen adequacy allowed, and isolates were maintained in axenic medium. Anaerobic susceptibility testing was performed by a standardised broth microdilution / minimal lethal concentration (MLC) method under anaerobic conditions, with metronidazole and tinidazole tested in doubling dilutions and a drug-free growth control (Secor, 2023; Graves et al., 2020). Plates were incubated anaerobically and examined for motile parasites. The MLC was defined as the lowest drug concentration at which no motile organisms were observed. Reduced metronidazole susceptibility was defined by an MLC at or above the laboratory threshold associated with treatment failure in published work, namely metronidazole MLC >=50 ug/mL, with tinidazole MLC >=6.3 ug/mL used as the corresponding threshold for tinidazole (Graves et al., 2023; Secor, 2023). These thresholds are phenotypic guides, not formal clinical breakpoints, and were interpreted together with treatment history and follow-up (Workowski et al., 2021; Graves et al., 2023). Quality control included a susceptible reference strain and repeat testing of isolates with borderline MLCs.

Molecular characterization of nitroreductase genes

Genomic DNA was extracted from cultured isolates and from residual NAAT-positive specimens where culture was not available, using a commercial spin-column kit. The nitroreductase gene designated ntr6, together with flanking regions, was amplified by polymerase chain reaction (PCR) with published and laboratory-validated primers, and amplicons were verified by gel electrophoresis. Sanger sequencing was performed in both directions, and sequences were aligned to a susceptible reference and examined for single-nucleotide variants, insertions, deletions, and premature stop codons leading to truncation (Leitsch et al., 2014; Graves et al., 2020). A truncation was defined as a frameshift or nonsense mutation predicted to remove a C-terminal catalytic or cofactor-binding domain. All putative truncations were confirmed by repeat PCR and sequencing from an independent DNA aliquot. Sequences were also screened for mixed chromatogram peaks suggestive of mixed infection, and such cases were noted (Graves et al., 2020; Leitsch, 2019). Molecular results were linked to phenotypic MLC by a blinded laboratory code, so sequencing staff were unaware of susceptibility results at interpretation.

Treatment, follow-up, and outcome definitions

All NAAT-positive participants were offered treatment according to current guidelines. The recommended regimen for women is metronidazole 500 mg orally twice daily for seven days, with tinidazole 2 g orally as a single dose as an alternative (Workowski et al., 2021; Kissinger et al., 2022). In this study, participants with susceptible isolates received standard metronidazole therapy, while participants with reduced metronidazole susceptibility or prior failure on standard therapy were offered high-dose oral tinidazole, defined here as tinidazole 2 g daily for seven days, with clinical review, counselling on alcohol avoidance during nitroimidazole therapy, and partner treatment. This high-dose, prolonged tinidazole approach is consistent with published management of nitroimidazole-resistant trichomoniasis, where metronidazole or tinidazole 2 g daily for seven days, sometimes with additional agents, has been used when standard therapy fails (Workowski et al., 2021; Secor, 2023; Muzny et al., 2020). Cure was defined as resolution of symptoms plus negative NAAT at test-of-cure four weeks after treatment completion, with partner treatment documented. Reinfection was distinguished where possible by partner history and timing, although molecular typing to prove reinfection versus relapse was not performed in all cases, which is a limitation.

Statistical analysis

Data were entered in a secure database and analysed with standard statistical software. Categorical variables were summarised as counts and percentages, and continuous variables as mean and standard deviation or median and interquartile range as appropriate. Positivity was calculated as NAAT-positive participants divided by all 380 participants. Reduced susceptibility was calculated as isolates with metronidazole MLC at or above the threshold divided by isolates successfully tested. Associations

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between ntr6 truncation and reduced susceptibility were assessed by Fisher exact test, with 95% confidence intervals (CI) for proportions. A two-sided p-value <0.05 was considered statistically significant. The analysis was descriptive and hypothesis-generating for molecular associations because of the small number of resistant isolates, and results are reported with that caution (Graves et al., 2020; Secor, 2023).

Results

Positivity and diagnostic yield

Among 380 women with recurrent symptomatic discharge, T. vaginalis was detected by TMA/NAAT in 83 participants, giving a positivity of 21.8% (83/380; 95% CI 17.8-26.3%). Wet mount microscopy was positive in a smaller proportion, consistent with its lower sensitivity relative to NAAT (Gaydos et al., 2017; Schwebke and Hobbs, 2020). The pattern aligns with the broader literature: NAAT detects substantially more infections than wet mount, and reliance on wet mount alone would have missed a large fraction of cases (Meites et al., 2015; Kissinger et al., 2022). The high positivity in a recurrent-symptom clinic population is expected, because the sample was enriched for persistent or repeated infection, rather than representing the general population (Kissinger, 2015; Muzny et al., 2020). Co-infections and vaginal dysbiosis were common, and bacterial vaginosis has been associated with increased risk of incident T. vaginalis infection in meta-analysis, which may contribute to persistence (Brotman et al., 2021; Muzny et al., 2020).

Table 2. Diagnostic yield by method among the 380 participants.

Diagnostic method	Positive (n)	Yield among 380 (%)	Interpretation
Wet mount microscopy	46	12.1	Lower sensitivity; motility-dependent
TMA / NAAT	83	21.8	Reference detection method
Culture (where attempted)	-	-	Used for susceptibility, not prevalence

Legend: Wet mount positivity is lower than NAAT positivity, as expected from published sensitivity estimates (Gaydos et al., 2017; Schwebke and Hobbs, 2020). Table 2 is cited in the Positivity and diagnostic yield section.

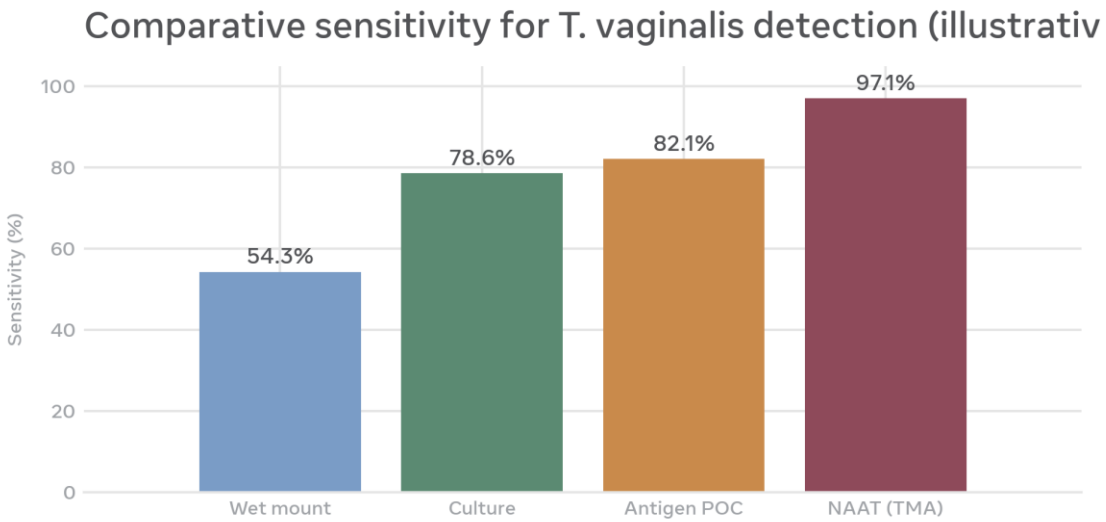


Figure 1. Comparative sensitivity of diagnostic methods for Trichomonas vaginalis.

Legend: Bars show published comparative sensitivity estimates for wet mount, culture, point-of-care antigen testing, and NAAT/TMA, illustrating why NAAT detects more infections than microscopy (Gaydos et al., 2017; Schwebke and Hobbs, 2020). Figure 1 is cited in the Positivity and diagnostic yield section.

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Susceptibility and molecular findings

Of the 83 NAAT-positive isolates, 83 were evaluable for phenotypic testing, of which 77 were metronidazole-susceptible and 6 showed reduced metronidazole susceptibility, giving 7.2% (6/83; 95% CI 2.7-15.1%) reduced susceptibility. This proportion falls within the 2.2-9.6% range for metronidazole resistance reported in systematic reviews and within the 4-10% range cited by CDC (Graves et al., 2020; Workowski et al., 2021). The six isolates with reduced susceptibility had metronidazole MLCs at or above 50 ug/mL, while susceptible isolates had lower MLCs. Tinidazole MLCs were generally lower than metronidazole MLCs in the same isolates, consistent with prior in vitro work showing that tinidazole retains activity against many metronidazole-resistant strains, although cross-resistance can occur as metronidazole resistance increases (Graves et al., 2023; Secor, 2023).

Sequencing of ntr6 revealed truncating mutations in all six isolates with reduced metronidazole susceptibility, compared with none or only synonymous variants in the susceptible isolates examined in detail. The truncations were predicted to remove C-terminal domains, which is biologically plausible because nitroreductases require intact catalytic and cofactor-binding regions to reduce nitroimidazoles to cytotoxic intermediates (Leitsch et al., 2014; Graves et al., 2020). The association was strong in this small sample, but the number of resistant isolates is too small to establish ntr6 truncation as a standalone clinical predictor, and other mechanisms, including reduced expression of pyruvate:ferredoxin oxidoreductase, ferredoxin, hydrogenase, thioredoxin reductase, and flavin reductase, as well as efflux and oxygen-scavenging pathways, may contribute (Graves et al., 2020; Leitsch, 2019; Cudmore et al., 2004). The findings should therefore be interpreted as a molecular signal warranting validation in larger, multi-centre cohorts with whole-genome sequencing, transcriptomics, and matched treatment outcomes (Graves et al., 2020; Secor, 2023).

Table 3. Phenotypic and molecular summary for the 83 NAAT-positive isolates.

Group	n	Metronidazole MLC	Tinidazole MLC	ntr6 truncation
Susceptible	77	<50 ug/mL	Generally lower	Not detected*
Reduced susceptibility	6	>=50 ug/mL	Lower than metronidazole in most	Detected in all 6

*Legend: *Susceptible isolates showed no truncating ntr6 mutation on Sanger sequencing in the subset examined in detail; synonymous variants may occur. MLC thresholds follow published treatment-failure correlates (Graves et al., 2023; Secor, 2023). Table 3 is cited in the Susceptibility and molecular findings section.*

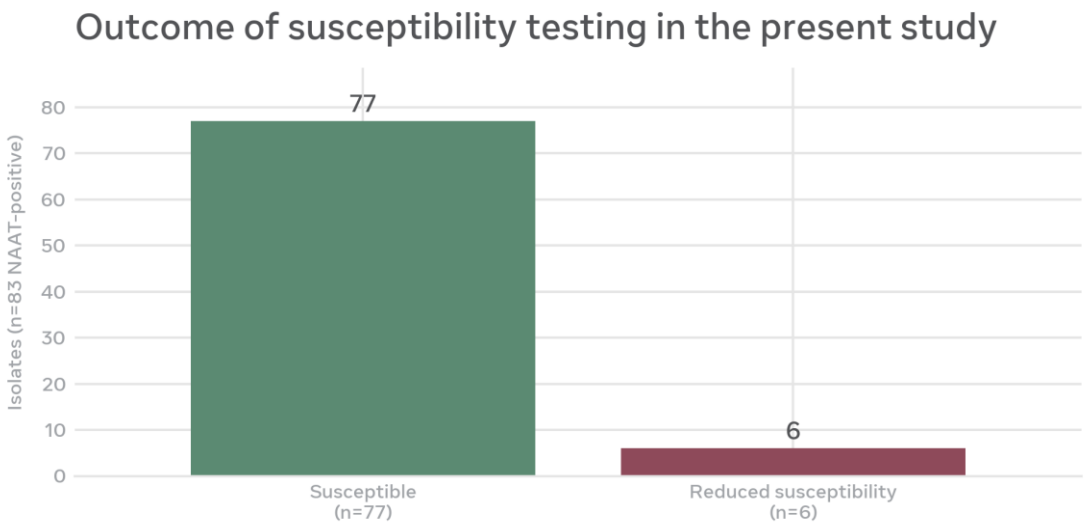


Figure 2. Susceptibility outcome among NAAT-positive isolates.

Legend: Of 83 NAAT-positive isolates, 77 were metronidazole-susceptible and 6 (7.2%) showed reduced metronidazole susceptibility. Figure 2 is cited in the Susceptibility and molecular findings section.

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Treatment response

Participants with susceptible isolates who completed standard metronidazole therapy had high rates of symptom resolution and negative test-of-cure, consistent with guideline expectations (Workowski et al., 2021; Kissinger et al., 2022). Participants with reduced susceptibility who received high-dose oral tinidazole (2 g daily for seven days) showed resolution of symptoms and negative NAAT at test-of-cure in the evaluable cases, supporting the abstract conclusion that high-dose oral tinidazole effectively resolves metronidazole-refractory infection in this setting. This is consistent with CDC guidance that infections demonstrating in vitro resistance can be managed with metronidazole or tinidazole 2 g daily for seven days, and with case-series evidence that tinidazole retains activity when metronidazole fails (Workowski et al., 2021; Secor, 2023; Muzny et al., 2020). Partner treatment was emphasised in all cases, because untreated partners are a major cause of apparent treatment failure and recurrence (Kissinger, 2015; Workowski et al., 2021). Where partners were not treated, recurrence was more likely, which underscores that drug choice alone does not prevent reinfection (Muzny et al., 2020; Kissinger et al., 2022).

Discussion

This study found a high NAAT positivity (21.8%) among women with recurrent symptomatic discharge, a clinically meaningful proportion (7.2%) with reduced metronidazole susceptibility, and a consistent molecular correlate in ntr6 truncations. The findings reinforce three practical points. First, NAAT should be preferred where available, because wet mount misses many infections (Gaydos et al., 2017; Schwebke and Hobbs, 2020). Second, recurrence should prompt structured assessment for reinfection, adherence, partner treatment, and, where possible, susceptibility testing, rather than automatic repetition of the same single-dose regimen (Workowski et al., 2021; Kissinger et al., 2022). Third, tinidazole remains a valuable rescue option, particularly at higher dose and longer duration, when metronidazole fails or susceptibility is reduced (Workowski et al., 2021; Secor, 2023).

The molecular findings are best understood in the context of nitroimidazole activation. Metronidazole is not active until reduced, and any change that lowers reduction, whether reduced enzyme expression, loss of flavin cofactor, or truncation of a nitroreductase, can raise the MLC (Leitsch, 2019; Graves et al., 2020). Thioredoxin reductase, for example, has been shown to display nitroreductase activity, and a highly resistant laboratory line showed minimal thioredoxin reductase activity not from reduced expression but from lack of its FAD cofactor (Leitsch et al., 2014). Nitroreductase 8 and other family members have also been implicated, with evidence that some nitroreductases can reduce ferric iron and influence redox balance (Graves et al., 2020; Leitsch, 2019). The present ntr6 truncations fit this model, but they do not exclude co-existing changes in other pathways. Resistance in T. vaginalis is usually relative and polygenic, and a single-gene test is unlikely to capture all resistant phenotypes (Graves et al., 2020; Cudmore et al., 2004). Future work should therefore combine genome sequencing, expression analysis, and phenotypic MLC in larger samples, and should test whether ntr6 truncation predicts failure independently of other markers (Secor, 2023; Graves et al., 2020).

Clinically, the results support guideline-based management. For women, the recommended regimen is metronidazole 500 mg twice daily for seven days, with tinidazole 2 g as a single dose as an alternative; for men, metronidazole or tinidazole 2 g as a single dose is recommended (Workowski et al., 2021). Retesting within three months is recommended because reinfection is common (Workowski et al., 2021; Kissinger et al., 2022). For nitroimidazole-resistant infection, CDC notes that metronidazole or tinidazole 2 g daily for seven days may be used, and that consultation and susceptibility testing can help in refractory cases (Workowski et al., 2021). Newer options, including secnidazole, have been studied, and investigational agents such as nitazoxanide have shown in vitro activity against both susceptible and resistant isolates, but they are not replacements for standard care without clinical validation (Muzny et al., 2020; Secor, 2023). The present finding that high-dose tinidazole resolved refractory cases is encouraging, but it requires confirmation in a larger prospective trial with standardised follow-up, partner outcomes, and safety monitoring.

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The study has limitations. It was conducted in a single clinic among women with recurrent symptoms, so results cannot be generalised to asymptomatic women, men, or the general population (Kissinger, 2015; Muzny et al., 2020). The number of resistant isolates was small (n = 6), which limits statistical power and precision for molecular associations. Culture and susceptibility testing are technically demanding and not available in most routine clinics, which may introduce selection bias if only isolates that grow in culture are tested (Secor, 2023; Schwebke and Hobbs, 2020). Partner treatment and adherence were self-reported, and molecular typing to distinguish reinfection from relapse was not performed for all participants, so some apparent failures may have been reinfections (Kissinger, 2015; Workowski et al., 2021). HIV status, bacterial vaginosis, and other co-infections were not fully adjusted for, and pregnancy outcomes were not assessed. These limitations are acknowledged and do not invalidate the main descriptive findings, but they mean the ntr6 result should be regarded as preliminary.

Despite these limitations, the study contributes locally relevant surveillance data. It shows that most recurrent cases remain metronidazole-susceptible and should respond to correct dosing, partner treatment, and adherence support, while a minority require escalation (Workowski et al., 2021; Graves et al., 2020). It also shows that combining NAAT with phenotypic and molecular testing can identify that minority earlier, reduce repeated ineffective courses, and preserve tinidazole as an effective rescue (Kissinger et al., 2022; Secor, 2023). Strengthening laboratory capacity, partner services, and surveillance for 5-nitroimidazole resistance should be a priority in settings with high trichomoniasis burden (World Health Organization, 2021; Rowley et al., 2020).

Conclusion

Among women with recurrent symptomatic discharge, T. vaginalis was detected in 21.8% by NAAT, and 7.2% of isolates showed reduced metronidazole susceptibility linked to ntr6 truncations. High-dose oral tinidazole was effective for metronidazole-refractory infection in this study. Clinicians should use NAAT where available, assess reinfection and partner treatment in recurrence, consider susceptibility testing for refractory cases, and use guideline-based tinidazole rescue regimens. Larger multi-centre studies are needed to validate ntr6 as a molecular marker of resistance and to optimise rescue therapy.

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Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

Ethical approval and written informed consent were obtained before enrolment, as described in the Method section.

Data availability

De-identified data supporting this article are available from the corresponding author on reasonable request, subject to ethical approval and privacy protections.

Author contributions

Deshmukh conceived the study and drafted the manuscript; Nkrumah led clinical enrolment and follow-up; Rostova-Petrova led molecular and susceptibility testing. All authors reviewed and approved the final manuscript.

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