Diagnosis and Treatment of Metronidazole-Resistant *Trichomonas vaginalis* Infection

76

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1 Introduction

Trichomonas vaginalis is the causative agent of trichomoniasis. There is an estimated prevalence of 276.4 million *T. vaginalis* infections worldwide. Important complications due to *T. vaginalis* infections are increased transmission of HIV, and infant morbidity due to preterm birth, low birth weight, and vertical transmission. Metronidazole and tinidazole are 5-nitroimidazole drugs used for treatment of *T. vaginalis* infections. *T. vaginalis* infections not responding to 5-nitroimidazole drugs used for standard therapy is a concern for adult sexual health due to persistence of infection and its complications as well as the risk for increased spread of disease due to clinical symptom relief following treatment without microbiological cure. At least a low level of metronidazole resistance is likely in 2–6 % of infections globally. Tinidazole resistance is strongly correlated to metronidazole resistance ($r=0.8709$, $P<0.0001$). Despite significant improvements in diagnostics in the past decade, new treatments are lacking. Alternative treatments tested in vitro rarely progress to clinical trials. So far, no consistently effective, non-nitroimidazole treatments are available to combat metronidazole-resistant *T. vaginalis* infections.

2 Trichomonas vaginalis

Trichomonas vaginalis is one of the four protozoan species of the family Trichomonadidae known to parasitize humans. Members of this family are characterized by their variable morphology, being spheroid or ovoid in form in axenic culture, but assuming an ameboid shape on contact with other cells [1, 2]. Trichomonads reproduce by longitudinal binary fission and lack a cystic stage, although large, round "pseudocysts" have been known to form under unfavorable conditions. All Trichomonadidae possess five anterior flagella, four of which are free moving. The fifth recumbent flagellum is anchored along the organism as a part of the undulating membrane. This membrane extends along at least half the length of the organism, and is supported by a noncontractile costa. Motility, described as "bobbing" or "quivering," is characteristic of this family of organisms [3, 4].

T. vaginalis is the only trichomonad known to cause disease in humans. It is the causative agent of trichomoniasis. *T. tenax*, usually found in the mouth, has been implicated in respiratory infection but its pathogenicity has never been confirmed [5]. *Pentatrichomonas hominis* [6] and *Trichomitus fecalis* have generally been isolated from the lower gastrointestinal tract. However, to date only one case of *T. fecalis* has been confirmed, leaving its identity as a human parasite in question [7].

Nutritionally, *T. vaginalis* is a fastidious organism. Lacking pathways for de novo synthesis of purines [8], pyrimidines [9], fatty acids, and sterols [10], the protozoan relies on salvage pathways to provide the necessary components of lipid and nucleotide metabolism. Amino acid synthesis and conversion is also thought to be limited. Carbohydrates are the preferred source of energy for metabolism. However, metabolic pathways for using amino acids, especially arginine, threonine, and leucine, as energy sources also exist [11], and energy generation using arginine probably takes place even if carbohydrates are available [12].

Energy metabolism takes place in the cytoplasm (for amino acids and carbohydrate glycolysis) and in an organelle called the hydrogenosome (for adenosine triphosphate (ATP) production via substrate-level phosphorylation). The hydrogenosome is analogous in structure and function to the mitochondrion in higher eukaryotes, although it lacks cristae and cytochromes [13, 14]. In the hydrogenosome, pyruvate is decarboxylated by an enzyme called pyruvate:ferredoxin oxidoreductase (PFOR). Ferredoxin serves as a terminal

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electron acceptor for PFOR, eventually leading to the production of acetate [15]. The fermentative metabolic processes of *T. vaginalis* also lead to the production of H_2, CO_2, lactate, and glycerol, the proportions of which vary depending on whether the organism grows in the presence or absence of oxygen [16].

A microaerophilic organism, *T. vaginalis* grows well under anaerobic conditions; however, some strains can tolerate oxygen well enough to be grown in ambient air. Optimum conditions are generally considered to be at 37 °C in moist air with 5 % CO_2 for growth in both axenic and tissue culture (this microaerophilic environment is similar to that found in the human vagina). Interestingly, *T. vaginalis* aerotolerance has often been found to reflect a particular strain's susceptibility to metronidazole, the drug most commonly used to treat trichomoniasis [17, 18].

3 Epidemiology

3.1 Prevalence and Transmission

With a prevalence of 276.4 million people infected worldwide, trichomoniasis is the most common nonviral sexually transmitted infection (STI) [19]. It is ubiquitous, being found in all races and cultures, but is especially prevalent among the underprivileged, injection drug users, individuals with multiple sex partners, and those who exchange sex for money [20]. It is estimated that at least one million new cases emerge in the United States yearly, many in African Americans [20, 21]. Globally, *T. vaginalis* infection is most prevalent in Africa and Asia, with infection rates reaching 40–60 % in some populations [19, 22].

Trichomoniasis has long been considered a disease of women, but the disease can also cause significant morbidity in men. Prevalence rates are highest in men with partners diagnosed with vaginal trichomoniasis [23]. Previous studies had shown that less than 5 % of the cases of nongonococcal urethritis are attributable to *T. vaginalis* [24]. However, a recent decline in the rates of chlamydial infection in the United States has been accompanied by an apparent increase in the frequency of *T. vaginalis* infection. Up to 17 % of male patients with nongonococcal, nonchlamydial urethritis are now confirmed to be suffering from trichomoniasis [25]. A similar rate of *T. vaginalis* infections was reported in men with urethritis attending a STI clinic in Malawi [26]. It is not yet clear, however, whether this trend represents a bona fide increase in the rate of trichomoniasis, or an improvement in diagnosis of the disease.

The prevalence of *T. vaginalis* infection in women has been found to vary significantly among different populations. Studies have shown that the rate of infection in women attending family planning clinics is about 5 % [27]. Reports from STI clinics indicate that anywhere from 1 to 40 % of female patients are identified with trichomoniasis [28]. The highest rates of infection are found in sex trade workers and women incarcerated in correctional facilities, where 50–75 % of these groups are infected with *T. vaginalis* [27].

The rate of transmission of *T. vaginalis* differs between sexes. Studies have shown that 15–70 % of men who have contact with an infected female partner will develop infection [29, 30]. Women exposed to the parasite via an infected male partner have a 65–100 % chance of developing trichomoniasis [30, 31]. *T. vaginalis* has been found to be able to survive for short periods of time outside of a host if sufficient moisture is maintained. Viable specimens have been obtained from body fluids (urine, semen, and vaginal exudates) 3–6 h after being emitted from the body [32, 33]. Live trichomonads have also been isolated from warm, damp washcloths 24 h after incubation [34], and from insufficiently chlorinated swimming pool water for up to 48 h [35, 36]. However, there have been no confirmed cases of trichomoniasis caused by exposure to contaminated objects.

Few nonsexual modes of transmission have been documented. Shared bathing water was implicated, though unconfirmed, as a source of infection in adolescent girls in Ndola, Zambia [37]; iatrogenic transmission is suspected in a female patient of a traditional healer who was diagnosed with *T. vaginalis* infection following the healer touching the female patient's genitals using his fingers [38]; lastly, perinatal transmission has been reported in a number of cases wherein clinical presentation is often respiratory disease [39–41].

3.2 Association with Human Immunodeficiency Virus and Other STIs

Patients with trichomoniasis are at an increased risk of contracting other STIs. This can be due to lifestyle risk factors (e.g., poverty or promiscuity), but may also be a reflection of the fact that *T. vaginalis* cytotoxicity towards urogenital tract epithelial cells (and the increase in vaginal pH commonly seen in infections of women) helps to create an advantageous niche for other sexually transmitted infectious organisms [42]. It is also possible that a preexisting STI could increase the likelihood of developing a trichomonal infection upon exposure to the parasite. One clinical study reported that 30 % of women diagnosed with *T. vaginalis* infection were accompanied by at least one other STI [43].

Similar to other STIs, *T. vaginalis* infection significantly increases the risk of contracting human immunodeficiency virus (HIV) (odds ratio 2.74, 95 % CI 1.25–6.00; relative risk 2.57, 95 % CI 1.05–4.02; hazards ratio 2.05, 95 % CI 1.43–4.65) [44]. Reasons for increased risk include damage to the mucosal surface, disruption of normal flora and pH facilitating viral penetration and survival, and an increased

number of immune cells at the genital mucosa enabling infection of these cells by HIV [44–46]. Another issue to consider is that *T. vaginalis* coinfection with HIV significantly increases HIV-1 RNA shedding in women (odds ratio 4.07, 95 % CI 1.78–9.37) [47]. Given that *T. vaginalis* and HIV are endemic in similar areas of the world, this means that prevention of trichomoniasis could be an important step in reducing global HIV/AIDS rates.

4 Clinical Aspects

4.1 Trichomoniasis in Men

Trichomoniasis in men is usually an asymptomatic carrier state [24, 48–51]. When symptomatic infection does occur, it presents as a mild urethritis. Clinical symptoms are similar to nongonococcal urethritis and include small amounts of clear or purulent discharge, and discomfort or a burning sensation during urination or after sexual intercourse. Rare cases of acute male trichomoniasis are characterized by more severe manifestations of urethral symptoms [52]. An extragenital *T. vaginalis* infection causing bilateral conjunctivitis has been reported in an adult male. Cause of the infection was linked to ocular exposure to genital secretions or fluids of a recent sex partner. No diagnosis of *T. vaginalis* in the sex partner was conducted [53].

The incubation period for *T. vaginalis* infection in men is usually less than 10 days, although longer incubation periods do occur [52]. Spontaneous resolution of both unapparent and symptomatic infection is common [49]. Studies using more sensitive diagnostic techniques are required to verify this data. One study showed that 70 % of untreated, symptomatic men had cleared the parasite within 2 weeks [29]. However, it has also been found that some cases of persistent nongonococcal urethritis, particularly those that have responded poorly to antibiotic therapy, may in fact be caused by resilient or resistant strains of *T. vaginalis*.

Prostatitis is the most common complication associated with trichomoniasis. Balanoposthitis, epididymitis, and other inflammations of the external genitalia are also frequently seen. Associations of trichomoniasis with prostate cancer remain undetermined [54, 55]. There is also evidence linking persistent *T. vaginalis* infection to urethral disease and infertility [56–58].

4.2 Trichomoniasis in Women

Unlike infection in men, trichomoniasis in women is usually persistent. Incubation periods range from 4 to 28 days [34]. Establishment of symptomatic infection usually involves a rise in the normal vaginal pH of 4.0–4.5 to a pH of 5.0 or higher (some of the virulence factors of *T. vaginalis* have been found to be inhibited at normal vaginal pH) [59]. This rise in pH is probably attributable to a concomitant decrease in acid-producing vaginal *Lactobacillus*, although the mechanism by which lactobacilli are inhibited or eliminated has not yet been elucidated but may be related to phagocytosis by trichomonads as demonstrated in vitro [60]. The symptoms of trichomoniasis are known to worsen during menses. This is likely a reflection of the fact that iron is an important mediator of many of the parasite's metabolic and pathogenic pathways (particularly cellular adherence) [61]. Nearly all cases of urogenital trichomoniasis are found in women of reproductive age, but it is not known if this is due to the unsuitability of the vaginal environment in premenarche and postmenopausal women, or is simply a reflection of the parasite's niche as an STI.

Asymptomatic infection rates are as high as 80 %, but about 30 % of women with an unapparent infection will develop symptomatic trichomoniasis within 6 months [23, 62]. Symptomatic infection is rated as mild, acute, or chronic. Chronic infection generally shows a similar clinical presentation to the mild form of the disease, but lasts for an extended period (i.e., years) and/or shows antibiotic resistance. Mild *T. vaginalis* infection is characterized by pruritus, dyspareunia, and sometimes dysuria. Small amounts of mucopurulent vaginal secretion are often present. Acute trichomoniasis usually presents with vulvar and vaginal erythema, and 2 % of cases show characteristic small hemorrhagic spots on the vagina and cervix, known as strawberry cervix [63, 64]. Use of a colposcope will increase the diagnosis of a strawberry cervix to about 90 % of patients with acute symptoms [64]. Copious discharge is often yellow or green in color, malodorous, and mixed with mucus [63, 64].

T. vaginalis infection has been implicated as a cause of cervical erosion and in the development of cervical cancer, although carcinogenicity likely can be related to high rates of coinfection with human papilloma virus [65]. Other complications associated with trichomoniasis arise when the parasite invades tissues outside the vagina. Skene's and Bartholin's glands are often infected, and ascending infection has been associated with endometritis and infertility [66]. *T. vaginalis* infection can be especially hazardous for pregnant women, predisposing them to premature rupture of the placental membrane, premature labor, and low birth weight babies [64, 67, 68].

4.3 Diagnosis

Diagnosis of trichomoniasis is difficult to make on the basis of clinical presentation alone. The high frequency of asymptomatic infection contributes greatly to underdiagnosis of the disease as well as the lack of resources for diagnosis particu-

larly in areas with the highest prevalence of trichomoniasis. In addition, the symptoms of *T. vaginalis* infection are often similar to those found in bacterial urogenital infection. As previously mentioned, symptomatic trichomoniasis in men presents as nongonococcal urethritis. Many symptoms associated with trichomonal infection in women are also common to bacterial vaginosis. For example, in STIs with bacterial etiology, vaginal pH is elevated in 90 % of cases [69, 70] and a positive "whiff" test, the presence of a fishy odor when vaginal exudate is mixed with 10 % potassium hydroxide, may be present (as in 50 % of trichomoniasis cases) [69, 71]. As coinfection with other STIs is not uncommon, it is important that specific tests for trichomoniasis be undertaken to prevent misdiagnosis and inappropriate treatment.

Microscopic diagnosis of *T. vaginalis* in women is usually performed after sampling vaginal exudates from the posterior fornix with a sterile cotton-tipped applicator. For men, urethral swabs are the most sensitive sample for culture, although a fresh semen sample or urine is also frequently used [48]. If a sufficient number of trichomonal cells are present (at least 10^4 trichomonads/mL), immediate diagnosis may be possible by microscopic examination of a wet mount, but is not used for diagnosis of *T. vaginalis* in men because wet mount lacks sensitivity [72]. *T. vaginalis* cells are similar in size to leukocytes, but can be identified by their characteristic motility [73, 74]. Unfortunately, the reliability of this test is highly variable, and its sensitivity has been quoted in the literature as anywhere from 40 to 90 % [75]. Additionally, if the test is not performed immediately, specimens are usually kept moist in physiological saline or transport medium. Although this does not (in the short term) affect the viability of trichomonads, it does have a profound negative effect on their motility [76] and thus the ability to recognize the organism on wet mount evaluation.

The diagnosis of trichomoniasis most often used due to affordability and acceptable sensitivity (44–75 %) is cultivation of the organisms in axenic medium [72]. Diamond's TYM (trypticase-yeast extract-maltose) supplemented with serum and antibiotics to prevent growth of bacteria and yeast has been found to yield reasonable results. Alternatively, a commercial InPouch TV (Biomed Diagnostics, California, USA) culture medium is available for detection of *T. vaginalis*. The InPouch TV system has benefits over the aforementioned Diamond's TYM preparation. The InPouch TV system can be stored at room temperature, is contained within a clear plastic pouch that can be examined by microscopy without needing to sample the culture, does not require immediate incubation after adding a sample, and does not require warming before use (Biomed product insert). Vaginal specimens can be inoculated into medium immediately or after storage in saline, and growth of motile trichomonads confirms a positive diagnosis. Diagnosis of *T. vaginalis* via cultivation also has the advantage that cultivated trichomonads can be maintained for further testing (i.e., antibiotic susceptibility). The disadvantage of this technique is that trichomonads do not grow quickly, and a minimum of 3 days for samples from women and 5 days for samples from men should be allowed before rendering a negative diagnosis [72, 77].

A number of fixed staining techniques have also been employed in the diagnosis of trichomoniasis. These include Giemsa [78], acridine orange [79], and the Papanicolaou (Pap) smear [80], among others. Unfortunately *T. vaginalis* cells often lose their characteristic shape on fixation. Studies on the diagnostic utility of the conventional or liquid Pap smear have shown its sensitivity to range from 44 to 96 % [72, 81]. A note of caution with these fixed staining techniques, there is a high frequency of false positive results (probably due to the similarity in size and shape of *T. vaginalis* and leukocytes). Between 20 and 30 % of uninfected women will be falsely diagnosed as having trichomoniasis [82].

Nucleic acid amplifications tests (NAATs) are the most sensitive tests available for the diagnosis of *T. vaginalis* in both men and women [48]. NAATs may not be used in resource-limited settings due to the required cost, infrastructure, and training. The APTIMA *T. vaginalis* assay (Hologic Gen-Probe Inc, California, USA) is the first United States Food and Drug Administration (FDA) approved NAAT that has reported sensitivity ranging from 88 to 100 %, with specificity of 98–100 % [72]. Other validated in-house polymerase chain reaction tests have been reported to have similar sensitivity and specificity [72].

NAATs have important advantages and disadvantages over culture. The need for viable organisms is not required for NAATs. Specimen storage and processing requirements are not as stringent. However, NAATs can detect nonviable organisms and may result in a false report of persistent infection following treatment. The follow-up time from treatment to reduce the number of false positive reports of infection using NAATs was investigated by Williams and colleagues [83]. Three weeks following treatment, 85 % of female vaginal samples were negative for *T. vaginalis* [83]. Resolution of clinical symptoms following treatment and a positive NAAT should not be immediately ruled as a false positive. Subclinical infections due to treatment failure have been suggested to be a source of a persistent infection rather than due to reinfection [84]. No FDA approved point-of-care NAAT tests are available at this time.

Three non-amplified point-of-care molecular tests are available. The OSOM TV Trichomonas Rapid Test (Sekisui Diagnostics, California, USA) and Kalon TV agglutination test (Kalon Biological, Surrey, UK) are two commercially available *T. vaginalis* antigen detection tests. Sensitivities range from 77–98 % and 55–99 %, respectively, with speci-

ficities >90 % [72]. A nucleic acid probe hybridization test, Affirm VPIII Microbial Identification Test (Becton Dickinson, Maryland, USA), provides detection of *T. vaginalis*, *Gardnerella vaginalis*, and *Candida albicans. T. vaginalis* sensitivity of the AFFIRM VPIII test has been reported to be 64 % with specificity of 100 % [72]. OSOM and Affirm VPIII have been approved for use as a diagnostic tool in the United States by the FDA. A major disadvantage of the non-amplified molecular tests is a lack of validation for use in asymptomatic women or for use in men [72]. On the other hand, these tests require little training or added infrastructure.

A new point-of-care molecular diagnostic tool is currently under development and testing on clinical vaginal swabs has been reported. The detection limit was evaluated to be five *T. vaginalis* cells. The sensitivity and specificity were 95.5 % and 95.7 %, respectively [85, 86]. The test targets a genetic biomarker that is present in multicopy within and unique to the *T. vaginalis* genome. Three stages of the test lead to identification of *T. vaginalis*. DNA from the test sample is extracted, and if target *T. vaginalis* DNA is present, then the target biomarker is amplified. The amplified products are identified using an electrochemical endpoint detection method. Results can be realized within 30 min.

Finally, it should be noted that isolation of trichomonads to confirm infection in males is often unsuccessful. It is hypothesized that this is because certain aspects of the male genitalia (e.g., an oxidative environment [87], zinc in prostatic fluid [88]) create an inhibitory milieu in which parasite numbers are greatly limited. In the absence of sensitive tests, it is important to assume that any male partner of an infected woman likely harbors the parasite himself. Concurrent treatment of the sexual partner(s) to prevent reinfection is essential.

4.4 Treatment

Metronidazole has been the drug of choice for the treatment of *T. vaginalis* infection since its development in 1959. Derived from the *Streptomyces* spp. antibiotic azomycin, metronidazole (1-(β-hydroxyethyl)-2-methyl-5-nitroimidazole) is a member of the nitroimidazole family of prodrugs whose metabolic products have been found to effectively eliminate infection by a number of protozoa and Gram-negative bacteria [89]. Other members of this family, including nimorazole, ornidazole, secnidazole, and tinidazole, are used throughout the world for the treatment of trichomoniasis. A nitroimidazole designated EU11100 was synthesized. This drug was shown to be both less toxic than metronidazole and effective at a lower concentration, but to date no clinical trials have been published [90].

Infants who contract *T. vaginalis* during vaginal delivery from an infected mother usually do not require treatment because infection generally resolves within a few weeks as the infant's (maternal) estrogen levels wane. However, if infection becomes symptomatic or progresses past the 6th week of life, metronidazole is generally administered. Treatment is often a single 50 mg/kg dose, or a 10–30 mg/kg dose daily for 5–8 days [91]. Canadian guidelines recommend a dose of 15–20 mg/kg, divided into three doses daily for 7 days, or a single dose of 40 mg/kg (to a maximum of 2 g) for the treatment of trichomoniasis in children.

Oral metronidazole is the treatment of choice for trichomoniasis in adults. The recommended regimens are a single 2 g dose oral metronidazole, a single 2 g dose oral tinidazole, or 500 mg oral metronidazole twice a day for 7 days [92, 93]. The single-dose treatment is preferred, as adherence is better than with multiple doses, and the overall amount of drug taken is reduced. However, the incidence and severity of side effects does increase slightly with the larger single dosage. Metronidazole can also be administered intravenously. This method is often utilized when patients show some intolerance to the drug, as side effects tend to be less severe than with oral treatment. Intravenous metronidazole is administered in a dosage of 500 mg to 2 g over 20 min [91].

A number of topical intravaginal preparations have been used to alleviate the symptoms of trichomoniasis in women. These medications include clotrimazole, nonoxynol-9, and povidone-iodine creams and gels, arsenical pessaries, furazolidone, paromomycin preparations, and both cream and insert metronidazole preparations. There are no topical treatments for trichomoniasis in men [20, 94].

The usefulness of non-nitroimidazole vaginal creams and inserts as a cure is doubtful, and no studies have shown definitive proof of efficacy [95]. However, these treatments are effective for relief of symptoms. The exception is hamycin, a drug related to amphotericin B. Currently in use in India as a topical treatment for trichomoniasis, hamycin has been found to effectively eliminate infection with both metronidazole-sensitive and -resistant strains of *T. vaginalis*. However, both clinical trials and in vitro testing on tissue culture have shown that the level of toxicity displayed by the drug toward eukaryotic cells makes it a poor choice of treatment [96].

Vaginal administration of metronidazole has been shown to be relatively ineffective as a cure, eliminating infection in up to 50 % of cases [97–99]. This is probably due to the fact that trichomonads are not always confined to the vagina, frequently invading Skene's, Bartholin's, and other glands, as well as the urethra [66]. Bioavailability of metronidazole as a vaginal suppository compared to IV infusion range from 20 to 56 % (oral metronidazole bioavailability is >90 % compared to IV infusion) [100]. As such, a topical vaginal medication is inadequate in completely eliminating infection. However, in cases of recalcitrant *T. vaginalis* infection, vaginal preparations are often added to the treatment regimen to

increase the chances of effecting a cure by increasing local drug concentration, and because of their comparatively lower risk of side effects (compared to oral administration) [97, 101]. A recent randomized controlled trial compared oral single-dose 2 g metronidazole versus a high-dose metronidazole and miconazole vaginal suppository (750 mg metronidazole/200 mg miconazole nitrate) twice a day for 7 days to treat *T. vaginalis*. The vaginal suppository resulted in similar efficacy of clinical and microbiological cure versus oral single-dose metronidazole (80 % versus 90 %, respectively) [102]. The sample size was small, but is evidence for a potentially useful combinatorial drug therapy for treatment of *T. vaginalis* infections in women that results in less systemic adverse effects compared to oral metronidazole.

Metronidazole regimens are generally well tolerated, and side effects are rarely of a severity that would necessitate discontinuation of metronidazole therapy. Common side effects include nausea and vomiting, headache, insomnia, dizziness, drowsiness, and rash. Patients taking oral metronidazole have also complained of dry mouth and metallic taste during the course of treatment. More serious side effects such as peripheral neuropathy, palpitation, confusion, eosinophilia, and leukopenia are rare, and seem to be associated with the nitroimidazole family. Cessation of therapy leads to mitigation of side effects, and no long-term adverse events have been identified in humans [28].

Cure rates for oral and intravenous metronidazole therapy of trichomoniasis range from 85 to 95 % on the first course of treatment. This rate increases if sexual partner(s) are treated simultaneously to prevent reinfection [91]. Partner treatment is highly recommended given the frequency of asymptomatic *T. vaginalis* infection.

Single-dose metronidazole treatment of *T. vaginalis* with concomitant bacterial vaginosis or HIV and nevirapine-based antiretroviral therapy have been associated with higher rates of treatment failure [103–106]. Multi-dose treatment with metronidazole, in these cases, should be considered while taking into account patient-specific risk of nonadherence.

5 Metronidazole Resistance

5.1 Mechanisms

Two proposed mechanisms for metronidazole resistance will be discussed. In both mechanisms metronidazole resistance is classified as aerobic or anaerobic. The first mechanism proposed involves metronidazole activation via hydrogenosomes [107–113]. The second mechanism is flavin reductase-based [17, 114–116]. Metronidazole enters *T. vaginalis* by passive diffusion wherein the drug is reduced by single and double electron transfers that result in production of toxic metabolites [107, 117]. Potential toxic radicals could be nitro radicals, nitrosoimidazole, or hydroxylamineimidazole [118]. However, the pathway for reduction of metronidazole to its active metabolites is still under debate. The target of the toxic metabolites is not clear. One target could be DNA, where transient binding of the active drug leads to disruption and breakage of chromosomal strands, and rapid cell death (within 5 h) [119]. The DNA of *T. vaginalis* contains about 71 % adenine and thymine residues, and these AT-rich regions are proposed to be both the site of metronidazole activity and the reason for the drug's specificity [120]. It is also possible that metronidazole metabolites target and disrupt proteins and protein trafficking [17, 109].

The first mechanism of metronidazole resistance in the hydrogenosome involves activity of enzymes proposed to be responsible for metronidazole activation. Within this organelle, the drug competes with hydrogenase (the terminal enzyme of pyruvate decarboxylation) for ferredoxin-bound electrons. Metronidazole is reduced and toxic metabolites via the formation of nitro radicals are produced [107, 117].

Aerobic resistance could be a result of impaired oxygen-scavenging mechanisms that lead to a decrease in the metabolism of metronidazole due to oxygen competition for ferredoxin-bound electrons. Increased oxygen concentration and reduction via ferredoxin leads to a decrease in the amount of metronidazole being reduced (i.e., less production of active metabolites), and the oxidation of metronidazole metabolites back into prodrug by oxygen and oxygen radicals (termed "futile cycling") [112, 121]. Decreased ferredoxin activity has also been implicated in aerobic resistance [113, 122], although oxygen-scavenging deficiency alone may be responsible [123]. Since metronidazole enters *T. vaginalis* through passive diffusion, reduced metabolism of the drug into its active form will result in less overall trafficking into the cell, and lower efficacy. Aerobic resistance is responsible for nearly all cases of clinically resistant trichomoniasis.

Anaerobic resistance develops when hydrogenosomal proteins involved in the reduction of metronidazole are downregulated or absent. Studies using laboratory-produced resistant strains of *T. vaginalis* and the related cattle infectious trichomonad, *Tritrichomonas foetus*, have shown that the transcription of ferredoxin, PFOR, and hydrogenase is drastically reduced or completely eliminated in highly resistant strains [110, 111]. Anaerobically resistant *T. foetus* strains often have modified hydrogenosomes that are significantly smaller than those found in metronidazole-sensitive trichomonads, presumably reflecting their decreased activity [111]. Reduced hydrogenosome size has only been demonstrated in laboratory-induced metronidazole-resistant strains of *T. vaginalis* and not in clinically resistant or susceptible strains [124]. Unlike aerobically resistant trichomonads, which use oxygen to detoxify metronidazole, anaerobically resistant *T. vaginalis* is extremely sensitive to oxygen and may survive only in an anaerobic environment. It is

hypothesized that this is because PFOR and hydrogenase have roles in protecting the trichomonad from reactive oxygen radicals. In addition, *T. vaginalis* possesses hydrogenosomal oxidase- and peroxidase-reducing enzymes that help protect the parasite from cell damage due to toxic oxygen species [108]. Reduction of hydrogenosomal function may lead to a downregulation in the activity of enzymes that protect *T. vaginalis* from oxygen stress. The extreme sensitivity of anaerobically resistant *T. vaginalis* to oxygen likely explains why such strains are rarely involved in disease, as the urogenital environments of men and women are aerobic and microaerophilic, respectively.

A second mechanism of resistance has been proposed because PFOR-mediated activation of metronidazole which requires ferredoxin was unaffected by knock-out of ferredoxin genes and the modified strain remained sensitive to metronidazole [17]. In the second mechanism of metronidazole resistance, Leitsch and colleagues have reported on cytosolic flavin reductase (FR), previously named NADPH oxidase, which is a key enzyme for flavin-mediated redox reactions in *T. vaginalis* [17, 114–116].

Diminished or absence of FR activity has been documented in clinical metronidazole-resistant *T. vaginalis* isolates [115]. Mutations of FR induced in vitro have led to metronidazole resistance in sensitive isolates [17]. Additionally, the authors propose that changes in ferredoxin, PFOR, and hydrogenase activity could be a result of reduced activity of flavin-mediated redox reactions. Therefore, changes observed in the first mechanism described do not induce metronidazole resistance, but are a result of resistance [17, 114].

In a study to determine the role of flavin-mediated redox reactions, resistance to metronidazole was attained following the use of diphenyleneiodonium (DPI) flavin inhibitor on *T. vaginalis* isolates grown under anaerobic conditions. The trichomonads were not viable when treated with DPI under microaerobic or aerobic conditions [114] and so these findings could not be applied to aerobic resistance. Whether thioredoxin reductase that was completely inhibited by DPI or FR that was nearly completely inhibited by DPI was responsible for induced metronidazole resistance was unclear. In another study, assays of clinically resistant isolates have demonstrated reduction of FR activity, rather than thioredoxin reductase of which the activity remained unaffected compared to metronidazole-sensitive isolates [115]. Yet, the clinically resistant isolates were aerobically resistant rather than anaerobically resistant and levels of FR were not consistently directly associated with levels of aerobic resistance [115].

Leitsch and colleagues [116] identified seven full length genes of FR, denoted *FR1-FR7*. FR1 activity was significantly impaired in metronidazole-resistant strains. In a laboratory-induced anaerobically resistant strain, C1res, and a clinical anaerobically and aerobically resistant strain, B7268, FR1 activity was absent. Interestingly, sensitivity to metronidazole under aerobic conditions was mostly restored in B7268 when a plasmid carrying a functional *FR1* gene was transfected. This finding is evidence of a role of FR in aerobic resistance, which was not elucidated by the DPI inhibitor study.

Impairment of oxygen-scavenging mechanisms described above remains an explanation for aerobic metronidazole resistance. The mechanism that leads to impairment of oxygen scavenging is unclear. Flavin reductase and NADH oxidase are the only two known oxygen-scavenging mechanisms of *T. vaginalis*. Metronidazole impairs NADH oxidase function [116]. Thus isolates with impaired FR function and treated with metronidazole under aerobic conditions accumulate intracellular oxygen that causes futile cycling of metronidazole [116, 125]. Futile cycling results in restoration of the parent drug, metronidazole, eliminating toxic metabolites. Still, further studies are required to elucidate the role of flavin-mediated redox pathways and ascertain a direct mechanism of resistance.

Other mechanisms of resistance that have been proposed include malate-dependent electron transport within the hydrogenosome, single nucleotide polymorphisms in nitroreductase genes, and inactivation by hydrogenosomal iron-sulphur flavoproteins [126–128]. Lastly, there is a lack of data to explain differences in cross-resistance of metronidazole and tinidazole.

5.2 Diagnosis of Resistance

Infection with metronidazole-resistant *T. vaginalis* is generally suspected when two standard courses of treatment fail to cure, and noncompliance and reinfection can be ruled out. Current estimates are that 2–6 % of cases of trichomoniasis will be caused by parasites with some degree of resistance to metronidazole [59, 129–133]. Rates in specific regions can be significantly higher; a study of prevalence of in vitro metronidazole resistance in Papua New Guinea reported detection of metronidazole resistance in 17.4 % of 23 cases examined [134]. Low or moderately resistant trichomonads are the cause of most recalcitrant infections, although highly resistant organisms have also been isolated from clinical samples.

Metronidazole susceptibility tests for *T. vaginalis* are similar to drug susceptibility assays for other microorganisms. Susceptibility testing usually follows the procedure reported by Meingassner and Thurner [130]. A number of samples of axenic medium containing a range of metronidazole concentrations (0.2–400 μg/mL) are prepared. The trichomonal isolate is then inoculated into each drug-medium sample and incubated, for at least 48 h. Metronidazole susceptibility can then be assessed by calculating the minimum inhibitory concentration (MIC) and/or minimum lethal

concentration (MLC) of drug for the organism. Inhibitory and lethal concentrations are obtained by observing the parasites for motility after the incubation period. The samples containing immobile trichomonads are then inoculated into fresh drug-free medium, incubated (again for at least 48 h), and reexamined for live cells. The MIC is the lowest metronidazole concentration at which nonmotile parasites survived (i.e., proliferated after the second inoculation). The MLC is the lowest concentration at which all trichomonads were killed (i.e., no growth on secondary inoculation).

In vitro metronidazole susceptibility testing is usually performed under aerobic conditions. This is partly because aerobic testing better reflects the environment in which *T. vaginalis* infection is found, and partly because anaerobic testing does not always accurately reflect clinical presentation [135]. In addition, MIC and MLC values can be over five times higher in aerobic testing compared to anaerobic [22], thereby allowing better discrimination of the resistance results.

Currently, there is no standard in vitro assay for the determination of *T. vaginalis* susceptibility to metronidazole. Different researchers favor various techniques, under different conditions (aerobic vs. anaerobic), to calculate different results (MIC vs. MLC). A survey of the literature on aerobic susceptibility testing shows that a strain of *T. vaginalis* having an MIC lower than 10 μg/mL, or an MLC lower than 50 μg/mL is generally considered metronidazole susceptible. A trichomonad with an MLC of >400 μg/mL (MIC of >50 μg/mL) would represent a highly drug-resistant strain of the parasite. Unfortunately, there is no direct correlation between the results of in vitro susceptibility assays and recommended dosages for clinical metronidazole treatment [136]. In vitro testing does not necessarily reflect the level of in vivo metronidazole susceptibility of a clinical isolate or predict outcome of treatment [132, 137, 138]. Thus there can be difficulty in determining a continuing course of therapy if primary treatment fails [139]. However, in one study the majority of patients treated according to metronidazole susceptibility results were cured following the use of susceptibility testing results [137]. Susceptibility testing is not routinely available in most diagnostic laboratories.

5.3 Standard Treatment After Initial Treatment Failure

Infection caused by metronidazole-resistant *T. vaginalis* can often be cured with increased doses of the drug and an extended course of therapy. Standard dosages following treatment failure include 500 mg oral metronidazole twice a day for 7 days, or 2 g oral metronidazole or tinidazole once a day for 5 days. Not surprisingly, there is a greater rate of adverse events associated with an increased (often double) treatment dose. In an attempt to limit side effects, treatment of refractory infection often combines oral and vaginal metronidazole therapy, or involves intravenous administration of the drug [140]. Some success has also been reported in a combination of standard metronidazole treatment and arsenical or clotrimazole pessaries, or zinc sulfate or betadine (povidone-iodine) douches [139, 141, 142]. Although evidence as to the efficacy of these therapies as cures is somewhat anecdotal, it is known that the treatments do ameliorate the symptoms of acute trichomoniasis.

Cases of highly drug-resistant *T. vaginalis* infection are difficult to resolve, as very high doses of metronidazole are toxic to the patient. With no alternatives to nitroimidazole drugs available, patients suffering from recalcitrant trichomoniasis are sometimes resigned to recurrent infection, relying on palliative measures to control symptoms. Fortunately such cases are infrequent. Overall, the cure rate for refractory trichomoniasis is 80 % for the first course of extended/combined therapy, assuming patient compliance and no reexposure [143]. The Centers for Disease Control and Prevention recommends consultation with a specialist and susceptibility testing for recalcitrant *T. vaginalis* infections [93].

5.4 Alternative Treatments for Metronidazole-Resistant Infections

There are very few therapeutic alternatives for the treatment of *T. vaginalis* infection. The 5-nitroimidazole family of drugs represents the only therapies currently proven to safely and effectively treat trichomoniasis. Of the nitroimidazoles, metronidazole and tinidazole have superior trichomonicidal activity, with most studies showing tinidazole to have a cure rate equal to that of metronidazole, but being effective at a slightly lower dosage (1.5 g single dose) [143–146]. Reports of high dose oral tinidazole in combination with intravaginal treatments such as tinidazole, clotrimazole, paromomycin, or ampicillin have demonstrated cure of recalcitrant infections [147–150].

A comparison of in vitro susceptibility of resistant isolates to metronidazole and tinidazole showed a strong correlation between metronidazole resistance and tinidazole resistance ($r=0.8709$, $P<0.0001$) [151]. Therefore, there is a definite need for non-nitroimidazole-based treatments.

An intravaginal preparation, paromomycin, has reported cure of 15 of 29 patients with recalcitrant trichomoniasis [147, 150, 152–158]. Two patients with metronidazole-resistant *T. vaginalis* infections responded to combination therapy of high-dose oral tinidazole combined with paromomycin cream intravaginally for 2 weeks. Unfortunately, as was the case with hamycin (mentioned previously), side effects have been noted that include pain and ulceration of the genital mucosa, making it unlikely that paromomycin is an ideal treatment alternative [156, 159].

Povidone-iodine has failed to cure recalcitrant *T. vaginalis* in three patients [148, 160, 161]. Cure was reported for two patients [162, 163]. Combination of povidone-iodine pessaries with intravaginal metronidazole cured two patients [162]. Povidone-iodine failed as alternative treatment to overcome nitroimidazole allergy in one case [164]. Eight and 30 % of patients that failed "orthodox" treatment also failed povidone-iodine treatment in a study that compared two durations of povidone-iodine treatment [165].

Arsenic had been used as a treatment for trichomoniasis before metronidazole was available. Acetarsol (arsenical pessaries) cleared metronidazole-resistant *T. vaginalis* infection in 4/6 patients reported from four case reports [141, 142, 147, 157].

Acidification of the vagina using acetic acid or boric acid has been reported in a handful of case reports. Based on five patient cases, acetic acid has not been reported to provide relief of infection [152, 158, 160, 166]. Multiple rounds of boric acid were required for microbiological cure in two patients [152]. One successful treatment with boric acid was reported as an alternative treatment due to metronidazole allergy [164].

Evidence for the use of nonoxynol-9 for recalcitrant *T. vaginalis* infections is limited. Two curative and two failed treatments have been reported [147, 157, 160, 162]. There are three reported failures and one success of combination of furazolidone and nonoxynol-9 as an alternative treatment for allergy to metronidazole and metronidazole-resistant *T. vaginalis* [148, 149, 153, 164]. The best evidence that suggest lack of efficacy comes from a randomized trial that reported 17.6 % cure of metronidazole-sensitive *T. vaginalis* infections using nonoxynol-9 versus 100 % cure rate using metronidazole [167].

Nifuratel and furazolidone are nitrofuran-class drugs. Nifuratel has not been licensed for use in the United States, but is used as a gynecological treatment of trichomoniasis in other regions. The efficacy of nifuratel has been reported in studies from the 1960s and 1970s with variable efficacy (38–80 %) [168, 169]. Recent studies have reported effectiveness in vitro and in vivo [170, 171]. Goodhew and Secor [170] noted contact dermatitis as an adverse reaction. Also, Evans, and Catterall [172] reported three adverse events of facial rash and generalized urticaria. However, a randomized trial by Mendling et al. [171] reported non-inferiority of nifuratel and a comparable safety profile. Furazolidone, despite in vitro activity, is unlikely to provide microbiological cure of *T. vaginalis* infections [20, 137, 143, 148, 149, 153, 164, 173, 174]. Furazolidone is contraindicated for use during pregnancy and is also not approved for use in the United States due to genotoxic and carcinogenic effects [175, 176]. Despite numerous reports of treatment failures with furazolidone, this drug has been used as a last resort in cases of recalcitrant *T. vaginalis* infections that have failed other alternative treatments.

A number of compounds containing nitro groups similar to nitroimidazoles have been investigated for activity against *T. vaginalis*. Nitazoxanide is a 5-nitrothiazolyl proven to be active against a broad spectrum of parasites in vitro. The drug was shown to exhibit trichomonicidal activity against both metronidazole-sensitive and -resistant strains. In addition, the drug has been shown to have low toxicity (at least in vitro) [124, 177, 178]. Nitazoxanide treatment of *T. vaginalis* has no reported successes [148, 173]. Analysis of the nitrothiazole derivative, niridazole, has shown it to possess multiple modes of action that contribute to broad-spectrum antimicrobial activity. Although specific mechanisms of action have not yet been elucidated, both metronidazole-sensitive and -resistant strains of *T. vaginalis* were found to be inhibited by the drug [179]. However, toxicity is a major concern and no reports of niridazole treatment of *T. vaginalis* were found. Sulfimidazole possesses two functional groups: a sulfonamide and a 5-nitroimidazole. In vitro testing has shown the drug to be effective against both aerobic and anaerobic bacteria, and metronidazole-sensitive and -resistant *T. vaginalis*. It should be noted however that MLCs for resistant trichomonads were approximately five times higher than for sensitive strains, potentially reflecting *T. vaginalis* resistance to the activity of the 5-nitroimidazole group [180]. Drugs with sulfonamide groups have had very limited treatment success [160, 181].

Disulfiram, a drug used to treat alcoholism, and its metabolite ditiocarb have shown in vitro trichomonicidal activity against both metronidazole-sensitive and -resistant strains of *T. vaginalis*. This is interesting since metronidazole can induce reactions similar to those of disulfiram, specifically nausea and vomiting, if taken with alcohol [170, 182].

This review does not provide treatment guidelines for patients infected with metronidazole-resistant *T. vaginalis* infections nor has an extensive list of all anecdotal treatments reported in literature. Some of the cases reported above and the dosages of the successful treatment have been summarized by Seña et al. [95]. However the successful case reports may be influenced by the previously failed regimens, combinations of therapy use and patient not returning for a late follow-up who had initial symptomatic improvement.

A vast number of studies, beyond the scope of this chapter and the highlights provided above, have been published that report in vitro susceptibility of *T. vaginalis*. For example, a screening of 1040 drugs from the US Drug Collection Library was conducted by Goodhew and Secor [170]. Two non-nitroimidazole drugs, disulfiram and nithiamide, were identified that had the best efficacy to inhibit growth of *T. vaginalis* in vitro, but were not as effective as metronidazole. Other drugs, plant-derived and microorganism-derived products tested in vitro have been summarized by Seña et al. [95]. Although preliminary in vitro research has been conducted on the trichomonicidal activity of a large number of

drugs, testing rarely proceeds to clinical trials. With rates of metronidazole-resistant *T. vaginalis* infection on the rise and current alternative treatments being unreliable, it is imperative that effective alternative therapies become available.

5.5 Infection Prevention

Infection control of sexually transmitted trichomoniasis is the same as for other STIs. Condoms are effective in preventing the spread of disease, and reduced transmission has been shown in women using either oral (hormonal) or prophylactic vaginal (i.e., nonoxynol-9) contraception [183]. Circumcision has not been proven to be an effective method to prevent *T. vaginalis* infections in males, but male circumcision may indirectly reduce prevalence in females (prevalence risk ratio 0.52, 95 % CI 0.05–0.98) [44, 184].

As *T. vaginalis* parasites can be passed from mother to newborn during vaginal birth, treatment of pregnant women to prevent perinatal infection is an option. Previously there have been concerns about metronidazole teratogenicity, based on studies showing mutagenicity in bacteria and carcinogenicity in mice [185, 186]. This led to the reluctance to treat pregnant women, or to limiting treatment to the second or third trimester. Several meta-analyses have shown, however, that children born to mothers treated with metronidazole showed no increase in birth defects compared to controls [187–190]. Additionally, it would seem beneficial to treat infections because there is a proven association between trichomoniasis and pregnancy complications such as preterm labor and low birth weight infants. Paradoxically, treatment may carry a risk of increased preterm labor. Four studies have been conducted that report on pregnancy outcomes following metronidazole treatment [191–194]. Generalizability of the findings is impeded by each study using a cohort with distinct population characteristics. Nonetheless, a recommendation to treat pregnant women infected with *T. vaginalis* is complicated by one of the four studies reporting a significant increase in risk of preterm labor. The other three studies report no significant change in risk of preterm labor. All four studies report no significant change in risk of low birth weight deliveries [195, 196].

Currently, there is no vaccine available against *T. vaginalis* infection and sufficient criteria for the infection to be reportable have not been met [197, 198]. However, the existence of a successful vaccination model in mice [46, 199], as well as a vaccine already commercially available for prevention of related *T. foetus* infection in cattle [200, 201], gives hope that eventually the disease will be preventable. Given the relationship between trichomoniasis and other STIs, especially HIV, the development of a vaccine would be an excellent step in preventing morbidity and mortality due to this and other sexually transmitted infections.

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