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Trichomonas vaginalis Metronidazole Resistance Is Associated with Single Nucleotide Polymorphisms in the Nitroreductase Genes $ntr4_{Tv}$ and $ntr6_{Tv}$

Teresa E. Paulish-Miller,[a] Peter Augostini,[b] Jessica A. Schuyler,[a] William L. Smith,[a] Eli Mordechai,[a] Martin E. Adelson,[a] Scott E. Gygax,[a] William E. Secor,[b] David W. Hilbert[a]

Femeris Women's Health Research Center, Medical Diagnostic Laboratories, Hamilton, New Jersey, USA[a]; Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA[b]

Metronidazole resistance in the sexually transmitted parasite *Trichomonas vaginalis* is a problematic public health issue. We have identified single nucleotide polymorphisms (SNPs) in two nitroreductase genes ($ntr4_{Tv}$ and $ntr6_{Tv}$) associated with resistance. These SNPs were associated with one of two distinct *T. vaginalis* populations identified by multilocus sequence typing, yet one SNP ($ntr6_{Tv}$ A238T), which results in a premature stop codon, was associated with resistance independent of population structure and may be of diagnostic value.

Trichomonas vaginalis is a flagellated, anaerobic protozoan and is the most common nonviral sexually transmitted pathogen, with ~3.7 million Americans infected (1). Patients suffering from infection are treated with metronidazole, a prodrug that is reduced to its active nitro radical anion form by anaerobic metabolism. Metronidazole resistance has been observed in 4.3 to 9.6% of clinical *T. vaginalis* isolates in the United States (1–3). The mechanism of metronidazole resistance in *T. vaginalis* is unknown. Laboratory-generated resistance is associated with downregulation of enzymes thought to reduce metronidazole, such as pyruvate-ferredoxin oxidoreductase (PFOR) and ferredoxin, as well as shrinking of the hydrogenosome, a mitochondrion-related organelle where these enzymes are located in *T. vaginalis* (4, 5). However, resistant clinical isolates harbor normal-sized hydrogenosomes and do not exhibit reduced transcription of the PFOR or ferredoxin genes (4, 5). In addition, disruption of the gene encoding ferredoxin did not cause a resistant phenotype (6). Other studies have demonstrated that laboratory-generated resistance is associated with reduced thioredoxin reductase activity and free flavins, both of which are proposed to reduce metronidazole as well (7, 8), and decreased flavin reductase activity has been observed in resistant clinical isolates (9). Lastly, another study identified 11 nitroreductase genes in the *T. vaginalis* genome (6), at least one of which was capable of metronidazole reduction (10). We hypothesized that loss of function of one or more of these nitroreductase proteins could mediate metronidazole resistance in some *T. vaginalis* isolates. To test this hypothesis, we obtained 200 *T. vaginalis* isolates from the American Type Culture Collection ($n = 5$) and the Centers for Disease Control and Prevention ($n = 195$). The characteristics of these isolates are described in Table S1 in the supplemental material. Metronidazole minimal lethal concentration (MLC) values were determined by the method of Narcisi and Secor (11). Briefly, isolates were grown in 0.2 to 400 μg metronidazole under aerobic conditions at 37°C for 48 h, at which point viability was determined by checking for motility by microscopy. Metronidazole resistance in *T. vaginalis* is categorized as low (MLC, 50 to 100 μg/ml), moderate (MLC, 200 μg/ml), or high (MLC, ≥400 μg/ml) (3, 12).

In preliminary experiments, we PCR amplified and sequenced the ntr_{Tv} genes from between 24 and 50 isolates in order to identify polymorphisms that were associated with resistance. For DNA isolation, *T. vaginalis* was grown anaerobically at 37°C in modified basal Diamonds medium (13) with 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% heat-inactivated horse serum. To extract DNA, ~10^6 trichomonads were pelleted by centrifugation and resuspended in breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris [pH 8], 1 mM EDTA). Then 200 μl of acid-washed glass beads (425 to 600 μm) and 200 μl of phenol-chloroform-isoamyl alcohol (25:24:1) were added, the sample was vortexed for 2 min, and 200 μl of Tris-EDTA (TE) was added. The phenol-chloroform extraction was repeated twice and DNA was precipitated with ethanol and sodium acetate, washed with ethanol, and resuspended in nuclease-free water. PCRs were performed in 40-μl volumes with 2.5 units FideliTaq (Affymetrix), 4 μl 10× buffer, 625 nM of each primer, 200 μM deoxynucleoside triphosphates (dNTPs), 2.5 mM $MgCl_2$, and 2 μl template. Reaction conditions were 95°C for 5 min; 35 cycles at 95°C for 1 min, 50°C for 1 min, and 68°C for 2 min; and then a final cycle at 68°C for 5 min. The primers used for PCR amplification and sequencing are listed in Table S2 in the supplemental material. Of the 40-μl reaction mixture, 20 μl was resolved on an agarose gel to confirm amplification, and the remaining 20 μl was purified using Wizard SV gel and the PCR Clean-Up system (Promega) and sequenced on an ABI3130 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. Each amplicon was sequenced with the same primers used for amplification, and both forward and reverse sequences were obtained for each amplicon.

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Address correspondence to David W. Hilbert, dhilbert@mdlab.com.
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TABLE 1 SNPs identified in ntr_{Tv} genes

Gene	SNP	Amino acid change	No. (%) of resistant isolates with:		No. (%) of sensitive isolates with:		P^a
			SNP present	SNP absent	SNP present	SNP absent	
$ntr3_{Tv}$	A176T	Q59L	1 (3.1)	31 (97)	0 (0.0)	33 (100)	
	G199A	G67R	21 (66)	11 (34)	22 (69)	10 (31)	
	G214A	G72R	0 (0.0)	32 (100)	2 (6.1)	31 (94)	
	G274A	D92N	9 (28)	23 (72)	4 (12)	30 (88)	
	A385G	S129G	31 (97)	1 (3.1)	33 (97)	1 (2.9)	
	T506C	L169P	0 (0.0)	32 (100)	2 (5.9)	32 (94)	
	A515C	E171A	0 (0.0)	32 (100)	1 (2.9)	33 (97)	
$ntr4_{Tv}$	T152A	V51D	3 (6.0)	47 (94)	5 (10)	45 (90)	
	G208C	E70Q	3 (6.0)	47 (94)	5 (10)	45 (90)	
	G76C	D26H	30 (60)	20 (40)	16 (32)	34 (68)	0.0056
	C213G	Y71STOP	33 (66)	17 (34)	17 (34)	33 (66)	0.0016
	C318A	H106Q	24 (48)	26 (52)	12 (24)	38 (76)	0.014
$ntr6_{Tv}$	G259A	E87K	6 (12)	44 (88)	1 (2.0)	49 (98)	
	T458C	V153A	1 (2.0)	49 (98)	1 (2.0)	49 (98)	
	A238T	K80STOP	20 (40)	30 (60)	2 (4.0)	48 (96)	<0.0001
	G427C	E143Q	38 (76)	12 (24)	18 (36)	32 (64)	0.0001
	T476C	V159A	20 (40)	30 (60)	2 (4.0)	48 (96)	<0.0001
$ntr9_{Tv}$	C288A	F96L	1 (3.7)	26 (96)	0 (0.0)	28 (100)	
	T350C	V117A	4 (15)	23 (85)	0 (0.0)	27 (100)	
$ntr10_{Tv}$	T395C	V132A	0 (0.0)	17 (100)	1 (14)	6 (86)	

[a] *P* values determined by two-tailed Fisher's exact test.

We found that $ntr1_{Tv}$, $ntr2_{Tv}$, $ntr5_{Tv}$, $ntr7_{Tv}$, $ntr8_{Tv}$, $ntr11_{Tv}$, and $ntr12_{Tv}$ were largely nonpolymorphic compared with the published sequence of the type strain G3 (14). However, we did detect single nucleotide polymorphisms (SNPs) in $ntr4_{Tv}$, $ntr6_{Tv}$, $ntr9_{Tv}$, and $ntr10_{Tv}$ (Table 1). These SNPs are denoted throughout this report with the first letter indicating the nucleotide or amino acid present in genome of the reference strain G3, the number indicating the position of the change relative to the open reading frame, and the second letter indicating the variant nucleotide or amino acid. Of these SNPs (amino acid changes), G76C (D26H), C213G (Y71STOP), and C318A (H106Q) in $ntr4_{Tv}$ ($P \leq 0.014$) and A238T (K80STOP), G427C (E143Q), and T476C (V159A) in $ntr6_{Tv}$ ($P \leq 0.0001$) were associated with metronidazole resistance. As only these two genes harbored SNPs significantly associated with resistance, we focused on them for the remainder of the study.

Further analysis revealed that the metronidazole resistance-associated SNPs were frequently associated within both $ntr4_{Tv}$ and $ntr6_{Tv}$, thus generating haplotypes (Table 2). For example, in $ntr6_{Tv}$, A238T (K80STOP) and T476C (V159A) were found only together, along with G427C (E143Q) (22/100 isolates); however, G427C (E143Q) was found singly (34/100 isolates). Similarly, in $ntr4_{Tv}$, G76C (D26H) and C213G (Y71STOP) were always found together (51/100 isolates, 36 of which also contained C318A [H106Q]). In addition, $ntr4_{Tv}$ and $ntr6_{Tv}$ haplotypes were associated with each other ($P < 0.0001$). For example, 14/22 isolates with all three resistance-associated $ntr6_{Tv}$ SNPs also had all three resistance-associated $ntr4_{Tv}$ SNPs, and 28/35 isolates with the reference $ntr6_{Tv}$ sequence also had the reference $ntr4_{Tv}$ sequence.

Based upon our findings with regard to $ntr4_{Tv}$ and $ntr6_{Tv}$, we extended our analysis to the other 100 isolates in our collection.

TABLE 2 Association of $ntr4_{Tv}$ and $ntr6_{Tv}$ haplotypes

$ntr4_{Tv}$ haplotype[a]	$ntr6_{Tv}$ haplotype (no. of isolates positive) (resistant [$n = 50$], susceptible [$n = 50$])									
	Reference[b]		G259A		G427C		A238T/G427C/T476C		Total	
	R	S	R	S	R	S	R	S	R	S
Reference	4	24	6	2	3	4	2	0	15	30
G76C/C213G	0	0	0	1	5	3	5	1	10	5
G76C/C213G/C318A	0	3	0	0	10	9	13	1	23	13
T152A/G208C	2	2	0	0	0	0	0	0	2	2
Total	6	29	6	3	18	16	20	2	50	50

[a] The $ntr4_{Tv}$ haplotype is displayed vertically, and the $ntr6_{Tv}$ haplotype is displayed horizontally. Numbers refer to the specific counts of resistant and sensitive isolates where the combination of haplotypes is found.
[b] Reference sequence refers to that found in the sequenced G3 isolate. R indicates metronidazole resistant (MLC ≥ 50 μg/ml), and S indicates metronidazole susceptible.

TABLE 3 Association of $ntr4_{Tv}$ C213G (Y71STOP) and $ntr6_{Tv}$ A238T (K80STOP) SNPs with metronidazole resistance

Phenotype	No. (%) of isolates with indicated genotype			
	$ntr4_{Tv}$ reference and $ntr6_{Tv}$ reference[a]	$ntr4_{Tv}$ C213G (Y71STOP) SNP and $ntr6_{Tv}$ reference	$ntr4_{Tv}$ reference and $ntr6_{Tv}$ A238T (K80STOP) SNP	$ntr4_{Tv}$ C213G (Y71STOP) SNP and $ntr6_{Tv}$ A238T (K80STOP) SNP
Total	85 (43)	76 (39)	2 (1.0)	34 (17)
Susceptible	56 (57)	38 (39)	0 (0)	4 (4.1)
Resistant[b]	29 (29)	38 (38)	2 (2.0)	30 (30)
Low	4 (18)	13 (59)	0 (0)	5 (23)
Moderate	10 (33)	10 (33)	0 (0)	10 (33)
High	15 (32)	15 (32)	2 (4.3)	15 (32)

[a] Reference sequence refers to that found in the sequenced G3 isolate.
[b] Resistance is defined as low (MLC, ≤100 μg/ml), moderate (MLC, 200 μg/ml), or high (MLC ≥400 μg/ml); isolates with MLC values between 100 and 400 μg/ml were categorized as moderate.

We focused on the $ntr4_{Tv}$ C213G (Y71STOP) and $ntr6_{Tv}$ A238T (K80STOP) SNPs, as they showed the strongest association with resistance for each gene (Table 1) and are predicted to cause nonsense mutations, thus providing a plausible biological mechanism for why they would be associated with metronidazole resistance. To this end, we developed quantitative real-time PCRs (qPCRs) to rapidly detect the presence of either the SNP of interest or the corresponding reference sequence. Reactions were performed in a 20-μl volume using iTaq master mix (Bio-Rad), 1 μM each primer, 200 nM probe, and 2 μl of template DNA. Reactions were performed on the MX3000P qPCR system (Agilent) using the following cycling conditions: 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 63°C for 45 s. The qPCR results were 100% concordant for the 100 isolates whose $ntr4_{Tv}$ and $ntr6_{Tv}$ genes had been sequenced. The combined results of sequencing and qPCR assays are listed in Table 3. The presence of metronidazole resistance was highly associated with the ntr_{Tv} genotype ($P < 0.0001$) (chi-square test). Neither SNP was present in 57% of susceptible isolates and 29% of resistant isolates, whereas both SNPs were present together in 30% of resistant isolates and only 4.1% of susceptible isolates ($P < 0.0001$ for both comparisons) (two-tailed Fisher's exact test). In contrast, the combination of $ntr4_{Tv}$ C213G (Y71STOP) and an intact $ntr6_{Tv}$ gene was present in 39% of susceptible isolates and 38% of resistant isolates. Lastly, two isolates (both resistant) harbored $ntr6_{Tv}$ A238T (K80STOP) alone and three isolates could be not be genotyped with regard to both genes.

However, among resistant isolates, there was no association between the ntr_{Tv} genotype and the degree of resistance. In addition, we analyzed the distribution of MLC values in each genotypic group and found that MLC values were significantly associated with the ntr_{Tv} genotype ($P < 0.0001$, Kruskal-Wallis test) (Fig. 1). MLC values were lower in the isolates lacking both SNPs than in

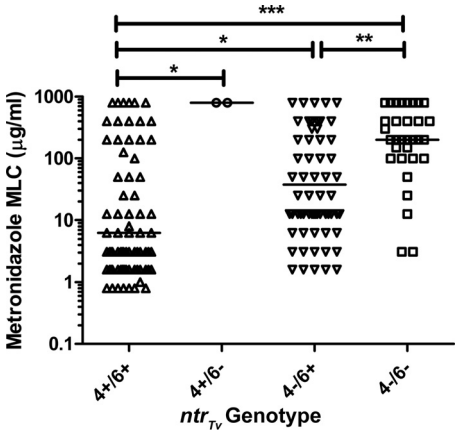

FIG 1 Metronidazole susceptibility in 197 isolates stratified by ntr_{Tv} genotype. Intact open reading frames (ORFs) are represented by +, and the presence of stop codons is indicated by −. Horizontal bars represent median values. Isolates with a range of MLC values (e.g., 100 to 200 μg/ml) are plotted using their mean MLC values. Isolates with an MLC value of >400 μg/ml are assigned a value of 800 μg/ml for display purposes. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

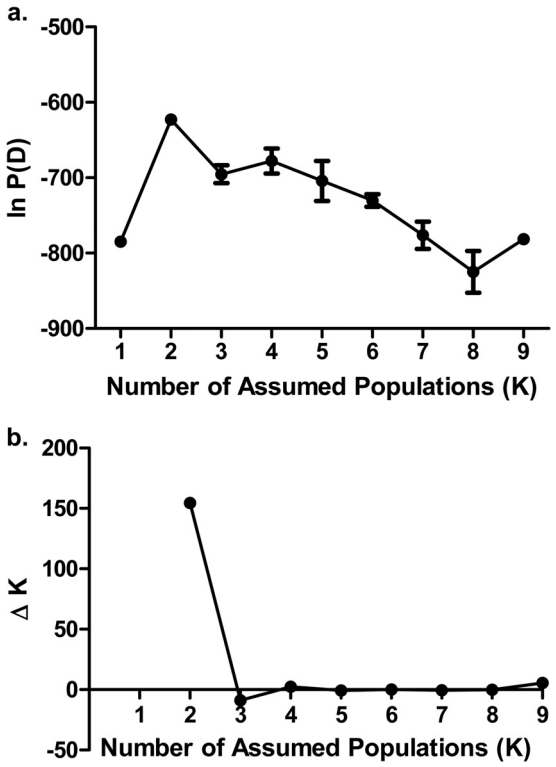

FIG 2 Determination of *T. vaginalis* population using Structure 2.3 to analyze SNP data from 100 isolates. (a) Mean values ± standard error of the means (SEM) ($n = 5$) for log probability of assumed population structure. (b) Second-order rate of change in likelihood (ΔK) between the number of assumed populations.

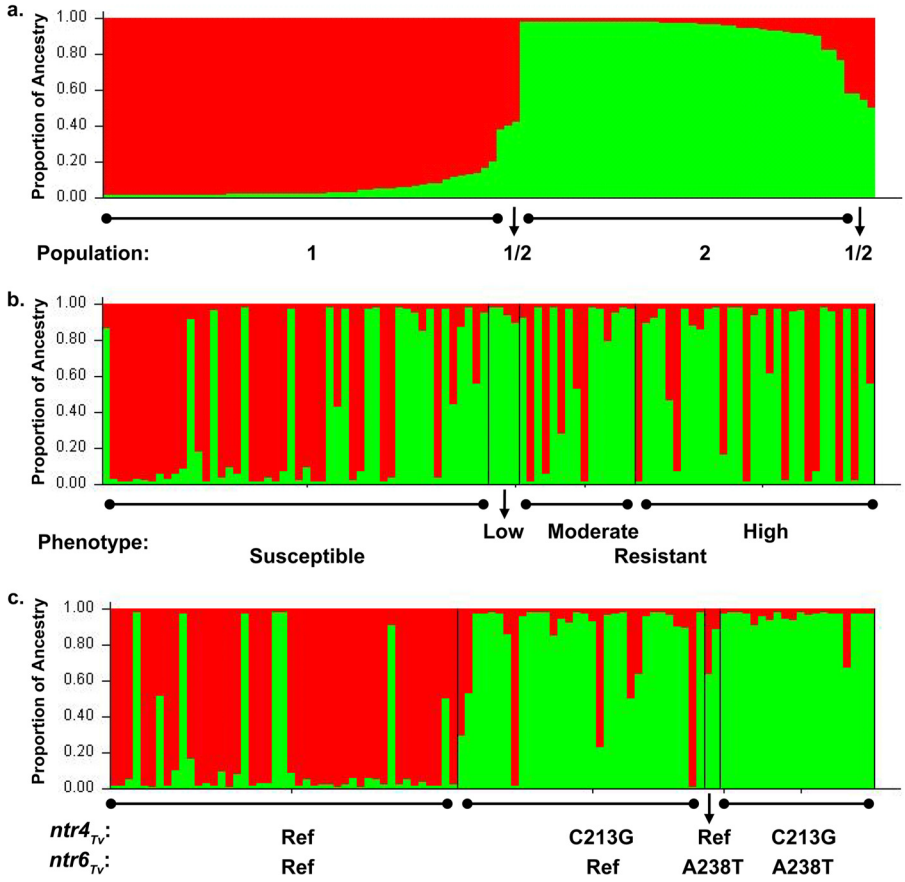

FIG 3 *T. vaginalis* population structure. Red represents ancestry from population 1, and green represents ancestry from population 2. (a) Total population sorted by *Q* (ancestry vector) (100 isolates), (b) population sorted by ascending MLC values (100 isolates), and (c) population sorted by ntr_{Tv} genotype (99 isolates, 1 excluded due to undetermined $ntr6_{Tv}$ genotype).

the isolates in each of the other groups ($P \leq 0.05$), especially the isolates harboring both SNPs ($P < 0.001$) (Dunn's posttest used for all pairwise comparisons). In turn, isolates with both SNPs had higher MLC values than those harboring only $ntr4_{Tv}$ C213G (Y71STOP) ($P < 0.01$).

Although we had identified SNPs with strong associations with metronidazole resistance, it is important that they be interpreted in light of the *T. vaginalis* population structure. Prior studies using microsatellite genotyping and multilocus sequence typing (MLST) have shown that *T. vaginalis* has a two-type population structure, with one population highly similar to an inferred ancestral population (type 1) and a second population that is more divergent and exhibits a higher degree of metronidazole resistance (type 2) (10, 14). To determine the population structure of our isolate collection, we utilized the MLST methodology (10). Briefly, we PCR amplified and sequenced seven different loci using template DNA from 100 isolates (50 each metronidazole resistant and susceptible), using the primers listed in Table 1. Reaction mixtures were the same as those used for ntr_{Tv} gene amplification. Reaction conditions were as follows: 95°C for 5 min; followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 68°C for 2 min; followed by 68°C for 5 min. PCR products were analyzed both by gel electrophoresis and by purification using Wizard SV gel and the PCR Clean-Up system (Promega) followed by sequencing on the ABI3130 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. Both forward and reverse sequences were obtained for each amplicon. Each allele for the seven different loci was assigned a numerical identifier, and these data were then analyzed using the program Structure 2.3 (15). Each run consisted of 100,000 burn-in simulations followed by data collection on 100,000 simulations; five independent runs were performed for each assumed population structure. To determine the population structure, the program was run using an assumed population structure of 2 to 9, and the $\ln P(D)$ (log probability) and ΔK (second-order rate of change in log probability) were determined. We found that an assumed population structure of 2 resulted in the highest log probability and that the transition from 2 to 3 populations resulted in the highest value for ΔK (Fig. 2). Based on these results, our isolate collection represented 2 populations, similar to those of the *T. vaginalis* collections analyzed in other studies (Fig. 3a) (10, 14).

The populations were analyzed with respect to the fixation index (F_{ST}), a 0-to-1 scale of population differentiation due to genetic structure, where 0 represents free interbreeding between populations and 1 indicates that all genetic differences between the populations are due to population structure, and the populations share no genetic diversity. Of the two inferred populations, population 1 was highly similar to an inferred ancestral population (where $F_{ST} = 0.018 \pm 0.0066$), whereas the other population (population 2) was highly divergent from the inferred ancestral

TABLE 4 Association of metronidazole resistance and ntr_{Tv} genotypes with inferred populations

Population	Characteristic (no. [%])							
	Total	$ntr4_{Tv}$ C213G (Y71STOP)	$ntr6_{Tv}$ A238T (K80STOP)	Metronidazole susceptible	Metronidazole resistant[a]			
					Total	Low	Moderate	High
1	43 (100)	2 (4.7)	0 (0)	29 (67)	14 (33)	0 (0)	5 (12)	9 (21)
2	50 (100)	44 (88)	19 (38)	18 (36)	32 (64)	4 (8)	11 (22)	17 (34)
1/2	7 (100)	4 (57)	2 (29)	3 (43)	4 (57)	0 (0)	3 (43)	1 (14)

[a] Resistance is defined as low (MLC ≤ 100 μg/ml), moderate (MLC > 200 μg/ml), or high (MLC ≥ 400 μg/ml); isolates with MLC values between 100 and 400 μg/ml were categorized as moderate.

population ($F_{ST} = 0.51 \pm 0.020$) (mean ± standard error). Based upon this analysis, we infer that populations 1 and 2 correspond to population types 1 and 2 identified by microsatellite analysis (14). Using an inferred ancestry cutoff of 0.75, we assigned 42 isolates to population 1 and 51 isolates to population 2, and 7 isolates consisted of an admixture of both populations (≥0.25 inferred ancestry from both populations).

The prevalence of metronidazole resistance was highly dependent upon population structure (Fig. 3b); 33% of isolates in population 1 (14/43 isolates) were metronidazole resistant compared to 64% (32/50 isolates) of isolates in population 2 ($P = 0.0032$, two-tailed Fisher's exact test) (Table 4). These results are consistent with the prior microsatellite typing study that found the more recently diverged *T. vaginalis* population (type 1) had an elevated mean metronidazole MLC (14). The $ntr4_{Tv}$ C213G (Y71STOP) and $ntr6_{Tv}$ A238T (K80STOP) SNPs were also both strongly associated with population structure (Fig. 3c); both were far more prevalent in population 2 (88% and 38%, respectively) than in population 1 (4.7% and 0%, respectively) ($P < 0.0001$ for both comparisons, two-tailed Fisher's exact test). Accordingly, we found that the group of isolates lacking both SNPs had only 20% population 2 ancestry, whereas isolates with either $ntr4_{Tv}$ C213G (Y71STOP) or both SNPs had 85% and 95% population 2 ancestry, respectively. These results indicate that the observed association between these SNPs and metronidazole resistance (Table 3) may be a secondary effect due to their association with population 2, which is in turn associated with resistance. However, within population 2, the $ntr6_{Tv}$ A238T (K80STOP) SNP was present in 17/38 (45%) of resistant isolates and only 2/22 (9.1%) of susceptible isolates, indicating a significant association with resistance even after controlling for population structure ($P = 0.0084$, two-tailed Fisher's exact test). Analysis of the distribution of MLC values within population 2 also revealed a significant difference between isolates harboring the $ntr6_{Tv}$ A238T (K80STOP) SNP and those strains that did not possess this SNP (Fig. 4).

In summary, we have identified stop codons in genes encoding two putative nitroreductases that are associated with metronidazole resistance. However, the *T. vaginalis* genome encodes 11 putative nitroreductases, of which more than one may reduce metronidazole. In the initial study characterizing these genes, the authors found that of the 11 genes, the 3 that were transcribed at the highest level were $ntr4_{Tv}$, $ntr6_{Tv}$, and $ntr10_{Tv}$ (10). As the presence of stop codons in $ntr4_{Tv}$ and $ntr6_{Tv}$ was strongly associated with resistance, we speculate that in these strains $ntr10_{Tv}$ may be expressed at a low level or lack sufficient nitroreductase activity to convert metronidazole to its active form. It is important to note that although we have identified an association between stop codons in these genes and resistance, there is not sufficient evidence to conclude that these changes are the actual cause of resistance; additional studies modulating expression of these genes in *T. vaginalis* will be necessary to further test that hypothesis. Additionally, metronidazole resistance does occur in isolates with intact $ntr4_{Tv}$ and $ntr6_{Tv}$ genes, indicating that multiple resistance mechanisms may be responsible for resistance. For example, one study linked resistance to infection of *T. vaginalis* by *Mycoplasma* (16); however, the mechanism by which infection would impart resistance is unclear, and this association was not found in a subsequent study (17). Another study found that infection by a double-stranded RNA virus was associated with a lower mean metronidazole MLC value (2); however, the virus is also strongly associated with population 1, being present in 73% of isolates in this population compared to only 2.5% of isolates in population 2 (18). Further studies will be required to determine the relative contribution of population ancestry, ntr_{Tv} genotype, and other factors to metronidazole resistance.

Lastly, the $ntr6_{Tv}$ A238T (K80STOP) SNP may have clinical utility in identifying metronidazole-resistant *T. vaginalis* in a rapid culture-independent fashion. As this SNP was present in 32/99 (32%) resistant isolates and only 4/98 (4.1%) susceptible isolates, its detection has a sensitivity of 32%, specificity of 96%, positive predictive value of 89%, and negative predictive value of 58%. Therefore, this SNP may be useful as a confirmatory test for suspected metronidazole resistance in treatment-refractory cases, especially in light of a recent study finding that identification of metronidazole resistance provided useful information to clini-

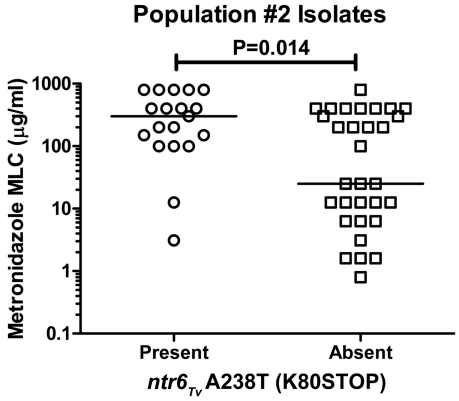

FIG 4 Distribution of metronidazole MLC values within 50 population 2 isolates by presence or absence of $ntr6_{Tv}$ A238T (K80STOP). Horizontal bars represent median values. Isolates with range of MLC values (e.g., 100 to 200 μg/ml) are plotted using their mean MLC values. Isolates with an MLC value of >400 μg/ml are assigned a value of 800 μg/ml for display purposes. *P* value determined by Mann-Whitney *U* test.

cians and led to improved patient outcomes (18). Our future efforts will focus on investigating the cellular role of $ntr4_{Tv}$ and $ntr6_{Tv}$ in metronidazole susceptibility, as well as the identification of additional resistance markers to improve culture-independent detection of *T. vaginalis* metronidazole resistance in the clinic.

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