

Metronidazole Susceptibility and TVV-infection of *Trichomonas vaginalis* from Metro Manila and Angeles City, Philippines

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ABSTRACT

Background. Metronidazole susceptibility and the presence of *Trichomonas vaginalis* virus (TVV) are the phenotypes found to be significantly correlated with the microsatellite-based genotypes of *T. vaginalis*. These phenotypes were assessed in *T. vaginalis* isolates from select urban areas to determine preliminary “type” of Philippine *T. vaginalis*.

Methods. Culture and microscopy were used to detect *T. vaginalis* in vaginal swab samples collected from women attending social hygiene clinics in Metro Manila and Angeles City, Philippines. Screening of TVV on *T. vaginalis* was performed using RNA gel electrophoresis and RT-PCR. A modified protocol for metronidazole susceptibility assay was used to determine the aerobic minimum lethal concentration (MLC) of metronidazole in axenized *T. vaginalis* isolates.

Results. A total of 42 *T. vaginalis* were screened for the presence of TVV and assayed for metronidazole susceptibility. TVV was detected in 13 of the isolates. All but one of the samples was susceptible to metronidazole.

Conclusion. This is the first study to assess the in vitro metronidazole susceptibility of Philippine *T. vaginalis* isolates. The isolates are generally susceptible to metronidazole even with the presence of TVV. The metronidazole susceptibility and presence of TVV are not enough to classify the isolates into type 1 or type 2.

Key Words: *Trichomonas vaginalis*, *Trichomonas vaginalis* virus, Metronidazole

INTRODUCTION

Metronidazole is the drug of choice for the standard treatment of trichomoniasis. It is a 5-nitroimidazole compound with 85-95% efficacy against trichomoniasis, the most widespread non-viral sexually transmitted infection (STI) worldwide with approximately 276.4 million incident cases every year.^{1,2} *Trichomonas vaginalis*, the causative agent of human trichomoniasis, may harbour double-stranded RNA virus classified as *Trichomonas vaginalis* virus (TVV). The endosymbiont TVV can cause upregulation of human pro-inflammatory reaction to the protozoon and has been associated to metronidazole susceptibility.^{3,4} Metronidazole susceptibility and the presence of TVV have been noted to be of high association to the microsatellite-based genotypes of *T. vaginalis*: type 1 is sensitive to metronidazole and often TVV-infected whereas type 2 is resistant to metronidazole and TVV-free.⁵

In the Philippines, a network of social hygiene clinics (SHC) provides a weekly STI screening and treatment of female “entertainment workers” in over 140 cities and reports on the prevalence of *T. vaginalis*.⁶⁻⁹ Our group has recently

Presented at the Philippine Society of Parasitology, Inc. Scientific Meeting, March 15, 2014, Manila, Philippines.

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reported the presence of TVV in Philippine *T. vaginalis* isolates, however, studies on the protozoon's metronidazole susceptibility is still lacking.¹⁰ With the availability of data on the TVV-infection and metronidazole susceptibility of *T. vaginalis*, typing of Philippine isolates based on these phenotypes can be performed. This study determined the presence of TVV and metronidazole susceptibility of *T. vaginalis*.

METHODS

Sample Collection

Ethical approval for this study was obtained from the Ethics Review Committee of the Philippine Department of Health. Seven SHCs in three cities in Metro Manila and two SHCs in Angeles City participated in this study (Figure 1). The participants from Metro Manila were female SHC attendees that were employed in spas, massage parlors and entertainment clubs while Angeles City participants were registered female sex workers. Women attending the SHCs were informed and had consented into giving vaginal swab samples prior to swab collection by the assisting certified medical personnel.

The swab samples were inoculated in tubes with BI-S-33 medium supplemented with 10% heat-inactivated horse serum, 500 µg/ml streptomycin and 500 U/ml penicillin.¹¹ The inoculated samples were then transported to the laboratory within 24 hours and incubated at 37°C for 24 to 72 hours. Microscopic examination was done every 24 hours to detect growth of *T. vaginalis*. Samples with motile *T. vaginalis* were subcultured and axenized by continuous passage in the same medium under similar conditions.

TVV Detection through RNA Gel Electrophoresis and RT-PCR

The total RNA of axenized, 24-h old *T. vaginalis* was extracted using the TRIzol reagent (Ambion, Life Technologies, Carlsbad, CA, USA). The manufacturer's protocol was followed in the extraction of RNA. TVV screening on protozoans from Metro Manila and Angeles City was performed through RNA gel electrophoresis.¹² Briefly, the denaturation reaction consisting of 2 µL RNA, 2 µL 10X MOPS (3-(N-morpholino)-propanesulfonic acid) electrophoresis buffer in DEPC-treated water, 4 µL formaldehyde, 10 µL formamide and 1 µL ethidium bromide in DEPC-treated water (200 µg/mL) was incubated for 60 min at 55°C. After incubation, samples were chilled for 10 min in iced water, centrifuged, and added with 2 µL of formaldehyde gel-loading buffer. The gel-loading buffer is made up of 50% glycerol diluted with DEPC-treated water, 10 mM EDTA, pH 8, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF. The samples were then loaded to 1.5% agarose gel with 2.2 M formaldehyde (1.5 g agarose, 72 mL sterile distilled water, 10 mL 10X MOPS electrophoresis buffer, 18 mL formaldehyde) submerged in 1X MOPS electrophoresis buffer. Electrophoresis was set at 4-5 V/cm for 4-5 hours. The gel was then visualized under UV light and samples presenting a band of 4.0- 5.5 kb was considered positive for TVV. The screening of TVV in samples from SHC in Angeles City was done using reverse-transcription polymerase chain reaction (RT-PCR). The OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany) and TVV species-specific primers were used to amplify the complementary DNA (cDNA) of the target viral genes.¹³ The RT-PCR reaction mix (50 µL) consists of 1X Q-buffer, 400

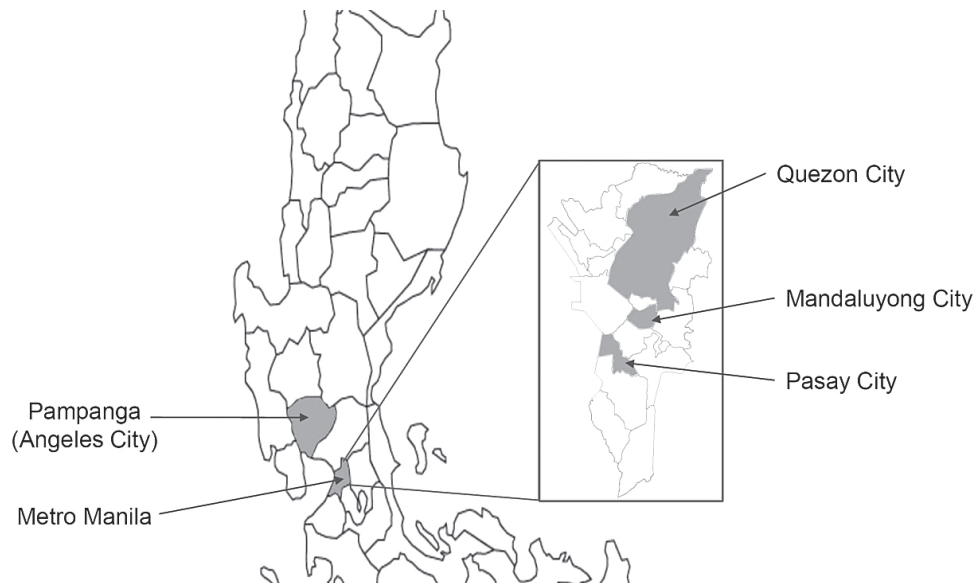


Figure 1. Location of sampling sites (shaded) in Luzon Philippines: Angeles City, Pampanga and Metro Manila. The detailed map on the right shows the three cities in Metro Manila that participated in this study.

µM of each dNTP, 0.6 µM of each primer, 2 µL of enzyme mix, and 1 µL of total RNA extract. Reverse transcription was conducted at 45°C for 30 min. The amplification of cDNA was performed using the following conditions: 95°C for 15 min for initial activation, 35 cycles of 94°C for 10 sec, 50°C for 1 min, 68°C for 4 min; and 68°C for 10 min for final extension. Amplicons were visualized under UV light in 1.5% agarose gel with SYBR Safe stain (Invitrogen, Life Technologies, CA, USA). Samples presenting an amplicon size of around 500 bp were considered positive for TVV.

Metronidazole Susceptibility Assay

A modified protocol of metronidazole susceptibility assay in aerobic condition was adapted to microplate setting and performed in triplicates.^{14,15} Briefly, axenic *T. vaginalis* were cultured for 24 hours then quantified using hemocytometer prior to the assay. The estimated density of *T. vaginalis* was 1 x 10⁶ cells/ml. In a microtiter plate, each well was filled with 150 µl of *T. vaginalis* culture added with 50 µL of BI-S-33 medium incorporated with decreasing dose of metronidazole (from 400 to 0.2 µg/mL). DMSO served as the drug carrier and negative control. The motility of *T. vaginalis* in each well was observed under light microscope after incubation at 37°C for 48 h. The minimum lethal concentration (MLC) was determined as the lowest concentration of metronidazole in which no motile cells was observed.

RESULTS

A total of 1,171 vaginal swabs were collected from women attending SHCs in Metro Manila and Angeles City. Culture method revealed that 111 samples were positive for *T. vaginalis*. Axenization of the cultures through sequential subculture and antiprotozoal assay was successfully done on 42 samples. Figure 2 shows the matrix of the samples in this study.

Twelve concentrations of metronidazole were considered in determining the MLC values of the 42 axenic *T. vaginalis* cultures. Table 1 shows the metronidazole MLC values of the isolates under aerobic conditions. All but one of the isolates was susceptible to metronidazole. Samples that have aerobic MLC of ≥50 µg/mL metronidazole were considered resistant strains.¹⁶ Forty one samples were susceptible to metronidazole (MLC: 0.8-25 µg/mL) and one sample has low resistance to metronidazole (MLC: 100 µg/mL).

Of the 42 isolates tested for metronidazole susceptibility, 13 were found to harbor TVV. The virus was detected in both metronidazole-susceptible and -resistant isolates as shown in Table 1.

DISCUSSION

Two clinical phenotypes, metronidazole susceptibility and presence of TVV, were noted to be highly associated

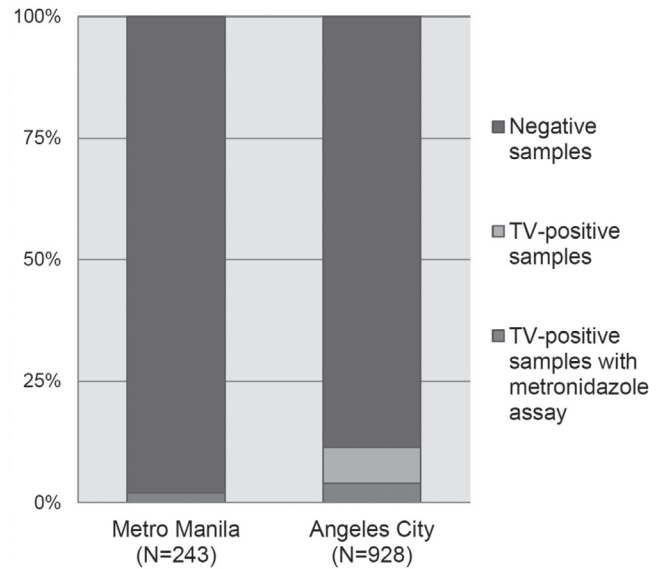


Figure 2. Matrix of Metro Manila and Angeles City samples. The percentage distribution of swab samples on the sampling locations with and without *T. vaginalis* and those subjected to metronidazole susceptibility assay is shown.

to the microsatellite-based, two-type population structure of *T. vaginalis*.⁵ The high association of these phenotypes to the genotypes suggests that they may be used in typing *T. vaginalis*, however, the results of this study suggests otherwise. Typing of Philippine *T. vaginalis* using these phenotypes was not possible since TVV was detected in both metronidazole-susceptible and -resistant *T. vaginalis* (Table 1). The results are in agreement with earlier studies noting the lack of association between TVV and metronidazole-resistance in *T. vaginalis*.^{17,18}

The nitroimidazoles – metronidazole and tinidazole – are the only antimicrobials known to be effective against

Table 1. Metronidazole MLC of *T. vaginalis* isolates under aerobic conditions

Metronidazole MLC* (µg/mL)	No. of <i>T. vaginalis</i>	
	TVV-infected	TVV-free
0.2	-	-
0.4	-	-
0.8	2	3
1.6	6	2
3.1	2	14
6.3	2	5
12.5	-	3
25.0	-	2
50.0	-	-
100.0	1	-
200.0	-	-
400.0	-	-
Total	13	29

**T. vaginalis* with metronidazole MLC ≥ 50 µg/ mL under aerobic conditions is considered resistant.

trichomoniasis and are the only drugs with FDA approval for oral and parenteral treatment of trichomoniasis.¹⁹ With the very limited choice of drug against trichomoniasis, the emergence of nitroimidazole-resistant *T. vaginalis* is alarming especially in areas with relatively high rates of nitroimidazole-resistant *T. vaginalis* strains. Resistance to metronidazole, believed to be acquired through a series of mutations, affects the gene expression of proteins needed for drug activation. Mutations lead to drug resistance in a multistep fashion of aerobic to anaerobic resistance.²⁰ This study showed that *T. vaginalis* from women in select regions in the Philippines were generally susceptible to metronidazole. The number of metronidazole-susceptible isolates with only a single case of low-level metronidazole-resistance indicate that cases of trichomoniasis in Metro Manila and Angeles City can still be treated with metronidazole.²¹ However, it must be noted that the treatment of participants with metronidazole-susceptible and -resistant *T. vaginalis* is not under the scope of this study. Proper SHC authorities were notified of the results and the authors were kept blind on the action of the SHC physician and the response of the participants.

TVV was detected only in *T. vaginalis* from Angeles City. This suggests that high-risk individuals have high chances of having both the protozoon and its endosymbiont virus - found to upregulate the inflammatory reaction in an in vivo host-protozoon-virus-bacteria environment.³ Female sex workers from Angeles City are high-risk individuals while employees of spa and clubs from Metro Manila are classified as medium-risk individuals. The medium-risk group is the bridging subpopulation since they may have sexual contact with individuals that have low- or high-risk of STI exposure while high-risk individuals experience high rates of STI exposure.² Sex workers are often exposed and not in control of risk factors like multiple sex partners, unprotected sexual intercourse, and unsafe working conditions.²²

This study was restricted by the viability and axenization of the protozoon as shown in the matrix of the samples (Figure 2). The axenization and maintenance of *T. vaginalis* in culture was done by continuous passage in broth medium with antibiotics. However, loss of viability of the protozoon was encountered in the laboratory. Bacterial contamination is a major problem in the culture of *T. vaginalis* even with the addition of antibiotics in the medium.²³ Upon axenization, immediate nucleic acid extraction and metronidazole susceptibility assay must be performed. Additionally, typing of *T. vaginalis* using other markers and a collaborative study with medical staff to establish the prevalence and clinical factors or consequences of harboring metronidazole-resistant *T. vaginalis* are warranted.

In summary, susceptibility to metronidazole was observed in both TVV-infected and TVV-free *T. vaginalis*, thus, phenotype-based typing of Philippine *T. vaginalis* isolates was not made. In general, Philippine *T. vaginalis* isolates have zero to low resistance to metronidazole.

Acknowledgments

We thank the staff of all participating social hygiene clinics for their technical assistance. This work was supported by a research grant from the Office of the Vice Chancellor for Research and Development (OVCRD) of the University of the Philippines Diliman (Grant No. 121217 PNSE).

Statement of authorship

All authors have approved the final version submitted.

Authors disclosure

All the authors declared no conflicts of interest.

Funding Source

This study was funded by the Office of the Vice Chancellor for Research and Development (OVCRD) of the University of the Philippines Diliman (Grant No. 121217 PNSE).

REFERENCES

- Cudmore SL, Delgaty KL, Hayward-McClelland SF, Petrin DP, Garber GE. Treatment of infections caused by metronidazole-resistant *Trichomonas vaginalis*. Clin Microbiol Rev. 2004;17(4):783-93.
- World Health Organization. Strategies and laboratory methods for strengthening surveillance of sexually transmitted infection. 2012. Switzerland.
- Fichorova RN, Buck OR, Yamamoto HS, et al. The villain team-up or how *Trichomonas vaginalis* and bacterial vaginosis alter innate immunity in concert. Sex Transm Infect. 2013;89:460-6.
- Snipes LJ, Gamard PM, Narcisi EM, Beard CB, Lehmann T, Secor WE. Molecular epidemiology of metronidazole resistance in a population of *Trichomonas vaginalis* clinical isolates. J Clin Microbiol. 2000;38:3004-9.
- Conrad MD, Gorman AW, Schillinger JA, et al. Extensive genetic diversity, unique population structure and evidence of genetic exchange in the sexually transmitted parasite *Trichomonas vaginalis*. PLoS Negl Trop Dis. 2012;6(3):e1573.
- Wi T, Ramos ER, Steen R, et al. STI declines among sex workers and clients following outreach, one time presumptive treatment, and regular screening of sex workers in the Philippines. Sex Transm Infect. 2006;82(5):386-91.
- Wi TC, Saniel OP, Ramos ER, MacNeil J, Neilsen G. RTI/STI Prevalence in Selected Sites in the Philippines. Family Health Int. 2014. Philippines.
- Ong VA, Rivera WL. Prevalence of *Trichomonas vaginalis* in vaginal swabs from sex workers in Angeles City, Pampanga, Philippines as detected by PCR. Trop Med Health. 2010;38(1):29-34.
- Queza MI, Rivera WL. Diagnosis and molecular characterization of *Trichomonas vaginalis* in sex workers in the Philippines. Pathog Glob Health. 2013;107(3):136-40.
- Rivera WL, Justo CAC, Relucio-San Diego MACV, Loyola LM. Detection and molecular characterization of double-stranded RNA viruses in Philippine *Trichomonas vaginalis* isolates. J Microbiol Immunol Infect. 2017; 50, 669-676. doi: 10.1016/j.jmii.2015.07.016.
- Diamond LS, Harlow DR, Cunnick CC. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans R Soc Trop Med Hyg. 1978;72(4):431-2.
- Sambrook J, Russel D. Molecular cloning: A laboratory manual. 3rd ed. USA: Cold Spring Harbor Laboratory Press. 2001.
- Goodman RP, Freret TS, Kula T, et al. Clinical isolates of *Trichomonas vaginalis* concurrently infected by strains of up to four Trichomonasvirus species (Family Totiviridae). J Virol. 2011;85(9):4258-70.

14. Meingassner JG, Thurner J. Strain of *Trichomonas vaginalis* resistant to metronidazole and other 5-nitroimidazoles. *Antimicrob Agents Chemother.* 1979;15:254-7.
15. Nix DE, Tyrrell R, Muller M. Pharmacodynamics of metronidazole determined by a time-kill assay for *Trichomonas vaginalis*. *Antimicrob Agents Chemother.* 1995;39:1848-52.
16. Krashin JW, Koumans EH, Bradshaw-Sydnor AC, et al. *Trichomonas vaginalis* prevalence, incidence, risk factors and antibiotic-resistance in an adolescent population. *Sex Transm Dis.* 2010;37:440-4.
17. Flegr J, Zaboř P, Vanacova S. Correlation between aerobic and anaerobic resistance to metronidazole in trichomonads: application of a new computer program for permutation tests. *Parasitol Res.* 1998;84(7):590-2.
18. Vanacova S, Tachezy J, Kulda J, Flegr J. Characterization of trichomonad species and strains by PCR fingerprinting. *J Eukaryot Microbiol.* 1997;44(6):545-52.
19. Workowski KA, Bolan GA. Sexually transmitted diseases treatment guidelines, 2015. *MMWR Recommend Rep.* 2015;63(3):1-110.
20. Rasoloson D, Vanacova S, Tomkova E, et al. Mechanisms of in vitro development of resistance to metronidazole in *Trichomonas vaginalis*. *Microbiology.* 2002;148:2467-77.
21. Kirkcaldy RD, Augostini P, Asbel LE, et al. *Trichomonas vaginalis* antimicrobial drug resistance in 6 US cities, STD Surveillance Network, 2009-2010. *Emerg Infect Dis.* 2012;18(6):939-43.
22. World Health Organization. Prevention and treatment of HIV and other sexually transmitted infections for sex workers in low- and middle- income countries: Recommendations for a public health approach. 2012.
23. Garber GE. The laboratory diagnosis of *Trichomonas vaginalis*. *Can J Infect Dis Med Microbiol.* 2005; 16: 35-8.