Diagnosis and molecular characterization of *Trichomonas vaginalis* in sex workers in the Philippines

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Trichomonas vaginalis is a pathogenic protozoon which causes the sexually transmitted infection, trichomoniasis. The absence or non-specificity of symptoms often leads to misdiagnosis of the infection. In this study, 969 samples consisting of vaginal swabs and urine were collected and screened from social hygiene clinics across the Philippines. Of the 969 samples, 216 were used for the comparative analysis of diagnostic tools such as wet mount microscopy, culture, and PCR utilizing universal trichomonad primers, TFR1/2 and species-specific primers, TVK3/7 and TV1/2. PCR demonstrated higher sensitivity of 100% compared to 77% of the wet mount. PCR primer set TVK3/7 and culture had the same and the best expected average performance [receiver-operating characteristic (ROC): 0.98]. Prevalence of infection in the sample population was 6.8%.

Keywords: Culture, Microscopy, PCR, Philippines, *Trichomonas vaginalis*

Introduction

Trichomonas vaginalis is a pathogenic protozoon that primarily infects the human genitourinary tract and can thrive in vaginal discharges, urine and urethral secretions causing the sexually transmitted infection (STI), trichomoniasis.¹ *T. vaginalis* infection generally shows non-specific signs and symptoms. The strawberry appearance of the cervix, termed as colpitis macularis, with small punctuated hemorrhagic spots on the vaginal and cervical mucosa are observed by routine examination in only 2% of infected patients.¹ In men, *T. vaginalis* trichomoniasis is largely asymptomatic. The duration of infection is 10 days or less and is associated with less than 5% of urethral infections.^{1,2} Trichomoniasis is one of the most common non-viral STIs in the world and has also been shown to be a risk factor for the transmission of the human immunodeficiency virus (HIV).^{3,4}

The most common method of detecting trichomonads is the wet mount because the procedure is simple, rapid and inexpensive but has low sensitivity. The culture method offers more sensitivity but with a longer turnaround period of at least 7 days to obtain the final result.⁵ Current rapid methods in the specific

detection of *T. vaginalis* are serologic-based but may exhibit cross-reaction with other trichomonads.⁶ We have recently reported the prevalence of *T. vaginalis* in vaginal swabs from sex workers in Angeles City, Philippines using PCR utilizing *T. vaginalis*-specific primers. Almost 10% of the 377 women sampled tested positive.⁷

The World Health Organization 2001 Report⁸ estimated 170 million trichomoniasis cases per year worldwide, with South and Southeast Asia having the highest estimate at 76.5 million cases. In the Philippines, the 2003 Sentinel for Sexually Transmitted Infections Etiologic Surveillance System Report of the Department of Health stated that the national aggregate of trichomoniasis is 1.0% of the 229 148 consultations in the sentinel sites.⁹

This present study therefore aimed to detect *T. vaginalis* and determine the prevalence of infection from sex workers in the Philippines utilizing PCR using universal and species-specific trichomonad primers.

Materials and Methods

Collection of samples

A total of 969 vaginal swab and urine samples were collected from five local government-funded social hygiene clinics (SHC) across the Philippines (Fig. 1) that provide periodic screening of sexually transmitted

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Figure 1 Location of study sites across the Philippines.

infections and diseases (STI/STD) for registered sex workers. The subjects were male (35/969 or 3.6%) and female (934/969 or 96.4%) sex workers aged 18–52 years. Trained SHC staff obtained swabs from the vaginal posterior fornix of the subjects. Male subjects submitted ~30 ml urine specimens (collected no less than 3 hours after previous micturition) in sterile containers provided. Samples were processed only from individuals with written consent. This study was approved by the Ethics Review Committee of the Philippine Department of Health and the respective city health offices.

Processing of samples

Each vaginal swab was dipped in a test tube with 1 ml of 0.85% normal saline solution, thoroughly swirled before discarding the swab and centrifuged at 500g for 3 minutes at room temperature. The supernatant was discarded leaving ~200 µl of the fluid in which the pellet was resuspended and 50 µl of the suspension was inoculated in 5 ml of Diamond TYM medium.¹⁰ One drop from this suspension was utilized for wet mount microscopy. One ml medium was added into the remaining suspension and kept at 4°C for genomic DNA extraction.

The urine samples were centrifuged for 5 minutes at 3500g at room temperature. The supernatant was discarded leaving ~100 µl of the fluid in which the sediment was resuspended. An aliquot of 50 µl of the

sample was inoculated to 5 ml of growth medium for culture and the rest was processed for wet mount microscopy. One milliliter of medium was added to the remaining suspension and maintained at 4°C for genomic DNA extraction.

Wet mount microscopy was performed under ×400 magnification and 20 fields were examined. This was performed within 15 minutes from obtaining the specimen as well as samples from cultures at 2, 5, and 7 days post-inoculation. Cells with characteristic morphology and motility of trichomonads were considered positive.

PCR and comparative analysis of diagnostic tools

DNA was extracted according to the procedure of Rivera *et al.*¹¹ PCR reaction mixture and amplification condition procedures using three primer sets: the universal trichomonad primer set TFR1/2,¹² and the species-specific primer sets TVK3/7,^{13–15} and TV1/2¹⁶ were followed according to the manufacturer's instructions (New England BioLabs, Ipswich, MA, USA) in TC-412 thermocycler (Techne, Cambridge, UK). PCR mixture without DNA from samples was used as the negative control.

For comparative detection between wet mount and PCR, a minimum sample size of 216 was computed using Epi Info™ 6 system (Centers for Disease Control and Prevention, Atlanta, GA, USA) with criteria of 70% and 87% sensitivity for wet mount and PCR, respectively, with confidence levels of 95% and 80% power against culture as the reference. The 216 samples were randomly selected from the 969 vaginal swabs and urine samples. Comparative analysis of the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and receiver-operating characteristic (ROC) of each test methods was calculated using the SPSS version 13 statistical software (SPSS Inc., Chicago, IL, USA). PPV estimates the probability that a specimen is *Trichomonas* positive, while NPV estimates the probability that the specimen is *Trichomonas* negative. Comparative analysis between wet mount and PCR was based on culture as the gold standard. Since three primer sets were used in the study to detect trichomonad and only *T. vaginalis* was detected, the three primer sets and culture were compared with each other using an expanded gold standard. Specimens were considered positive if they were positive by culture or any of the three PCR primer sets.

Results

Prevalence of T. vaginalis infection

Based on the total samples included in the study and the total positive specimens for *T. vaginalis* detected by wet mount, culture, or PCR, the prevalence of *T. vaginalis* in the study sites was 6.8% (66/969). Almost

Table 1 Performance of wet mount and PCR primer set TFR1/2 in 216 samples analyzed using culture as the reference

Diagnostic tool	No. of positives (13*)	% sensitivity (95% CI)	% specificity (95% CI)	PPV	NPV
Wet mount	10	77% (46.2–95%)	100% (98.2–100%)	100%	99%
PCR using primer set, TFR1/2	15 [‡]	100% (75.3–100%)	99% (96.5–99%)	87%	100%

Note: *Number of positive specimens by culture.

[‡]two (2) samples were PCR positive but wet mount and culture negative.

half of the total positive cases (43.9%) were patients 18–22 years old followed by 23–27 years old (30.3%), 28–32 years (13.6%), 33–37 years (6.1%), 38–42 years (4.5%), and 43–47 years (1.5%).

Comparative detection by wet mount and PCR

A total of 216 samples were used for comparative analysis between wet mount microscopy and PCR with culture as the gold standard. Wet mount, PCR utilizing trichomonad universal primer set TFR1/2, and culture methods detected 15 samples positive for the presence of trichomonads. Only 10 samples were positive by wet mount and 13 by culture (Table 1). Interestingly, two samples observed to be trichomonad negative by wet mount and culture was found to be trichomonad positive in PCR with universal primers. Only 1 of the 35 urine samples was positive for trichomonads. The wet mount method had a sensitivity of 77% and a specificity of 100%, while the sensitivity and specificity of PCR were 100% and 99%, respectively. The wet mount method had a PPV and an NPV of 100% and 99%, respectively, while PCR had a PPV of 87% and an NPV of 100% (Table 1).

Most cultures became positive 2 days post-inoculation. Two more cultures were observed to be trichomonad positive after 5 days. No viable cells grew after 7 days. Positive cultures were further maintained at room temperature and subsequently subcultured in fresh medium and incubated at 35°C. Results for wet mount and culture were concordant.

To compare wet mount and PCR methods, areas under ROC were used to represent expected performances. For the 216 clinical specimens, the average performance of PCR (ROC: 1.0) is better than that of wet mount (ROC: 0.88) in detecting the presence of *T. vaginalis*.

Comparative detection of PCR primer sets and culture with expanded gold standard

A comparison in the ability to detect *T. vaginalis* utilizing the three primer sets (TFR1/2, TVK 3/7, or

TV1/2) and culture was performed using an expanded gold standard (defined as a positive culture result and/or a positive PCR test).

Of the 969 samples, a total of 66 samples were tested *T. vaginalis*-positive as detected by both culture and PCR methods. A total of 58 cultures (87.9%) became positive at 2 days post-inoculation. At 5 days post-inoculation, six cultures (9.1%) were positive from which only two cultures were positive as detected by PCR as mentioned above. The universal primer TFR1/2 yielded PCR products from 52 isolates, the specific primers TV1/2 and TVK3/7 yielded 44 and 64 products, respectively. The sensitivity of PCR using primers TVK3/7 was found to be comparable to that of culture at 97%, while only 66.7% of the total positive specimens were amplified by primer set TV1/2, making it the least sensitive primer set utilized. No differences in the specificities and predictive values were observed (Table 2).

The average performance of TFR1/2 was 0.89, and both TVK3/7 and culture yielded the same average performance value of 0.98. TV1/2 primer set had an ROC value of 0.83. At 5% level of significance, the expected average performances of primer sets were significantly different from each other having a *P*-value of less than 0.05.

Discussion

In the Philippines, services are rendered more on the diagnosis, treatment, and control of sexually transmitted pathogens such as *Neisseria gonorrhea* and *Chlamydia trachomatis* than *T. vaginalis*. The prevalence of trichomoniasis is often neglected because there are no readily available specific procedures for screening *T. vaginalis* infection. This study demonstrated that PCR and culture methods are more sensitive than wet mount microscopy which Philippine SHCs routinely utilize.

The most commonly used method for detecting *T. vaginalis* infection is direct visualization through

Table 2 Performance of PCR primer sets: TFR1/2, TVK3/7, TV1/2, and culture in 969 samples analyzed using an expanded gold standard

Diagnostic tool	No. of positives (66*)	% sensitivity (95% CI)	% specificity (95% CI)	PPV	NPV
Culture	64	97% (89.5–99.6%)	100% (99.6–100%)	100%	100%
PCR using primer set, TFR1/2	52	78.8% (67.0–87.9%)	100% (99.6–100%)	100%	100%
PCR using primer set, TVK3/7	64	97% (89.5–99.6%)	100% (99.6–100%)	100%	100%
PCR using primer set, TV1/2	44	66.7% (54.0–77.8%)	100% (99.6–100%)	100%	98%

Note: *Total positive specimens detected either by culture or any of the three primer sets.

microscopy or the wet mount method. This is an inexpensive and simple technique but relies on the continued viability of the organism.¹⁷ This technique therefore is not feasible in most SHCs where samples from large populations of patients are screened simultaneously. In this study, the sensitivity of the wet mount method is 77% which is comparable to that reported by previous researchers.^{18–21} Wet mount technique requires at least 10^4 organisms per milliliter of vaginal fluids in order to detect a positive sample.²² The tube wet mount method described in this study was an alternative to the glass slide method commonly used in laboratories. In the tube wet mount method, centrifugation of the sample concentrated the specimen and enhanced *T. vaginalis* detection.

Culture is considered the gold standard in detecting *T. vaginalis* infection. However, such method is not cost-effective, laborious and time-consuming. A density of $10\text{--}10^4$ trichomonads per milliliter of sample is needed for a culture to be rendered positive.²¹ Furthermore, culture could not detect non-viable cells affected by the transport process of the samples.²³ The type of formulations to be used for cultivating *T. vaginalis* should also be taken into account. Schmid *et al.*⁵ determined that Diamond TYM medium proved to be superior to other media used for *T. vaginalis* cultivation. In this study, Diamond TYM medium was used for maintaining *T. vaginalis* isolates. Growth was observed at 3, 5, and 7 days post-inoculation consistent with the study by Smith.²⁴ The sensitivity of culture in this study was 97% and specificity was 100% consistent with previous reports.^{13,18–21,24–26}

In the comparative analysis between PCR and wet mount with culture as the gold standard, two samples were found to be PCR positive but culture negative. If the gold standard was culture alone, these two samples would have been considered false positive. According to Crucitti *et al.*,¹³ PCR has a detection limit lower than that of culture, and is capable of identifying non-viable as well as non-motile organisms. One of the two samples found to be PCR positive but culture negative was a urine sample. According to Hobbs *et al.*,²⁵ DNA amplification technique such as PCR is more sensitive than culture in detecting *T. vaginalis* in urine samples. The transport of specimens is likely to be the cause of the negative result in culture. As observed by Smith,²⁴ temperature fluctuation may have an effect on the growth of trichomonads. This was an unavoidable circumstance as the samples were needed to be transported from the different sampling sites to the laboratory.

Based on the expanded gold standard, primer set TVK3/7 had the highest sensitivity among the three

trichomonad primers used in this study. Primer set TV1/2 had the lowest sensitivity. This is identical to the results obtained by Crucitti *et al.*¹³ No differences were observed in their specificities. The ROC values of the different methods used in this study reflected the average performances of each method. Culture and primer set TVK3/7 had the highest average performance among the methods used which meant that they had the most accurate detection capability. The respective ROC value was congruent with the respective sensitivity of each method analyzed. Even though wet mount technique was the least accurate of the methods evaluated, the ROC value of wet mount clearly showed that it is better in detecting trichomonad than merely observing for symptoms of the disease.

The prevalence of trichomoniasis in this study was calculated to be 6.8%. This finding was higher than the previously reported national aggregate of 1% by the HIV AIDS Technical Report 2003. Though the report is 10 years ago, it can be inferred from this study that there is a rising trend of STI/STD incidence and possibly including HIV cases since trichomoniasis is also implicated in HIV infection.

In males, *T. vaginalis* infection is mostly asymptomatic and of short duration therefore generally left undetected.¹ The complications of undiagnosed and untreated cases of trichomoniasis and the association with other infections or diseases warrant the need to consider the importance of laboratory diagnosis in trichomonad detection. Such studies on *T. vaginalis* infection in males are urgently needed.

This study hopes that health policies on screening STI/STDs would be re-evaluated. Consequently, specific laboratory diagnosis of *T. vaginalis* must be included in the management of STI/STD in the Philippines. PCR can efficiently detect asymptomatic cases and is therefore highly recommended and may be complemented with tube wet mount and culture especially for high risk individuals. This is due to the growing threat of trichomoniasis to the public health. Lastly, this study could help in enhancing cooperation among health institutions including local government units, health departments, non-government organizations, research, and the academe to improve the prevention of the increasing cases of STI/STDs in the country.

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