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Comparison of microscopic examination and PCR in the diagnosis of Trichomoniasis in patients attending the sexually transmitted disease and acquired immune deficiency syndrome control program in Kandy, Sri Lanka

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Abstract

Trichomoniasis is a sexually transmitted disease (STD) in humans. It is caused by a motile pathogenic protozoan, *Trichomonas vaginalis*. Clinical diagnosis of trichomoniasis is not reliable due to nonspecific clinical presentation, which overlaps with the clinical features of other sexually transmitted diseases. Therefore, confirmation of suspected clinical cases by laboratory tests is essential. The diagnosis is usually based on wet mount. The sensitivity of this method is lower than polymerase chain reaction (PCR). Therefore, in the present study we evaluated PCR as a diagnostic tool for diagnosis of trichomoniasis in Sri Lanka for the first time.

Female patients attending the sexually transmitted disease and acquired immune deficiency syndrome (STD/AIDS) control program in Kandy were included in the study. Patients' demographic data, clinical status and three vaginal swabs were obtained from the each patient separately. Two vaginal swabs were used for wet mount and permanent staining respectively. Other vaginal swab was used to isolate genomic DNA. PCRs were performed using the genus and species specific primers.

151 patients were studied during the period from May 2015 to November 2015. Out of these, majority of patients (87/151) were aged between 15 to 35 years. 97 patients were clinically symptomatic. Of 151 samples, three were positive for trichomoniasis by direct smears. 8 samples were positive for PCR confirming the etiological diagnosis as *T. vaginalis*.

The findings of the study suggest that PCR can be used to diagnose clinically suspected trichomoniasis patients in STD clinics in Sri Lanka.

Keywords: Trichomoniasis, diagnosis, microscopic examination, polymerase chain reaction and Sri Lanka

Introduction

Trichomonas vaginalis (*T. vaginalis)* is a highly motile, aero tolerant, haploid eukaryotic parasite which inhabits the human urogenital tract epithelium (Conrad et al., 2012). According to WHO global incidence and prevalence estimates of sexually transmitted infections (STI) in

2008, *T. vaginalis* infection was the commonest non-viral STI in humans. Men and women are equally affected; however men are usually asymptomatic and at times presented with urethritis, prostatitis, epididymitis, and penile discharge. *Trichomonas* infection in women is usually symptomatic and can present with odorous vaginal discharge, vulval irritation, itching, dysuria, abdominal pain, and dyspareunia (Javanbakht et al., 2013; Petrin et al., 1998; Hobbs and Sena**,** 2013). Trichomoniasis enhances acquisition and transmission of human immunodeficiency virus (HIV) infection (McClelland et al., 2007). It also predisposes to complications such as preterm birth, low birth weight, infertility, endometritis, pelvic inflammatory disease and cervical metaplasia (Javanbakht et al., 2013; Saleh et al., 2014).

In 2008, WHO has estimated 187 million cases of *Trichomonas* infection globally. The prevalence of trichomoniasis in our neighboring country, India is 8.46% (Madhivanan et al., 2009). The prevalence of trichomoniasis in Sri Lanka is limited. Studies carried out in Sri Lanka have reported prevalence ranged from 4.4% to 8.5% (Perera, 1994; Herath et al., 2012; Iddawela et al., 2007). However, a recent research done by Samarawickrema et al. (2014) showed a much lower prevalence (2.3%) of trichomoniasis in Sri Lanka.

Diagnosis of trichomoniasis based on clinical examination is not sufficient due to nonspecific clinical presentation, which overlap with the clinical features of other sexually transmitted diseases (Hobbs and Sena, 2013; Fule and Tankhiwale, 2012). Therefore, confirmation of suspected clinical diagnosis by laboratory method is essential for proper diagnosis and management of this infection.

Wet mount preparation is easy, rapid and inexpensive method for diagnosis of *T. vaginalis* infection (Queza and Rivera, 2013). Direct observation of the pear-shaped trophozoites with their characteristic tumbling motility is considered 100% specific for the organism. However, the negative results cannot be excluded the trichomoniasis because of its low sensitivity (38% to 82%) (Draper et al., 1993).

Culture in microaerophilic conditions has been considered the "gold standard" for the diagnosis of *T. vaginalis* (Lazenby, 2011). However culture is time consuming and if it is not inoculated immediately, the viability of organism is lost and culture becomes ineffective (Lazenby, 2011; Patil et al., 2012; Riley et al., 1992). And also, density of 10^2 organisms per milliliter of sample is needed for a culture to be rendered positive (Garder, 2005).

DNA amplification technique such as polymerase chain reaction (PCR) is more sensitive than culture in detecting *T. vaginalis* (Queza and Rivera, 2013). Therefore, it is highly recommended and may be complemented with wet mount and culture especially for high risk individuals (Queza and Rivera, 2013). Advantages of PCR assays over other available diagnostic methods include high sensitivity (Saleh et al., 2014; Queza and Rivera, 2013) specificity (Hobbs and Sena, 2013), capability of identifying non-viable as well as non-motile organism, less time consuming compared to culture (Paul et al., 2012) and ability to detect the parasite in urine (Lee et al., 2012).

In Sri Lanka, PCR has not been used to diagnose *Trichomonas* infection. Therefore, the present study was focused on to establish a PCR method to diagnose trichomoniasis and to determine the socio demographic factors responsible for transmission of trichomoniasis in Sri Lanka.

Materials and methods

Study site and sample collection

Samples were collected at Sexually Transmitted Disease and Acquired Immune Deficiency Syndrome Control Program

(STD/AIDS control program) in Kandy from May, 2015 to November, 2015. STD/AIDS control program in Kandy is responsible for treatment and prevention of all the sexually transmitted infections including AIDS in Kandy district. 151 female patients age between 15 to 55 years, attending the STD/AIDS control program in Kandy were included in this study. Written informed consent was obtained from patients. Patients who refused vaginal examination and those who received metronidazole or tinidazole within one week prior to sampling were excluded. Patients' demographic data and clinical status were obtained from each patient using interviewer administered questionnaire.

Three vaginal swabs were collected from the posterior fornix of each patient. This was done by a clinician using a sterile Cusco's speculum which was routinely carried out at the STD/AIDS control program in Kandy. One swab from posterior fornix was used to prepare wet mount. Second was used to prepare smear for Giemsa staining. Remaining swab was used for PCR. It was placed in 0.5 ml of sterile normal saline (0.9%) in a sterilized centrifuge tube (15 ml). Centrifuge tubes were kept at 4^0C until transport to laboratory. Samples were stored at-20^0C until use.

Ethical clearance was obtained from the ethics review committee, Faculty of Allied Health Sciences, University of Peradeniya.

Wet mount preparation

Wet mounts were prepared using a drop of normal saline (0.9%) on microscopic slides. Wet mounts were examined immediately under high power (x40) magnification to detect motile pear-shaped trophozoites. The presence of pear-shaped trophozoites with characteristic jerky or tumbling motility was considered as positive for *Trichomonas vaginalis* infection.

Giemsa staining

Smears were prepared on a glass slide using second swab. They were air dried and fixed using methanol. Then, smears were stained with Giemsa. Stained smears were observed under (x100) magnification, to detect violet, pear-shaped trophozoites. The presence of violet, pear-shaped trophozoites that slightly larger than white blood cells, measuring 9x7 µm size with five flagella were considered as positive for *Trichomonas vaginalis.*

Genomic DNA extraction

Genomic DNA extraction was performed for all collected samples. It was done using commercial DNA extraction kit (Invitrogen, USA), according to the manufacture's instruction.

PCR amplification

PCR amplification was done at the Department of Parasitology, Faculty of Medicine, University of Peradeniya.

The internal transcribed spacer (ITS-1/5.8S/ITS-2) genomic region of the genus *Trichomonas* was amplified with primer sets (genus specific primer) NS5 (forward primer 5-GTA GGT GAA CCT GCG GAA GGA TCA TT-3) and NC2 (reverse primer 5-TTA GTT TCT TTT CCT CCG CT-3) (Kleina et al., 2004). Positive samples were further analyzed using *T. vaginalis* species specific primers targeting *T. vaginalis* ribosomal DNA (rDNA) (TVITS F: 5´- ACA CCG CCC GTC GCT CCT AC -3´ and TVITSR: 5´ AAT TTG CAT TCA AAG ATT AAC-3´) (Kazemi et al., 2010).

PCR reactions were performed in a total volume of 25 µl using 5.00 µl of genomic DNA, 1.5µl of 10x PCR buffer, 1.5µl of 10 pmol forward primer, 1.5 µl of 10 pmol reverse primer, 2 µl of 2.5 mM dNTP, 2 µl of 50 mM $MgCl_2$ and 0.25 µl of 5 u/µl Taq DNA polymerase under the following conditions; initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30

seconds, annealing temperature at 55°C for 30 seconds, extension temperature at 72°C for 1 min and final extension at 72°C for 5 minutes.

PCR amplification was consisted of 30 cycles and performed in an automated thermo cycler [Amplitronix^{TM} 6 version 1.0.4 (USA)]. For each set of PCR reactions, negative and positive controls were included. Laboratory strain of *Trichomonas* and the sample that gave positive result for wet mount was used as a positive control. Sterile distilled water and wet mount negative and previously PCR negative samples were used as negative controls.

Gel electrophoresis

The amplified products were assessed by electrophoresis on a 1.5% agarose gel, using 100 voltage and 250 A current, for 30 minutes. The gel was stained with 0.5 µg/ml ethidium bromide (Vavantis-catalog number PC0707) for 15 minutes and viewed on UV transilluminator (Alpha Innotech – gel imaging systems for life sciences).

Samples containing amplified DNA product of 297 bp from the ITS -1/5.8S/ITS-2 genomic region of *Trichomonas* species and amplified DNA product of 313 bp targeting *T. vaginalis* ribosomal DNA (rDNA) were considered as positive for *T. vaginalis*.

Statistical analysis

Statistical analysis was done manually.

Results

Hundred and fifty one patients were studied during the period from May to November 2015. Age range of patients was from 15 to 55 years. The majority of them were between ages 15 – 35 years (87/151). Out of 151, 122 were married. Hundred and one were from Kandy district, and the rest were from other districts of Sri Lanka (Kurunagala, Kegalle, Trinchomalee). Of the study population, 45 had secondary education (Table 1).

Out of 151 patients, 97 were clinically symptomatic and the rest (54) were asymptomatic. 19 patients were commercial sex workers and 26 had multiple sex partners. Sixty nine patients had used contraceptive methods (Table 01). Of the study population, 8 (5.3%) were positive for *Trichomonas vaginalis* infection. Among positives, the highest percentage of *Trichomonas* positivity was found in unemployed and married women between 36-45 years, who had secondary education (Table 1).

Microscopy

Out of 151 samples, 3 (1.99 %) samples were positive for wet mount microscopy and Giemsa stained smear (Figure 1).

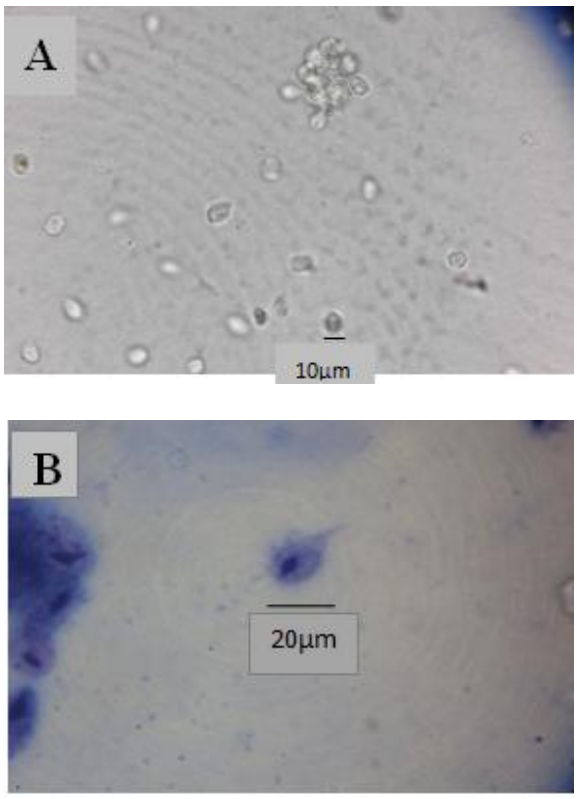

Figure 1: Light microscopic view (under x 40 magnification) of *T. vaginalis* trophozoite. [A – Wet mount (x40); B – Giemsa stain (x100)].

Table 1: Comparison of demographic characteristics of study population.

Variable	Number of patients Investigated (%)	Number of patients positive for trichomoniasis (PCR method) (%)
Age range		
15 – 25 years	43 (28.5 %)	01 (12.5%)
26- 35 years	44 (29.1 %)	02 (25%)
36 – 45 years	39 (25.8 %)	03 (37.5%)
46 – 55 years	25 (16.6 %)	02 (25%)
Level of education		
Primary education	15 (9.9 %)	02 (25 %)
Secondary education	45 (29.8 %)	06 (75%)
Tertiary education	35 (23.2 %)	-
No formal education	06 (4.0 %)	-
Marital states		
Single	19 (12.6 %)	01 (12.5%)
Married	122 (80.8 %)	06 (25%)
Widow	10 (6.6 %)	01 (12.5%)
Occupation (12 months)		
Unemployed	93 (61.6 %)	05 (62.5%)
Student	07 (4.6 %)	-
Commercial sex worker	19 (12.6%)	-
Retired	00 (0%)	-
Other	32 (21.2 %)	03 (37.5%)
Number of sex partners		
(last 3 months)		
One	98 (64.9 %)	03 (37.5%)
Two	27 (17.9 %)	02 (25%)
Multiple	26 (17.2 %)	03 (37.5%)

Polymerase Chain Reaction

Out of 151 samples 8 samples (5.3%) were positive for both genus and species specific primers (Figures 2 and 3).

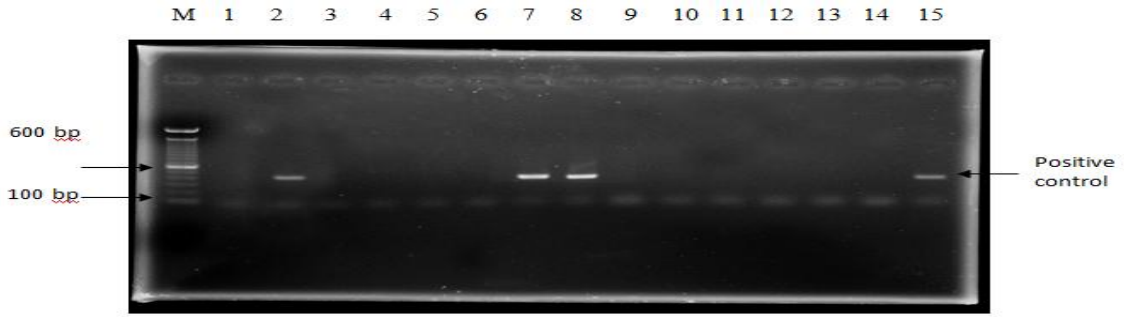

Figure 2: Gel electrophoresis photo of internal transcribed spacer (ITS -1/5.8S/ITS-2) genomic region of the genus specific PCR. Lane M - 100 bp DNA marker, Lanes 1, 3 to 6, 9 to 13 were negative samples, Lanes 2, 7, and 8 were positive samples, Lane 14 – negative control (distilled water), Lane 15 – positive control (DNA from wet mount positive sample).

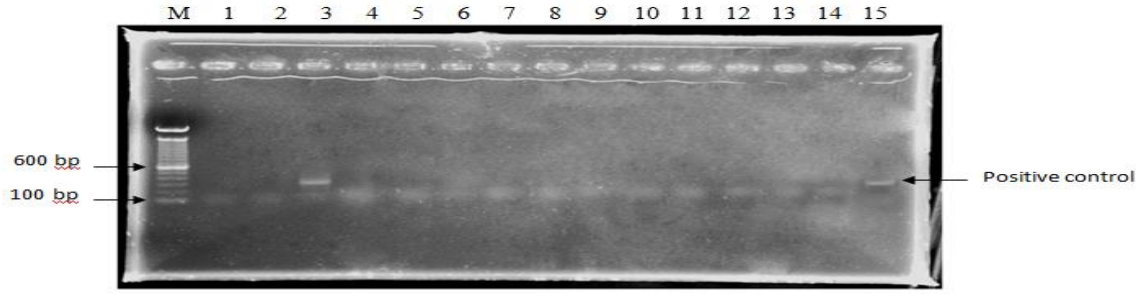

Figure 3: Gel electrophoresis photo of species specific PCR targeting ribosomal DNA. Lane M - 100 bp DNA marker, Lanes 1, 2, and 4 to 13 were negative samples, Lane 3 positive *sample,* Lane 14 – negative control (DNA from wet smear negative and previously PCR negative sample), Lane 15 – positive control (DNA from wet smear positive sample).

According to the present study, 3 samples (1.99%) were positive for both wet mount and Giemsa staining. 8 samples (5.3%) were positive for PCR, done using both genus and species specific primer sets. 5 cases which were negative for microscopy (both wet smear and Giemsa stained smear) were detected by PCR.

Discussion

Trichomoniasis is the most common non-viral sexually transmitted disease often neglected due to nonspecific clinical presentation and also due to lack of specific procedures for screening. Out of one hundred and fifty one, 8 were positive for trichomoniasis in the present study population and out of those positive patients majority were aged 35 to 55 years. This is in agreement with the study conducted by Fernando et al. (2012) in Colombo, Sri Lanka. According to the present study, all the positive patients had primary and secondary education. However, several studies have reported a high prevalence of trichomoniasis among less educated women living in developing countries (Opara et al., 2008). In Sri Lanka, proper sexual education program is not incorporated in to school curriculum. Therefore, the level of education cannot be considered as a risk factor for sexually transmitted diseases in Sri Lanka. The present study showed that the majority of patients attending the STD/AIDS control program were unemployed and they were more likely to be positive for *Trichomonas* infection than commercial sex workers. This may be due to increased awareness about STDs among commercial sex workers than others and also taking precautions such as usage of condoms. It is also possible that commercial sex workers who have recurrent infections are using metronidazole (Fernando et al., 2012).

The present study showed 1.99 % positivity by wet mount examination. Studies conducted using same diagnostic technique in Colombo (Banneheke et al., 2013) and Kandy (Iddawela et al., 2007) in Sri Lanka have reported the prevalence of 2.8% and 3.67% respectively.

Similarly, a study conducted in Southern India reported 1.7% positivity for wet mount (Dharma Vijaya et al., 2013). However, a study carried out on human immunodeficiency virus-infected individuals from South India, has documented 0.51% positivity (Paul et al., 2012). Variable results of wet mount examination could be due to low sensitivity of wet mount microscopy, high dependency on expertise of the microscopist, and delay in transporting and laboratory processing of samples (Queza and Rivera, 2013; Perazzi et al., 2010).

According to our study, positivity of Giemsa stained smears was 1.99% and it was similar to the positivity by wet mount. However, this method was time consuming compared to wet mount (Afzan et al., 2010). Therefore, permanent staining using Giemsa is not used in routine setting where high numbers of patients are present.

According to the present study, 5.3% of patients had *Trichomonas* infection based on PCR using NS5/NC2 and TVITS F and TVITS R primer sets. Similarly, studies conducted in South India and Philippines showed the 5.02% and 6.9% positivity of PCR respectively (Queza and Rivera, 2013; Paul et al., 2012). Using different primer sets (TFR1/2), another study has reported results (6.94% positivity for PCR and 4.63% positivity for wet mount) comparable to our study.

A study conducted in Belgaum showed 30% positivity for PCR using TVK3/TVK7 primer sets while wet mount microscopy gave only 18% positivity (Patil et al., 2012). Similar study conducted by Saleh et al. (2014) in India showed the 85.2% positivity for PCR done using primers targeting 67 bp region of a repeated sequence of the *T. vaginalis* genome, while wet mount showed 84.9% positivity.

In our study vaginal swabs were collected by a clinician from the posterior fornix during speculum examination. However, some studies have shown that the self-administered vaginal swabs are practical, rapid and easy to obtain and are effective as physician administered vaginal samples for the PCR detection of *Trichomonas* infection.

Limitation of the present study is that we did not perform culture, which is the "gold standard" for diagnosis of trichomoniasis to compare with the PCR assay. Further studies should be carried out to compare the PCR results with the culture.

The study findings suggest that detection of trichomoniasis using PCR technique is more sensitive than wet mount and can be used for diagnosis of *T. vaginalis* infection in STD clinics in Sri Lanka.

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Conflict of interests

The authors declare that they have no conflict of interests.

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