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Comparative Evaluation of Microscopy and Polymerase Chain Reaction Sensitivity in the Diagnosis of *Trichomonas vaginalis* Using Vaginal Swab Samples from Women in Ibadan, Oyo State, Nigeria.

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Abstract
Trichomoniasis in women remains a public health challenge due to high prevalence, inefficient and inaccurate diagnosis, especially in developing countries. This study aimed to determine the prevalence of trichomoniasis in women in Ibadan, using both microscopy and PCR techniques, and to investigate any possible association between Trichomonas vaginalis and Cervical Intraepithelial Neoplasia (CIN). Two vaginal swab samples were collected from 200 women (aged 20-60 years) attending the Obstetrics and Gynaecology Unit, University College Hospital, Ibadan, for Papanicolaou smear test. They were processed for microscopy and PCR analyses. Ninety-six vaginal swab samples gave quantifiable concentrations of DNA and were used for the analyses. The primer set Tv1/Tv2 was used for amplification of the T. vaginalis 18S ribosomal gene. Confidence intervals for sensitivity of the diagnostic techniques were determined using the Student's t-test. Microscopy detected 25% of T. vaginalis (95% CI, 1.16−1.34, P<0.001) while PCR detected 87.5% (95% CI, 1.75−1.93, P<0.001). Using PCR as a standard, the percentage sensitivity of microscopy was 28.57%. Kappa value was 0.08. These findings showed that PCR was more sensitive to T. vaginalis using vaginal swab samples compared to microscopy. They also demonstrated a positive association between T. vaginalis and CIN. Negative cases got by microscopy should be further tested using molecular diagnostic techniques such as PCR.

Keywords: *Trichomonas vaginalis*, Microscopy, PCR, Cervical, Intraepithelial Neoplasia

Introduction
Trichomonas vaginalis is an obligate extracellular flagellated anaerobic parasitic protozoan which causes trichomoniasis, the most prevalent non-viral sexually transmitted but treatable disease in the world [1,2,3,4]. *T. vaginalis* infection mainly follows intravaginal or intraurethral inoculation of the organism due to its site specificity. Lack of education and adequate screening programs have allowed *T. vaginalis* infection to go unreported in million people globally. As a result, incidence of *T. vaginalis* has increased by about 11.5% since 2005 and it is estimated that about 275-276.4 millions of people across the world are infected [5, 6]. Prevalence of trichomoniasis ranges from 12-28.5% across different populations which comprise obstetrics and gynaecology clinic attendees and commercial sex workers [7].

Although, *T. vaginalis* infects men and women in approximately the same proportion, women are regarded to be infected more by the pathogen. They are also regarded to be more affected by the burden of trichomoniasis than their male counterparts [8]. Trichomoniasis causes significant morbidity in women, most usually manifesting as symptomatic vaginitis [4]. It is also associated with several other reproductive health issues which comprise urethritis, epididymitis and infertility in men and, urethritis and cervicitis in women [9, 10]. Complications arising from this infection in women include preterm birth delivery, post-abortion infection and low-birth-weight babies [11,12]. Besides, trichomoniasis is a significant risk factor for sexual acquisition and transmission of human immunodeficiency virus (HIV) as 24% of HIV infections are directly correlated with trichomoniasis [13,14,15] and also to cervical intraepithelial neoplasia [16]. Furthermore, a significant association has also been observed with cervical cancer, unarguably the most significant health complication of *T. vaginalis* infection [17,18].

The cases of trichomoniasis are about 50% asymptomatic, having little or no clinical signs in women and more especially in men [8,19]. Clinical manifestations of trichomoniasis consist of vaginal discharge of abnormal colour with offensive odour, vulval and vaginal pruritus and/or erythema, colpitis macularis (strawberry cervix) characterized by punctate haemorrhagic lesions and increased vaginal pH (>5) [20,21].

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The symptoms of trichomoniasis are not specific enough for their diagnosis. Routine clinical diagnosis of trichomoniasis in women usually depends on direct microscopic observation of motile parasites in vaginal fluid in wet-mount preparations [22]. This technique is highly specific, but its sensitivity is only about 44−68% of positive culture samples, which currently is the gold standard laboratory test for *T. vaginalis* infection, both in women and in men [9,23]. Culture is more reliable than wet-mount microscopy but it needs frequent microscopic observation for 2-7 days [24]. Another disadvantage is the possibility of contamination. Although, culture is about 90% sensitive to *T. vaginalis* small numbers and non-viable parasites could make it give false negative culture test [25].

Several assays for diagnosing trichomoniasis based on PCR have been developed [26]. Examples of PCR targets include the ferredoxin gene [27], highly repeated DNA sequences [28], beta tubulin gene [29], 18S ribosomal gene [30]. In this study, the sensitivity of wet-mount microscopy and PCR to *T. vaginalis* obtained from vaginal swab samples was determined using *T. vaginalis* 18S ribosomal gene. The association between *T. vaginalis* and cervical intraepithelial neoplasia was also investigated.

Materials and Methods

Study Area

The study was carried out among attendees of Obstetrics and Gynaecology clinic, University College Hospital (UCH), Ibadan, Oyo state. Oyo state is located 3°5′ east of the Greenwish meridian and latitude 7°23′ north of the equator. It is a tropical rainforest zone with warm, dry season from November - April and rainy season from May - October.

Study Population

A total of 200 women, aged 25-60 years, attending the Obstetrics and Gynaecology clinic of the University College Hospital (UCH) in Ibadan, Oyo state, were recruited for this study after consent was obtained. Their socio-demographic and clinical details, structured into a questionnaire were obtained from each participant before the collection of vaginal swab samples.

Specimen Collection

Vaginal swab samples were collected in duplicate from each participant by clinicians using a sterile alginate swab and inserted into an air-tight polystyrene tube. One set of the samples was transported inside an iced insulated cooler to the laboratory within 20min of collection and was stored at -20°C prior to DNA extraction from *T. vaginalis*. Normal saline (1.5mL) was added to the second sample used for wet mount microscopic identification within 5min of sample collection. Pap smear test was also carried out on each participant to detect the presence of Cervical Intraepithelial Neoplasia I-III in each participant.

Extraction of DNA

DNA extraction from *T. vaginalis* was carried out using the Chelex extraction method.

Hundred microlitre (100µL) of the 10% Chelex solution was aliquot into clean mini tubes for each sample to be extracted. Aliquots (100µL) of 10% Chelex solution was dispensed into mini tubes after shaking the Chelex suspension. Two microlitre (2µl) of the tissue obtained from the vaginal swab was placed in individual Chelex tubes. The tissue was scraped across a new piece of sterile weighing paper with a sterile scalpel blade, flattened out several times till it forms a little flake (this enables more surface area of the tissue to be exposed to the Chelex). The flake was then placed into the Chelex tube and incubated at 100 °C for 20 min (with shaking after 10 min interval). The Chelex tubes were then taken out of the incubator after 20 min and centrifuged at maximum speed. Two microlitre (2µL) of the supernatant was used as amplification template for each PCR. Nanodrop was done using the Nanodrop spectrophotometer ND-1000 to determine the concentration and purity levels of the extracted DNA.

PCR primers

A set of primers (Tv1/Tv2) targeting conserved regions of the 18S ribosomal DNA of *T. vaginalis*, as designed by [30], was used. The primer sequences were selected from regions of the 18S ribosomal gene different from those of *Trichomonas tenax* (accession no. U37711), *Trichomonas foetus* (U17509), *Candida albicans* (M60302), *Giardia lamblia* (U09492), *Entamoeba gingivalis* (D28490), *Trypanosoma brucei* (AJ009149), and *Homo sapiens* (U13369) [30]. The primer sequences were as follows: Tv1, 5′ TAA TGG CAG AAT CTT TGG AG 3′, and Tv2, 5′ GAA CTT TAA CCG AAG GAC TTC 3′.

PCR amplification

PCR was performed with a thermal cycler (GeneAmp PCR system 9700). A standard PCR was carried out in a total volume of 25µl using thin-walled PCR tubes. The master mix consisted of 1× PCR buffer, 2.5mM $MgCl_2$, 200µM of each of the four deoxynucleoside triphosphates, 0.4µM of forward and reverse primers, and 1U of *Taq* DNA polymerase. A total of 25ng of genomic DNA extracted from *T. vaginalis* was used for each amplification. The tubes were kept in the thermocycler and programmed as: 2 min of denaturation at 94°C, followed by 40 cycles each consisting of 30sec of denaturation at 94°C, 45sec of annealing at 55°C, and 45sec of extension at 72°C. A final extension step at 72°C for 5min was also included. Each amplification cycle

comprised *T. vaginalis*-positive and -negative vaginal swab samples (sample with DNA extracted from *T. vaginalis*) and a blank containing distilled water.

The annealing temperature started at 55°C and was lowered two degrees for every one cycle until reaching 48°C, and then kept constant till the end of the cycling process.

Detection of PCR products

The primer set Tv1/Tv2 taken was designed to amplify a DNA product of 312bp. A 5-µl aliquot of PCR product was separated by horizontal gel electrophoresis at 100V in 3.0% agarose gel stained with ethidium bromide (0.5µg/ml), in Tris-borate-EDTA (TBE) buffer. The sizes of the amplified products were assessed by comparison with a commercial 50-bp DNA marker. PCR amplifications were visualized using a UV light transilluminator (Enduro^{TM} GDS, Labnet International, Inc.).

Statistical analysis

Confidence intervals for detection rate were determined using the Student's *t*-test. Agreement between PCR and microscopy in diagnosis of *T. vaginalis* in vaginal swab samples was calculated using the Kappa (κ) test. Statistical analysis was done using the SPSS v20 software.

Ethical Consideration

Ethical approval (UI/EC/13/0125) was obtained from the joint University of Ibadan / University College Hospital ethical committee. Informed consent was obtained from each participant prior to each sample collection. Participation was voluntary.

Results

A total of 200 vaginal swab samples were collected from consenting women, in Ibadan attending the Obstetrics and Gynaecology Unit, University College Hospital, Ibadan, for Papanicolaou smear test.

Amongst the participants, the age group 41-50 years had the highest number (46%) of participants, while the age group 20-30 years had the lowest number (10%) of enrolled participants (Table 1). Besides, married women formed 95% of the participants and divorced women 0.5% (Table 1).

Table 1: Socio-demographic parameters of the participants

Parameters	**Frequency**	**Percentage**
Age		
20-30	20	10.0
31-40	56	28.0
41-50	92	46.0
51 and above	32	16.0
Marital status		
Single	9	4.5
Married	190	95.0
Separated	1	0.5
Educational status		
No formal education	9	4.5
Student	8	4.0
Production	21	10.5
Professional	161	80.5
Retired	1	0.5
Ethnicity		
Yoruba	172	86.0
Igbo	18	9.0
Others	10	5.0
Smoking		
None	198	99.0
Active	1	0.5
Passive	1	0.5
Alcohol		
Yes	14	7.0
No	186	93.0

Ninety-six (96) samples which had quantifiable DNA concentration were eventually used for the study. The samples were tested for the presence of *Trichomonas vaginalis* by microscopy and polymerase chain reaction (PCR) with the primer set Tv1 and Tv2 that amplifies 18S ribosomal gene using the DNA extracted from the parasite.

In the clinical vaginal swab samples, wet-mount microscopy detected 25% (24 of 96) (95% CI, 1.16-1.34, $P < 0.001$) *T. vaginalis* in the samples and 75% of the samples were negative (Fig 1). PCR detected 87.5% (84 of 96) (95% CI, 1.75-1.93, $P < 0.001$) *T. vaginalis*. However, 9.4% of the samples were negative and 3.1% inconclusive (Fig 2). All PCR-positives were microscopy-positive. Also, all microscopy-positives were PCR-positives. The percentage sensitivity of microscopy when PCR is used as a standard was 28.57%. PCR detected 83.33% (60 of 72) of wet-mount 'false negatives'.

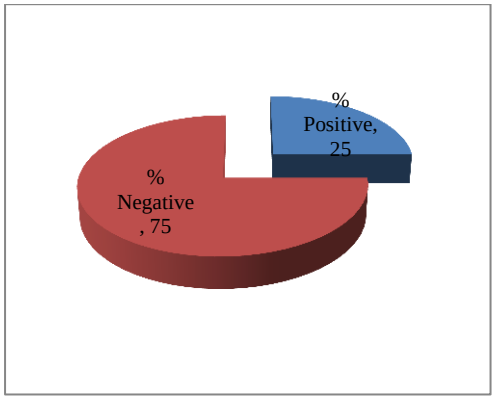

Fig 1: Percentage frequency of microscopy detection of *T. vaginalis* in the samples

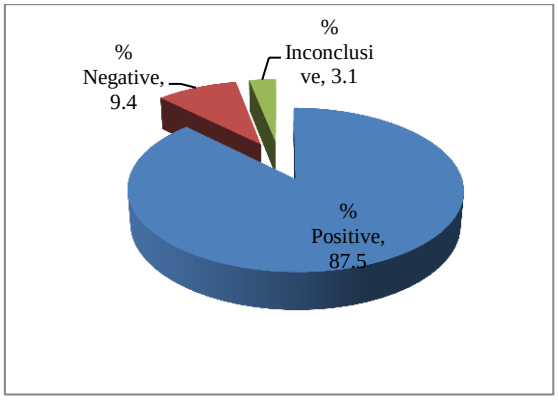

Fig 2: Percentage frequency of PCR detection of *T. vaginalis* in the samples

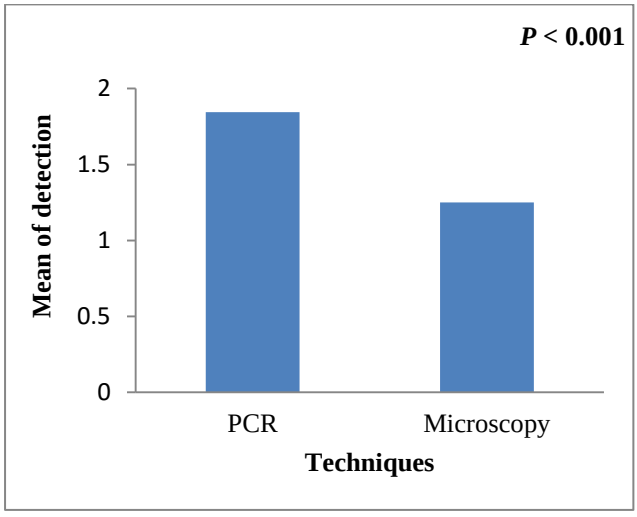

Figure 3: Mean of detection of PCR and microscopy to *T. vaginalis*

Mean of detection of PCR was 1.84 while that of microscopy was 1.25, $P < 0.001$. The degree of agreement (concordance) between PCR and microscopy in the diagnosis of *T. vaginalis* in vaginal swab samples using Kappa (κ) test was significant at 5% with κ value 0.08 ($\kappa = 0.08$, $P < 0.05$).

Relationship between T. vaginalis and possible development of Cervical Intraepithelial Neoplasia

Logistic regression was carried out on the two variables that have association with Pap smear (presence of abnormal discharge and microscopic findings). The result revealed that microscopy as a variable was important for the outcome of interest (Cervical Intraepithelial Neoplasia) based on the Wald test. The Wald test implied that those that were infected with *T. vaginalis* were 1.13 times more likely to have cervical intraepithelial neoplasia infection as compared to those that do not have *T. vaginalis* infection. The overall accuracy model (logistic regression model) to predict subject with cervical intraepithelial neoplasia was 91.6%, with a sensitivity of 33.3% and specificity of 97.3%.

Discussion

A comparative study of microscopy sensitivity with that of PCR was carried out for the detection of *T. vaginalis* in vaginal swab samples collected from 96 women attending the Obstetrics and Gynaecology unit, University College Hospital (UCH), for Papanicolaou smear test.

With the sensitivity of PCR shown to be 76−100% [31], this result is in agreement with some previous works done by other researchers. Works carried out by [32], demonstrated that the sensitivity of PCR was 80.95%. [33] reported PCR sensitivity of 84%. Studies carried out by [9] also strengthened this result. Their findings showed PCR sensitivity of 85.2%. Besides, works carried out by [34] showed 100% sensitivity of PCR to *T. vaginalis*.

On the whole, using PCR as a standard, the percentage sensitivity of wet-mount microscopy was 28.57% which is higher than (22%) obtained by [35]. A higher sensitivity (36%) was reported by [29]. Besides, works carried out by [36] showed wet-mount microscopy sensitivity to be 33.3%. Furthermore, [37] reported a wet-mount sensitivity of 56%.

Sexual partner is a significant risk to cervical intraepithelial neoplasia [38]. This study showed that *T. vaginalis* infection, which is a sexually transmitted parasite, was found only in participants with sexual partners while cervical intraepithelial neoplasia was also found only in participants with sexual partners, indicating that *T. vaginalis* infection could be a predisposing factor to cervical intraepithelial neoplasia. Multiple sexual partners increase the risk of *T. vaginalis*, thus increasing the chances of cervical intraepithelial neoplasia.

In retrospect, only 28.57% of participants with confirmed trichomoniasis were diagnosed by wet-mount microscopy. This finding shows that a more accurate molecular diagnostic test for *T. vaginalis*, such as PCR, is needed in order to prevent or reduce reproductive health complications [39]. Although, wet-mount preparation is a simple, rapid and relatively cheap diagnostic technique its sensitivity is very low. Its sensitivity is highly dependent on the state of the microscopy and the expertise of the microscopist. In addition, prompt transport and laboratory processing should be done within 30min of sample collection otherwise the trophozoite characteristic jerky movement will be lost by time. The diagnosis of *T. vaginalis* infection by PCR is a more sensitive method that should be integrated into a joint strategy for the screening of multiple sexually transmitted infections (STIs) [40]. Besides negative results obtained by wet-mount microscopy should further be tested with high molecular diagnostic technique such as PCR.

The discovery of positive association between *T. vaginalis* and cervical intraepithelial neoplasia has implicated *T. vaginalis* to be a predisposing factor to cervical intraepithelial neoplasia. So, for protection against cervical cancer and cervical intraepithelial neoplasia, *T. vaginalis* infection must be considered. Besides, more studies on the association between *T. vaginalis* and cervical intraepithelial neoplasia should be carried out.

In conclusion, the high prevalence of trichomoniasis recorded in this study might be a public health risk, more especially because HIV infection and transmission, and other STIs can be enhanced with *T. vaginalis* infection. Consequently, clinicians should routinely screen women visiting the obstetrics and gynaecology clinic for the trichomoniasis and appropriate treatment be administered early to prevent the spread of STIs and other health and social implications that could result from the infection. Additionally, negative results obtained by wet-mount microscopy should further be examined with other more sensitive diagnostic techniques such as PCR, nucleic acid assay testing (NAAT), DNA Assay, OSOM Trichomonas Rapid Test and Affirm VP III [41] to ascertain true prevalence rate of infection.

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