Prevalence and Detection of *Trichomonas vaginalis* in Human Immunodeficiency Virus-Infected Pregnant Women

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Word Counts

Summary: 30

Abstract: 192

Text: 2338

Number of references: 25

Number of figures: 1

Number of tables: 2

Conflict of Interest: The authors state no conflict of interest.

Funding: This work was supported by the National Institutes of Health [grant number 1-R21-HD084274-01A1]; the U.S. President's Emergency Plan for AIDS Relief [Cooperative Agreement: AID-674-A-12-00017]; and a travel grant from the UCLA Center for World Health.

Summary

Among HIV-infected pregnant women in South Africa, culture detected *T. vaginalis* in only a portion of PCR-positive cases, and cycle threshold values proved valuable in the interpretation of PCR results.

Abstract

Background

Trichomonas vaginalis is a STI associated with increased transmission of HIV and significant adverse birth outcomes; culture and PCR are commonly used in diagnosis.

Methods

Consenting HIV-infected pregnant women were recruited from clinics in South Africa and screened for *T. vaginalis* using PCR. PCR-positive women provided an additional sample for culture. We compared *T. vaginalis* detection between PCR and culture, and investigated how PCR cycle threshold (Ct) values differ among culture results.

Results

A total of 359 women were enrolled and 76 (20%) tested *T. vaginalis* PCR-positive. Cultures were obtained from 60 of the PCR-positive women, and 37 (62%) were culture-positive. The median baseline Ct of the PCR-positive/culture-positive group was 22.6 vs. 38.0 among those PCR-positive/culture-negative ($p<0.001$). Culture positive cases had lower Ct values (higher DNA load); a Ct value < 30 predicted positivity with a sensitivity of 97% and specificity of 96%.

Conclusions

Culture was positive in roughly half of PCR positive cases. The culture-negative cases had significantly higher Ct values, indicating a lower concentration of *T. vaginalis* DNA. A Ct

value of 30 provides a reliable threshold for predicting culture positivity. The clinical significance of culture-negative infections detected by PCR is still unclear.

Key Words: *Trichomonas vaginalis;* HIV; PCR; culture; cycle threshold;

Introduction

Trichomonas vaginalis is one of the most common sexually transmitted infections in the world. The World Health Organization (WHO) estimated that in 2012 there were 143 million new cases of *T. vaginalis*, more than the incidence of *Neisseria gonorrhoeae, Chlamydia trachomatis,* or syphilis infections. [1]

The high rates of *T. vaginalis* infection are particularly troubling in the context of adverse reproductive health and pregnancy outcomes. Women infected with *T. vaginalis* can experience a range of symptoms, including itching, vaginal discharge, and dysuria, as well as more significant complications such as pelvic inflammatory disease and fallopian tube pathology. [2, 3] *T. vaginalis* infection is also associated with an increased risk of acquiring HIV (risk ratio: 1.27-2.57), [4] as well as increased vaginal shedding of HIV. [3] A woman infected with *T. vaginalis* during pregnancy is more likely to experience preterm delivery, premature rupture of membranes, and delivery of low birthweight infants. [5, 6, 7] In addition, *T. vaginalis* infection during pregnancy has been associated with an increased risk of vertical transmission of HIV. [8]

T. vaginalis is a curable STI, making prompt detection and treatment appropriate interventions to mitigate infection-associated complications, particularly in high-risk populations. Estimates of the proportion of asymptomatic *T. vaginalis* infections are as high as 80% [5], and the US Centers for Disease Control and Prevention (CDC) recommends screening for *T. vaginalis* among HIV-infected women, especially HIV-infected pregnant women. [9] Three classes of detection methods of increasing sensitivity commonly used to screen for *T. vaginalis* are: wet-mount microscopy, culture, and nucleic-acid amplification tests (NAATs).

While NAATs are typically sensitive, specific, and have short turn-around times, their high sensitivity may result in the detection of low organism levels with limited clinical significance. In addition, remnant nucleic acids from non-viable organisms following treatment may be detected; this may be one reason why the CDC has no recommendation regarding test-of-cure after treatment for *T. vaginalis*. [9] As part of an ongoing study of STIs in pregnancy we compared *T. vaginalis* detection using culture and PCR, and assessed the value of PCR cycle threshold to predict culture positivity.

Materials and Methods

Study Participants

Participants were recruited from two clinics in the Soshanguve Township, Tshwane, South Africa from June 2016 through May 2017. All participants were HIV-infected pregnant women, >18 years old and <34 weeks gestation, and presenting for their first antenatal clinic visit for their current pregnancy. Women were assessed for eligibility, consented, and enrolled. Enrolled participants completed a baseline questionnaire, received basic antenatal care (ANC)

clinical services from the study nurse per South African national guidelines, [10] and were asked to provide a self-collected vaginal swab for STI testing. The specimens used for this study were collected from a subset of women from a large cohort study assessing the acceptability, feasibility, and impact on birth outcomes of point-of-care PCR diagnosis and treatment of *T. vaginalis*, *N. gonorrhoeae*, and *C. trachomatis*.

Ethics

The study protocol was approved by the institutional review boards of the University of Pretoria (IRB Approval #: 401/2015) and the University of California, Los Angeles (IRB Approval #: 15-001351). All participants provided informed written consent.

PCR Testing

The GeneXpert (Cepheid, Sunnyvale, California) diagnostic platform and Xpert® TV test cartridges were used to screen for *T. vaginalis*. [11] The Xpert® TV test cartridge includes three internal controls: a sample processing control which detects the presence of PCR reaction inhibitors; a sample adequacy control that confirms the presence of human genes in the specimen; and a probe check control that verifies the integrity of the probe and reagent preparation. The Xpert TV Assay's limit of detection is reported by the manufacturer as 5 cells/mL. [12] The cycle threshold (Ct) values of positive PCR runs were recorded.

HIV-infected pregnant women who tested positive for *T. vaginalis* were provided with metronidazole in a regimen similar to the South African [10] and U.S. CDC treatment guidelines. [9] A test-of-cure visit was scheduled for 3 weeks after treatment was initiated. Women with positive Xpert® TV tests were asked to provide an additional vaginal-swab for culture using a

FLOQSwab Dry Swab (Copan Diagnostics, Brescia, Italy). Women who tested positive for *T. vaginalis* were provided two options for partner treatment: a referral for treatment at the clinic, or a pill packet for *T. vaginalis* treatment.

T. vaginalis **Culture**

T. vaginalis culture was performed using the InPouch TVTM diagnostic device (Biomed, San Jose, CA, USA) per the manufacturer's instructions. [13] The InPouch TVTM limit of detection has been reported to be 4 organisms per mL [14] and 0.2 organisms per reaction. [15] Inoculated InPouch devices were incubated at clinic sites at 18^{o} - 37^{o}C, and transported to the University of Pretoria within 36 hours of inoculation.

Upon arrival at the University of Pretoria, culture devices were incubated at 37^{o}C and observed by wet-mount microscopy daily for motile trichomonads. An InPouch device was declared culture positive if motile trichomonads were observed within the first 7 days of incubation; if no motile trichomonads were observed in this time frame, the InPouch device was declared culture negative.

Data Management and Statistics

Study data were collected and managed using REDCap electronic data capture tools hosted at the Foundation for Professional Development (Pretoria, South Africa). [16] GeneXpert Ct values for PCR-positive/culture-positive and PCR-positive/culture-negative samples were compared to assess the parasite burden detected by PCR in the two groups.

Datasets were tested for normality using the Shapiro-Wilk test; data that were not normally distributed are reported here using median and inter-quartile range (IQR). Non-parametric comparisons of sample distributions were performed using the Mann-Whitney *U* test. All statistics were calculated using IBM SPSS Statistics for Windows (Version 24.0, IBM Corp. in Armonk, NY).

Results

T. vaginalis Screening at Baseline

A total of 359 HIV-infected pregnant women were enrolled, of whom all 359 (mean age: 30.1 years) provided self-collected vaginal swabs for PCR testing of *T. vaginalis*. Of these, 76 (20%) women tested positive for *T. vaginalis* (*T. vaginalis*-positive). Women who tested positive for *T.vaginalis* had similar demographic characteristics to *T. vaginalis*-negative women (Table 1). Of those *T. vaginalis*-positive women, 19 (25%) reported one or more of the following symptoms: dyspareunia, dysuria, abnormal vaginal discharge, or vaginal bleeding; 25 (33%) were observed to have vaginal discharge and/or bleeding on clinical exam; and 37 (49%) had no symptoms or signs upon examination. Of the 72 (95%) women who chose to inform their partner of their infection with *T. vaginalis*, 49 (68%) chose to provide their partner with a pill packet, while 23 (32%) chose to provide a clinic referral.

A total of 60 InPouch cultures were obtained from *T. vaginalis*-PCR-positive (PCR-positive) women during the first ANC visit, of which 37 (62%) were culture positive (Table 2). Women with positive cultures had similar demographic characteristics to culture-negative women (data not shown). The median Ct value of the PCR-positive/culture-positive group was

Table 1. Demographic characteristics of study participants, stratified by *T. vaginalis* PCR-status at baseline.

	T. vaginalis-PCR-Positive (*n* = 76)		*T. vaginalis*-PCR-Negative (*n* = 283)	
Age (years)	29.3	± 5.8	30.2	± 5.3
Est. Gest. Age (weeks)	19.5	± 6.7	17.6	± 6.9
Prior Pregnancies	1.8	± 1.4	1.9	± 1.3
On ART – *n* (%)	76	(100)	280	(99)
Level of Education – *n* (%)				
None	0	(0)	3	(1)
Below Matric	46	(61)	142	(50)
Matric Certificate	22	(29)	121	(43)
Degree/Diploma	5	(7)	12	(4)
No Response	3	(4)	5	(2)
Currently Employed – *n* (%)	28	(36)	120	(42)

Monthly Income				
None	6	(8)	28	(10)
< $76 USD per month	26	(34)	78	(28)
$76 USD - $380 USD per month	33	(43)	137	(48)
$381 USD - $760 USD per month	3	(4)	28	(10)
> $760 USD per month	1	(1)	7	(3)
No Response	7	(9)	5	(2)

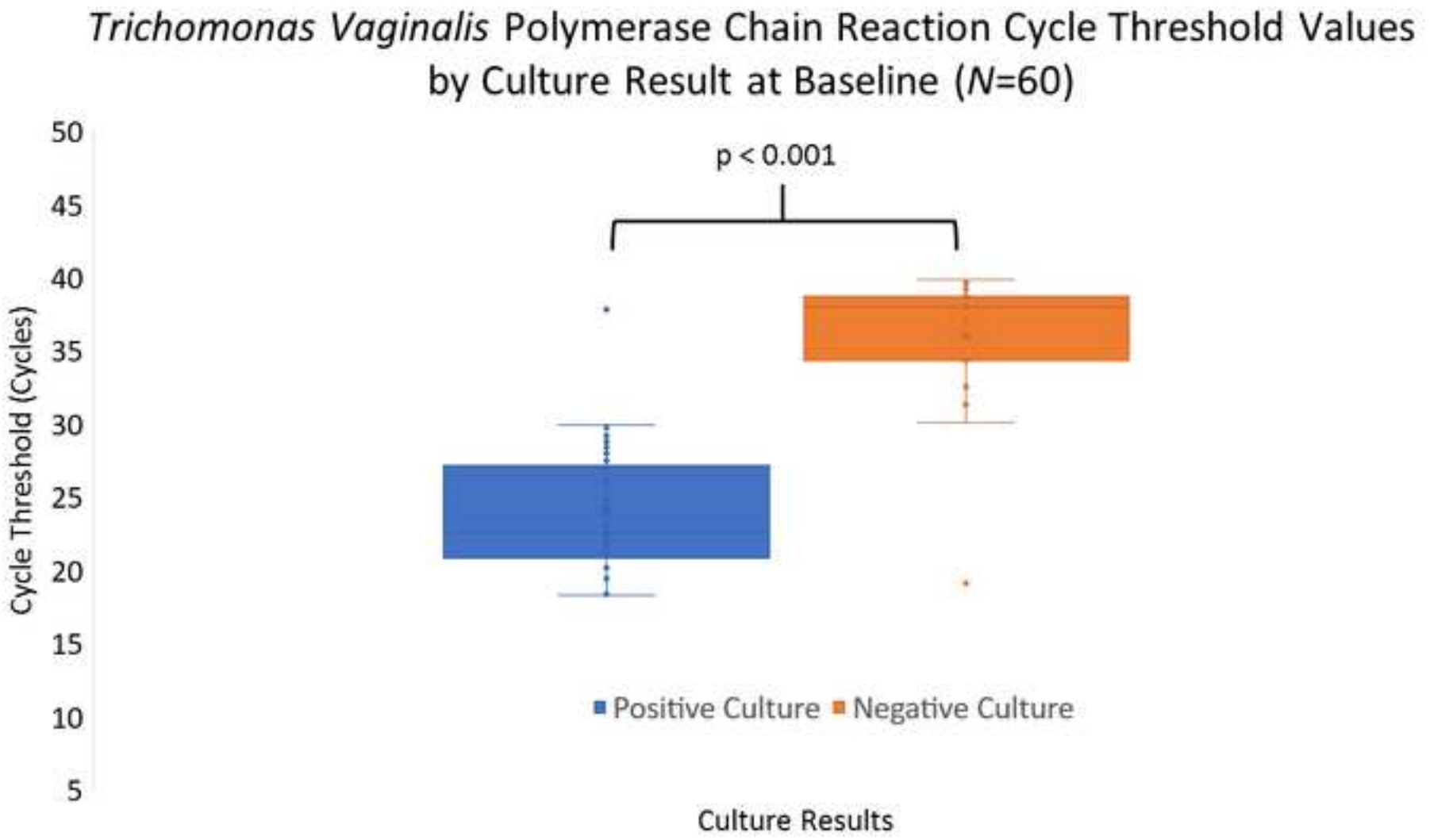
Plus-minus values are means ± standard deviation. Matric is roughly equivalent to high school graduation in South Africa. ART, Antiretroviral therapy; USD, United States Dollars.

Table 2. Number of women screened for *T. vaginalis*, polymerase chain reaction test results, and InPouch culture test results, at baseline and test-of-cure visits.

	Women Tested	***T. vaginalis* PCR Positive**	**Culture Samples Collected**	***T. vaginalis* Culture Positive**
Baseline	359	76	60	37
Test of Cure	59	13	10	5

Note that a culture sample was not obtained from all women who tested positive via PCR, and not all women testing PCR-positive for T. vaginalis *at baseline returned for a test of cure. PCR, polymerase chain reaction.*

Figure 1. Cycle threshold values for T. vaginalis PCR tests at baseline, by culture result. The nonparametric Mann-Whitney U test was used to compare sample distributions.



22.9 (IQR: 21.2 – 27.1), and the median Ct value of the PCR-positive/culture-negative group was 38.0 (IQR: 35.2 – 38.5). The difference in median Ct value between the two groups was 15.4 ($p < 0.001$; Figure 1). All culture-positive cases had a Ct value less than 30, with the exception of 1 case (Ct = 37.8). In contrast, all culture-negative cases had a Ct value less than 30, except for 1 case (Ct = 19.1). The area under the receiver operating characteristic curve was 0.95 ($p < 0.001$); a Ct value less than 30 correctly predicted culture positivity with a sensitivity of 97% and a specificity of 96% (Youden Index: 0.93).

T. vaginalis Positivity and Symptomatology at Baseline

Among the 34 women with self-reported symptoms and/or clinically-observed signs of an STI, the median Ct value was 24.9 (IQR: 21.3 – 30.1), while the median Ct of the 31 with no signs and/or symptoms was 29.1 (IQR: 22.3 – 37.3). The difference in median Ct value between the two groups was 4.3 ($p = 0.06$).

Women with symptoms and/or signs of an STI were culture-positive in 19 cases and culture-negative in 10 cases. Those without symptoms or signs were culture-positive in 18 cases and culture-negative in 11 cases. A chi-square analysis revealed no significant difference in the number of positive cultures between those with symptoms and/or signs and those without ($\chi 2 = 0.07$, $p = 0.78$).

T. vaginalis Testing at Test-of-Cure

Of the 59 PCR-positive women who returned for the 3-week test-of-cure (ToC) visit, 13 (22%) tested PCR positive again. Ten cultures were obtained from PCR-positive women at test-of-cure visits, of which 5 (50%) were culture positive (Table 2). The median Ct value of the PCR-

positive/Culture-positive group (*N* = 5) was 21.3 (IQR: 18.9 – 26.3), and the median Ct value of the PCR-positive/Culture-negative group (*N* = 5) was 37.5 (IQR: 34.3 – 37.8). Four of the five PCR-positive/Culture-negative cases had a Ct value greater than 30.

Discussion

We observed a high prevalence of *T. vaginalis* (20%) in a population of HIV-infected pregnant women seeking antenatal care at public health centers in South Africa. The prevalence was similar to other cohorts of HIV-infected women (30%) [17] and non-HIV infected pregnant women (41%) [18] in South Africa. The prevalence was somewhat higher, however, than in HIV-infected pregnant women in the Democratic Republic of Congo (18%) [19] and Nigeria (19%). [20] We found that approximately half of the *T. vaginalis* infections were symptomatic, consistent with current estimates suggesting high proportions of asymptomatic *T. vaginalis* infection. [5] Given the current policy of syndromic management of STIs in South Africa, and most of the world, only symptomatic women would be treated for STIs during antenatal care [10, 21]

The CDC recommendation to screen HIV-infected pregnant women for *T. vaginalis* indicates a need to determine the most effective screening method. The current gold standard in many settings for *T. vaginalis* diagnosis is culture. Our work shows that culture only detects *T. vaginalis* in roughly half of cases detected by PCR. We also found no association between signs/symptoms of STI infection and culture positivity. These findings suggest culture may miss many cases of *T. vaginalis*, even in symptomatic women, that would otherwise have been detected by PCR.

An additional benefit of a PCR screening method is the information on organism load provided by the cycle threshold (Ct) value. When we compared the Ct values of the PCR-positive/Culture-positive and PCR-positive/Culture-negative groups at baseline, we observed significantly higher Ct values in the PCR-positive/Culture-negative group. These findings are consistent with those observed by Caliendo and colleagues in 2005 amongst a cohort of HIV-uninfected young women, when they compared *T. vaginalis* detection with the InPouch to a laboratory-based PCR method; the authors found significantly higher Ct values in a PCR-positive/Culture-negative group compared to a PCR-positive/Culture-positive group. [15]

The ten cultures obtained from PCR-positive women at the test-of-cure visit showed a similar trend to that observed at baseline, with five positive cultures having a median Ct value 16.2 cycles greater than that of the five negative cultures. The most current Sexually Transmitted Disease Treatment Guidelines from the CDC make no recommendation on whether to implement test-of-cure after *T. vaginalis* treatment, though the guidelines do advise that NAAT "can be conducted as soon as 2 weeks after treatment." [9] In a study investigating how long *T. vaginalis* nucleic acid remains detectable after metronidazole treatment, Martin and colleagues found that *T. vaginalis* RNA was no longer detectable by 3 weeks post-treatment in a group of 34 women. [22] Although our sample size was small, the low rate of culture positivity at test-of-cure may suggest remnant *T. vaginalis* DNA persists at least as long as 3 weeks after treatment.

It is unclear what the clinical significance is of a woman testing positive for *T. vaginalis* using a PCR method but testing negative using culture. The sensitivity of NAATs allows for the detection of low levels of *T. vaginalis* organisms, as well as remnant protozoal nucleic acid in

injured or phagocytosed cells. [23] Thus, the PCR-positive/Culture-negative cases observed in our study (both at baseline and at test-of-cure) may represent detection of *T. vaginalis* DNA in a sample with fewer adverse clinical consequences. However, it is also possible that PCR-positive/Culture-negative cases are in fact clinically significant infections below the limit of detection of culture. Peterman and colleagues found evidence suggesting *T. vaginalis* may be undetectable by culture at one visit but then become detectable at a subsequent visit, without any intervening sexual contact. [24] Clearly, more work remains to elucidate the clinical significance of a PCR-positive/Culture-negative test. A potentially fruitful area of future research may lie in the use of viability PCR to differentiate between DNA of cells with intact membranes from free-floating DNA. [25]

One limitation of this study is that we did not perform wet mount microscopy or collect cultures from women who tested PCR negative for *T. vaginalis*. Although it is theoretically possible that some infections were missed, the generally accepted higher sensitivity of NAATs compared to both wet mount and culture suggests this is unlikely. [3] Another limitation is that we only collected samples at the 3-week test-of-cure. Future studies may focus on performing multiple consecutive tests and determining an appropriate Ct threshold for false-positives due to clinically irrelevant parasitic nucleic acids. If culture is assumed to be the gold standard for a clinically relevant infection, a Ct value less than 30 could yield a sensitivity of 97% and a specificity of 96%; however, the clinical significance of *T. vaginalis* DNA in culture negative cases is still unclear. Future studies which follow such PCR-positive/culture-negative cases in patients who do not receive treatment could shed light on this clinical question.

In summary, we found high rates of *T. vaginalis* infection (symptomatic and asymptomatic) among HIV-infected pregnant women in Soshanguve Township, South Africa. We have shown that culture detects *T. vaginalis* organisms in only a portion of PCR-positive cases, and demonstrated the potential utility of cycle threshold values in the interpretation of PCR results. We highlight a lingering question in the field, namely what is the clinical significance of *T. vaginalis* DNA detection among culture negative cases during STI screening and follow-up? Future work focused on answering this question could significantly improve our understanding of the most effective methods for *T. vaginalis* detection.

Acknowledgements

We thank Cepheid for the donation of test cartridges and loan of equipment. We would also like to thank our study nurses Chriselda Maginindane and Tshiamo Matlala, and study assistants Mpho Ngubeni and Bertha Theledi for their support and contribution to this study. Erika Morikawa was supported by the David Geffen School of Medicine's Dean's Office and the UCLA Center for World Health. We would also like to thank Andrew Biundo for facilitating the delivery of cultures from the clinic to the University of Pretoria. Finally, we would like to thank the patients for their participation in our study.

References

1. Newman L, Rowley J, Vander Hoorn S, et al. Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. PLOS ONE **2015**; 10:e0143304.
2. de Waaij DJ, Ouburg S, Dubbink JH, et al. Evaluation of Presto plus assay and LightMix kit Trichomonas vaginalis assay for detection of Trichomonas vaginalis in dry vaginal swabs. Journal of Microbiological Methods **2016**; 127:102-4.
3. Kissinger P. Trichomonas vaginalis: a review of epidemiologic, clinical and treatment issues. BMC Infectious Diseases **2015**; 15.
4. Kissinger P, Adamski A. Trichomoniasis and HIV interactions: a review. Sexually Transmitted Infections **2013**; 89:426-33.
5. Cotch MF, Pastorek JG, Nugent RP, et al. Trichomonas vaginalis Associated With Low Birth Weight and Preterm Delivery. Sexually Transmitted Diseases **1997**; 24:353-60.
6. Mann JR, McDermott S, Gill T. Sexually transmitted infection is associated with increased risk of preterm birth in South Carolina women insured by Medicaid. The Journal of Maternal-Fetal & Neonatal Medicine **2010**; 23:563-8.
7. Silver BJ, Guy RJ, Kaldor JM, et al. Trichomonas vaginalis as a Cause of Perinatal Morbidity. Sexually Transmitted Diseases **2014**; 41:369-76.
8. Gumbo FZ, Duri K, Kandawasvika GQ, et al. Risk factors of HIV vertical transmission in a cohort of women under a PMTCT program at three peri-urban clinics in a resource-poor setting. Journal of Perinatology **2010**; 30:717-23.
9. Workowski KA. Centers for Disease Control and Prevention Sexually Transmitted

Diseases Treatment Guidelines. Clinical Infectious Diseases **2015**; 61:S759-S62.

10. South African National Department of Health. Sexually Transmitted Infections: Management Guidelines 2015. **2015**.
11. Van Der Pol B. Clinical and Laboratory Testing for Trichomonas vaginalis Infection. Journal of Clinical Microbiology **2015**; 54:7-12.
12. Xpert® TV package insert. Cepheid, Sunnyvale, CA; **2014.**
13. InPouch™ TV package insert. Biomed Diagnostics, Inc., White City, OR; **2015.**
14. Borchardt KA, Smith RF. An evaluation of an InPouch TV culture method for diagnosing Trichomonas vaginalis infection. Sexually Transmitted Infections **1991**; 67:149-52.
15. Caliendo AM, Jordan JA, Green AM, et al. Real-Time PCR Improves Detection ofTrichomonas vaginalisInfection Compared With Culture Using Self-Collected Vaginal swabs. Infectious Diseases in Obstetrics and Gynecology **2005**; 13:145-50.
16. Harris PA, Taylor R, Thielke R, et al. Research electronic data capture (REDCap)—A metadata-driven methodology and workflow process for providing translational research informatics support. Journal of Biomedical Informatics **2009**; 42:377-81.
17. Kock M, Rukasha I, Dijkmans A, et al. P3-S7.07 Detection of Trichomonas vaginalis in HIV positive women in Pretoria, South Africa. Sexually Transmitted Infections **2011**; 87:A301-A.
18. Wilkinson D, Abdool Karim SS, Harrison A, et al. Unrecognized Sexually Transmitted Infections In Rural South African Women. Bulletin of the World Health Organization **1999;** 77:22-28.
19. Sutton MY, Sternberg M, Nsuami M, et al. Trichomoniasis in pregnant human

immunodeficiency virus–infected and human immunodeficiency virus–uninfected Congolese women: Prevalence, risk factors, and association with low birth weight. American Journal of Obstetrics and Gynecology **1999**; 181:656-62.

20. Oyeyemi OT, Fadipe O, Oyeyemi IT. Trichomonas vaginalis infection in Nigerian pregnant women and risk factors associated with sexually transmitted infections. International Journal of STD & AIDS **2015**; 27:1187-93.
21. World Health Organization. Guidelines for the Management of Sexually Transmitted Infections. Reproductive Health and Research. **2004.**
22. Martin DH, Burnett J, Taylor SN. O10.2 Trichomonas vaginalis nucleic acid clearance following treatment of hiv negative women. Sexually Transmitted Infections **2015**; 91:A47.1-A.
23. Wang Y, Wang Z, Zhang Y, et al. Polymerase chain reaction–based assays for the diagnosis of human brucellosis. Annals of Clinical Microbiology and Antimicrobials **2014**; 13.
24. Peterman Thomas A, Tian Lin H, Metcalf Carol A, et al. Undetected Trichomonas vaginalis Infections? Clinical Infectious Diseases **2009**; 48:259-60.
25. Janssen KJH, Hoebe CJPA, Dukers-Muijrers NHTM, et al. Viability-PCR Shows That NAAT Detects a High Proportion of DNA from Non-Viable Chlamydia trachomatis. PLOS ONE **2016**; 11:e0165920.