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Laboratory Diagnosis Of T. *Vaginalis* In Pregnant And Non-Pregnant Women

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ABSTRACT

Objectives: To determine the frequency of Trichomonas vaginalis (T. vaginalis) infection according to different clinical laboratory techniques in a population of pregnant and non-pregnant patients receiving care at reference hospitals in Goiânia, and to perform molecular identification of the parasite using polymerase chain reaction (PCR).
Methods: Traditional techniques were used: culture (gold-standard), wet mount microscopy and Papanicolaou (Pap) smears. Additionally, a stained preparation from cultured T. vaginalis was used. Molecular identification of T. vaginalis was made by PCR using the TVK3/TVK7 primer set.
Results: There was no statistically significant difference in the rates of T. vaginalis infection between the groups of pregnant and non-pregnant women: culture (20.8 versus 25.5%); wet mount microscopy (12.5 versus 7.1%); Pap smear (31.4 versus 48.9%); stained preparation from cultured T. vaginalis (37.5 versus 52.0%) and PCR (12.5 versus 7.1%). Accuracy was established by comparison with culture. Sensitivity with wet mount microscopy was greater in the pregnant group (50% versus 30% in non-pregnant women), while the sensitivity of the Pap smear was 100% in the pregnant women and 97% in the non-pregnant women. Specificity was 100% with wet mount examination in both groups, while the specificity of Pap smear was 83% in the pregnant women versus 64% in the non-pregnant women.
Conclusions: The prevalence of T. vaginalis in this study was high, both in the pregnant women and in the non-pregnant women. The comparative study showed the degree of effectiveness obtained with the techniques evaluated.

KEYWORDS: Trichomonas vaginalis; clinical laboratory techniques; women; pregnant women; polymerase chain reaction

Introduction

Trichomonas vaginalis (*T. vaginalis*) is a flagellated protozoan that infects the vagina. Its prevalence varies in accordance with the characteristics of the populations evaluated. The prevalence rates of *T. vaginalis* are higher in poor populations (10-40%) from countries such as Zimbabwe and Zambia (in Africa), Egypt (in the Middle East), the coastal regions of Peru, and northeastern Brazil (in Latin America). [1-4] Hygiene conditions and women's immune status are important characteristics to be analyzed when calculating the prevalence of this parasite since the frequency of this infection is highest in pregnant women and in women with pathologies that affect the immune system. [1,2]

There are three main clinical laboratory techniques routinely used in the diagnosis of *T. vaginalis*. With *wet mount microscopy*, specificity is 100%; however, the sensitivity of this exam is fairly poor due to the rapid loss of flagellar motility (within 30 minutes). [5] With *liquid culture*, sensitivity and specificity are considered high (around 95%), since culture provides the parasite with the ideal nutritional conditions for its development and multiplication. [6] *Papanicolaou (Pap) smears* are the most commonly used method for the detection of *T. vaginalis*. Specificity and sensitivity are only moderate with Pap smears (around 80%), since the presence of static trophozoite forms can be a confounding factor, making the effectiveness of this method highly dependent on the examiner's experience. [7]

Polymerase chain reaction (PCR) techniques are highly specific (98%) and allow a specific target DNA sequence to be amplified *in vitro*. Amplification techniques use specific primers to identify the DNA sequence of the parasite. The sensitivity of PCR of approximately 95%, according to the most recent studies, depends on the optimization and standardization of the protocols applied, demanding effort and dedication from investigators. [6]

Pregnancy may favor *T. vaginalis* infection, since this is a period in which immunity is physiologically reduced. The infection may trigger early onset placental insufficiency, principally in the second trimester of pregnancy, resulting in premature delivery of the newborn infant. [8, 9]

The objective of the present study was to compare the techniques of wet mount microscopy and Papanicolaou (Pap) smears with the gold standard, liquid culture, and to also perform molecular identification of *T. vaginalis* using the TVK3/TVK7 primer set in pregnant and non-pregnant patients receiving care at reference hospitals in Goiânia.

Materials and Methods

Population

This cross-sectional, experimental study involved 170 patients, 72 of whom were pregnant, while the remaining 98 were not. Low-risk pregnant patients of any age who were requesting gynecological examination comprised the group of pregnant women. The control group consisted of sexually active, non-pregnant women in an appropriate phase of the cycle to permit samples to be taken for testing.

Site
The patients were receiving care at two of the principal referral public healthcare centers in the state of Goiás, Brazil: the *Dona Iris Hospital and Maternity Unit*, a reference in women's healthcare and linked to the Municipal Health Fund, and the *Federal University of Goiás' Teaching Hospital*. Both institutes are part of the Brazilian National Health Service network in the city of Goiânia, Goiás, Brazil.

Ethical issues
The study protocol was approved by the internal review board of the Federal University of Goiás Teaching Hospital, accredited by the National Commission of Ethics in Research (CONEP). The participants signed an informed consent form and the study was conducted in accordance with the 2008 revision of the Declaration of Helsinki. [10]

Sampling
Medical teams at the respective hospitals collected the samples.

Sampling procedures
During a gynecological examination, cervicovaginal secretion samples were collected using a sterile swab against the posterior vaginal fornix, ectocervix and endocervix. This triple sample collection was not performed in the group of pregnant women due to the potential risk of bleeding and/or miscarriage.

Wet mount microscopy
For this exam, a sterile swab was used to collect samples. The swabs were immediately soaked in sterile saline solution (0.85%). Microscopic examination was performed within half an hour of sampling.

Liquid culture
Modified Diamond's medium, considered the gold standard, was used for the culture of *T. vaginalis*. [10] Sampling was performed as for wet mount microscopy, with the vaginal swabs immediately being transferred into the liquid medium. The samples were examined on a glass slide under a coverslip, both immediately following sampling. Later analyses were conducted at 24, 48 and 72 hours.

Stained preparation from cultured T. vaginalis
After a 72-hour period, the cultures in liquid medium were centrifuged at 3,500 rpm and their pellets were smeared onto glass slides. These smears were placed in an oven at 37ºC for air-drying fixation. Next, the preparations were stained using the Quick Panoptic method with Romanowsky stains.

DNA extraction
To extract the DNA, the tubes containing the swabs in liquid culture medium were shaken vigorously and the swabs were then removed. Next, the tubes were centrifuged at 3,500 rpm for 5 minutes. The supernatants were removed and the pellets were re-suspended in 10 ml of deionized water and centrifuged again, after which their supernatants were removed. The washed pellets were stored at -21ºC. DNA extraction was later performed using the QIAamp Mini Kit (QIAGEN, Valencia, CA).

PCR
To perform PCR and amplification of the target DNA, the following primers were synthetized: TVK3: TCTGTGCCCCGTCTTAAGTATGC and TVK7: ATTGTCGAACATTGGTCTTACCCTC from a conserved region of DNA from *T. vaginalis* containing 262 pb. The synthetized sequences were checked and aligned with the GenBank NCBI database using the basic local alignment search tool (BLAST) (blast.ncbi.nlm.nih.gov/). To amplify the DNA target sequence, a mix was prepared with 2.5 µl buffer (10x), 1.0 µl of $MgCl_2$ (50 mM), 0.5 µl of dNTP mix (10 mM), 6.5 µl of Milli-Q H_2O, 0.5 µl of Taq polymerase (5 IU), 1 µl of each primer and 10 µl of DNA extracted from the sample, resulting in a total volume of 25 µl. Amplification was performed using the PTC-0200 DNA Engine® thermal cycler (Bio-Rad, Hercules, CA). The amplification protocol was as follows: 2-minute initial denaturation at 96ºC, 33 cycles of 1 minute at 94ºC for denaturation, 1-minute annealing at 60ºC, 1 minute and 30 seconds for extension at 70ºC, and 30 minutes at 60ºC for the final extension of the target DNA. The amplicons were electrophoresed using the automated DNA sequencer MegaBACE 1000 (GE Health Care, USA). The genotyping analyses were performed using the Fragment Profiler® software program.

Quality control
Positive and negative controls were included in all the PCR runs. The DNA isolated from *T. vaginalis* (above 10 x 10^5 cells/ml), developed in vitro, was used as positive control. The negative control consisted of a mix of PCR with primers but without the addition of DNA.

Results
The frequencies of *T. vaginalis* found with liquid culture, the gold standard technique, were practically the same in the two groups: 20.8% in the group of pregnant women versus 25.5% in the group of non-pregnant women (Table 1). With the Pap smear technique (Figure 1A), the number of samples testing positive for *T. vaginalis* was higher (31.43% in the pregnant women versus 48.9% in the non-pregnant women).

In the stained preparations from cultured *T. vaginalis* (Figure 1B), the number of samples testing positive for the parasite was the highest in both groups (37.5% versus 52.0%). With wet mount microscopy (Figure 1C), the percentage in the two groups was similar to that found with PCR (12.5% versus 7.4%).

The DNA extraction technique and the protocol used for PCR were found to be effective, allowing confirmation of the presence of the parasite DNA in a large proportion of the samples that tested positive in culture. PCR positivity was 12.5% in the group of pregnant women and 7.1% in the non-pregnant women (Figure 2).

The effectiveness of wet mount microscopy and Pap smear was established by comparing these techniques with the gold standard liquid culture (Tables 2 and 3). The Pap smear technique was found to be fairly sensitive, with the presence of *T. vaginalis* being detected in 100% of cases; however, compared to the gold standard, the rate of false positives was high, particularly in the group of non-pregnant women, where specificity was 64% compared to 83% in the group of pregnant women. The sensitivity of wet mount microscopy was higher in the group of pregnant women (50%) compared to the non-pregnant women (30%); however, specificity was 100% in both groups.

Table 1: **Frequency of** *T. vaginalis* **infection according to three different diagnostic techniques in pregnant and non-pregnant women.**

Techniques	Pregnant Women	Non-Pregnant Women	Total
	T. vaginalis +	*T. vaginalis* +	
	n %	n %	n %
Wet mount microscopy	9 (12.50)	7 (7.14)	16 (9.41)
Liquid culture	15 (20.83)	25 (25.51)	40 (23.53)
Pap smear	22 (31.43)	48 (48.98)	70 (41.18)
Stained preparation from cultured T. vaginalis	27 (37.50)	51 (52.04)	78 (45.88)
PCR	9 (12.50)	7 (7.14)	16 (9.41)
Total	72 (100.00)	98 (49.6)	170 (100.00)

Table 2: **Accuracy of the techniques used to detect** *T. vaginalis* **in pregnant women receiving care at the** *Dona Iris Hospital and Maternity Unit***, Goiânia, Goiás, Brazil.**

Techniques		*Liquid Culture (gold standard)*			Measures of Accuracy	%
		Positive	Negative	Total		
Wet mount microscopy	Positive	7	0	7	Sensitivity	50
	Negative	7	58	65	Specificity	100
	Total	14	58	72	Positive predictive value	89
					Negative predictive value	80
Pap smear	Positive	14	10	24	Sensitivity	100
	Negative	0	48	48	Specificity	83
	Total	14	58	72	Positive predictive value	58
					Negative predictive value	100

Table 3: Accuracy of the techniques used to detect *T. vaginalis* in non-pregnant women receiving care at the *Dona Iris Hospital and Maternity Unit*, Goiânia, Goiás, Brazil.

Techniques		*Liquid Culture (gold standard)*			Measures of Accuracy	%
		Positive	Negative	Total		
Wet mount microscopy	Positive	7	0	7	Sensitivity	30
	Negative	16	75	91	Specificity	100
	Total	23	76	98	Positive predictive value	100
					Negative predictive value	82
Pap smear	Positive	21	27	48	Sensitivity	95
	Negative	1	49	50	Specificity	64
	Total	22	76	98	Positive predictive value	44
					Negative predictive value	98

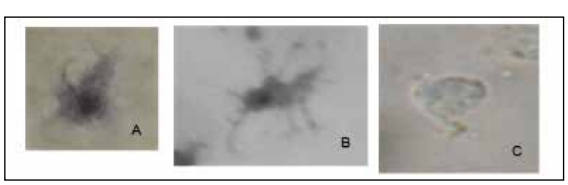

Figure 1. A: *T. vaginalis* **in a Papanicolaou smear under optical microscopy (x1,000). B:** *T. vaginalis* **on a smear of cultured material in liquid medium, stained using Romanowsky-type stain (Quick Panoptic), under optical microscopy (x1,000). C:** *T. vaginalis* **in a sample of vaginal secretion with saline solution on a glass slide under coverslip, using optical microscopy (x1,000).**

Discussion

The present study found a high prevalence of *T. vaginalis*, both in the group of pregnant women and in the non-pregnant women. The frequencies varied according to the technique used, with PCR being more effective in the group of pregnant women. Although not significant, this difference may reflect greater success in obtaining DNA from pregnant women compared to non-pregnant women.

The DNA extraction and PCR techniques successfully detected and confirmed positive cases found with liquid culture. The method of extracting DNA from *T. vaginalis* was performed directly from the liquid medium culture, as previously described by Kengne et al. in 1994, confirming the effectiveness and precision of the procedure. [12]

The results found with the PCR technique confirmed those reported by Lawing et al., with a percentage that was close to that achieved with the gold standard liquid culture.[13] PCR resulted in high specificity, amplifying the conserved region of the gene, with a mutation risk that was considered minimal or zero, as previously shown in the studies published by Khalaf & Kadhim and Crucitti et al. [14,15]

In the present study, even after the DNA from *T. vaginalis* had deteriorated, the molecular technique was able to detect its presence, albeit in very small quantities. With the MegaBACE 1,000 automated DNA sequencer used to perform the electrophoretic run, it was possible to detect the presence of the precise region of interest, collected in culture medium and confirmed in an electrophoretic run in polyacrylamide gel.

Kurewa et al. reported the presence of the parasite in 11.8% of pregnant women using only wet mount microscopy. Those results are in agreement with the findings of the present study in which a rate of 12.5% was found in a similar group using the same technique. [1]

Wet mount microscopy is a highly specific technique in which *T. vaginalis* is identified by its flagellar motility at a time when the parasite load in most cases is generally high. With this technique, the parasite was found within 30 minutes of sampling.

Stoner et al. found that, with wet mount microscopy, the flagella of *T. vaginalis* gradually lose motility within 30 minutes after collection. Exposure to oxygen is presumed to be the cause of this loss, since *T. vaginalis*, which is considered a facultative anaerobic parasite, fails to complete the citric acid cycle. [5]

In the study conducted by Saleh et al., sensitivity with wet mount microscopy was high, with high rates of positivity. Those results contradict the findings of most of the papers found in the literature. The high sensitivity found by those authors may be due to the use of glucose (5%) in the saline solution, with the sample being stored at body temperature (37ºC) and examined 10 minutes later. [16] In the present study, sensitivity was low with wet mount microscopy and this is in agreement with other studies in which the unmodified classic technique was used. [13]

Bearing in mind that culture for *T. vaginalis* is a technique that involves the maintenance and multiplication of the parasites in their trophozoite form and that these forms are distinguished from polymorphonuclear leukocytes by flagellar movement, we presume that after 48 hours (two days) the cultures in liquid medium should be centrifuged, the sediments placed as smears on glass slides and then stained using Romanowsky stains (Giemsa and/or Quick Panoptic). With this method, positivity for *T. vaginalis* was high.

Higher frequencies of *T. vaginalis* were found in the culture staining preparations and Pap smears. Because flagellar motility is not needed for them to be recognized, the trophozoite forms of the parasite are distinguished from cell remnants by the presence of their unique and slightly elongated nuclei that are perfectly visible and characterized using common staining techniques (Romanowsky-type stains) and with Papanicolaou staining.

The study conducted by Mahmoud et al. reported poor sensitivity (33%) with Giemsa staining, a Romanowsky-type dye. Those authors emphasized the fact that the trophozoites had been lost on the slide or damaged at the time of microscopic visualization. [17] Nevertheless, the results of the present study confirm that these dyes represent an important tool for visualizing cultured parasites.

The individual host conditions as well as the aggressiveness and the number of parasites are other factors that always affect the signs and symptoms of trichomoniasis. [18] For this reason, we believed that pregnancy could have resulted in a greater number of positive results compared to the control group, a hypothesis that, in the present study, was not confirmed.

The presence of *T. vaginalis* in relation to the immune status of patients was done by assessing the relative number of lymphocytes in full blood counts. The blood samples and the gynecological sampling were obtained on dates close to each other. In the group of pregnant women, a slight majority (54%) had a percentage of lymphocytes below 25%, a finding that is considered normal bearing in mind that the organism of pregnant women physiologically establishes an adaptive immunity, stimulating tolerance to fetal antigens (50% of the genetic material being paternal). [19] *T. vaginalis* infection was present in 47.6% of the group of immunosuppressed pregnant women (10/21) and in 37.5% (6/47) of the group of immunosuppressed non-pregnant women, i.e. those women with a percentage of lymphocytes below 25%.

Conclusions

The rates of *T. vaginalis* infection found in the present study were high, both in the group of pregnant women and among the non-pregnant women. The high frequency of the parasite was principally due to the poor socioeconomic conditions of the study population. [1,2,20]

The comparative study highlighted the degree of effectiveness of the clinical laboratory techniques. The DNA extraction and PCR techniques used for the molecular identification of *T. vaginalis* were also found to be effective.

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There was no statistically significant difference between the groups (p>0.05).

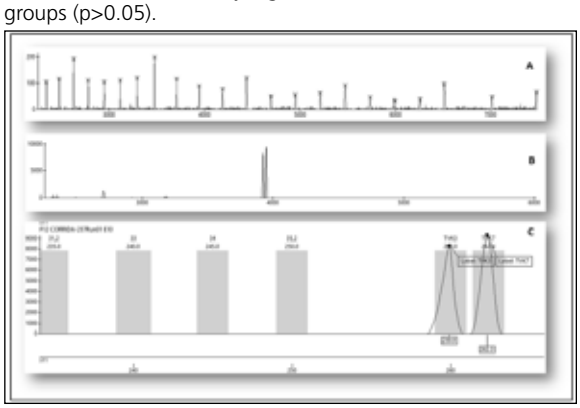

Figure 2: **Electropherogram (PCR).** *A:* **Internal lane standard (ILS 600) with 22 bands marked with carboxy-x-rhodamine every 20 pb, spaced at every 25 pb. The internal lane standard of 600 pb was used to attribute the sizes of each DNA fragment separated by capillary electrophoresis.** *B:* **Localization of the target DNA in the electrophoretic run performed in the MegaBACE 1000 automated sequencer.** *C:* **Electropherogram of genotyping, showing the presence and location of the TVK7 and TVK3 genes in the sample of target DNA.**

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