Molecular sensitivity threshold of wet mount and an immunochromatographic assay evaluated by quantitative real-time PCR for diagnosis of Trichomonas vaginalis infection in a low-risk population of childbearing women

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SUMMARY
Vaginal trichomoniasis is a sexually transmitted infection caused by Trichomonas vaginalis, a flagellated protozoan. Diagnosis of T. vaginalis infection is mainly performed by wet mount microscopy, with a sensitivity ranging from 38% to 82%, compared to culture, still considered the gold standard. Commercial immunochromatographic tests for monoclonal-antibody-based detection have been introduced as alternative methods for diagnosis of T. vaginalis infection and have been reported in some studies to be more sensitive than wet mount. Real-time PCR methods have been recently developed, with optimal sensitivity and specificity. The aim of this study was to evaluate whether there is a molecular sensitivity threshold for both wet mount and immunochromatographic assays. To this aim, a total of 1487 low-risk childbearing women (median age 32 years, interquartile range 27-37) were included in the study, and underwent vaginal swab for T. vaginalis detection by means of a quantitative real-time PCR assay, wet mount and an immunochromatographic test. Upon comparing the results, prevalence values observed were 1.3% for real-time PCR, 0.5% for microscopic examination, and 0.8% for the immunochromatographic test. Compared to real-time PCR, wet mount sensitivity was 40% [95% confidence interval (CI) 19.1% to 63.9%] and specificity was 100% (95% CI 99.7% to 100%). The sensitivity and specificity of the immunochromatographic assay were 60% (95% CI 36% to 80%) and 99.9% (95% CI 99.6% to 100%), respectively. Evaluation of the wet mount results and those of immunochromatographic assay detection in relation to the number of T. vaginalis DNA copies detected in vaginal samples showed that the lower identification threshold for both wet mount (chi-square 6.1; P=0.016) and the immunochromatographic assay (chi-square 10.7; P=0.002) was ≥100 copies of T. vaginalis DNA/5 μL of eluted DNA.

Keywords: Trichomonas vaginalis, wet mount, immunochromatographic assay, real-time PCR.

INTRODUCTION
Vaginal trichomoniasis is a sexually transmitted infection (STI) caused by Trichomonas vaginalis, a flagellated protozoan [1]. The prevalence of T. vaginalis infection is not completely known, since it is not a reportable disease, and also due to...
the highly variable characteristics of the populations evaluated by the different studies, and the different accuracy of the diagnostic methods employed [2, 3]. Culture is still considered the gold standard for diagnosis of T. vaginalis infection. The molecular real-time PCR method has been recently introduced for the laboratory diagnosis, with optimal sensitivity and specificity [4]. Compared with real-time PCR, culture showed 100% specificity, but 73.3% sensitivity [4]. Nevertheless, in routine laboratory practice, diagnosis of T. vaginalis vaginal infection is mainly performed by wet mount microscopy, with sensitivity ranging from 38% to 82%, compared to culture, depending by the expertise of the reader, temperature, evaporation of moisture, and the inoculum size, in that fewer than 10⁴ organisms/mL will not be seen [5]. Similar values of sensitivity for wet mount (60%) are observed also in comparison to PCR [4]. The immunochromatographic monoclonal-antibody-based detection technique can be an alternative diagnostic method for diagnosis of T. vaginalis infection, already found by some Authors as more sensitive than wet mount (83% vs 71.4%, P=0.004), being independent from both expertise of the laboratorian and other variables influencing the motility of the protozoa [6]. To date, a molecular threshold, defined as the number of T. vaginalis DNA copies below which wet mount or immunochromatographic assays would result negative, is not known. The aims of this study were: to evaluate if there is a molecular sensitivity threshold for both wet mount and an immunochromatographic assay used in the study; to evaluate the molecular epidemiology of T. vaginalis infection in a low-risk population of childbearing women; to evaluate the diagnostic accuracy of wet mount and immunochromatographic assay compared with real-time PCR used as reference method.

## MATERIALS AND METHODS

### Study design

This was a single-centre, cross-sectional, observational study conducted from January 2015 to October 2015. All female outpatients from Prenatal Medicine, Fertility Centre, or Microbiology ambulatories, independently of pregnancy or presence/absence of symptoms of vulvovaginitis, undergoing vaginal swabs for microbiological examination, were included in the study. Exclusion criteria were: antimicrobial therapy within 30 days prior of the evaluation, reported sexual activity or use of douches, vaginal medications or suppositories, feminine sprays, genital wipes or contraceptive spermicides in the 48 h prior to sample collection, language barrier, psychiatric conditions.

### Sample collection

Vaginal specimens were collected with the aid of a disposable vaginal speculum and by sterile swabs. A total of three swabs were collected for diagnosis of T. vaginalis infection in the following order of draw: one swab for wet mount examination, one for immunochromatographic testing, and one for real-time PCR assay. Other swabs were also collected for diagnosis of vaginosis or vaginal candidiasis.

### Wet mount examination

Wet-mount microscopy was performed according to standard procedures. Briefly, a swab with vaginal fluid was placed in a plastic tube containing 1 mL of saline. Within 10 minutes of collection, the swab was vigorously mixed in the saline, removed, and pressed onto a clean, dry microscope slide, along with one drop of exudate-saline solution. A coverslip was placed over the sample, and the slide was examined by bright field microscopy at 400x.

### Immunochromatographic assay

The immunochromatographic assay was performed by means of the commercially available JD’s Trichomonas vaginalis (T.V.) antigen rapid test kit (Taipei, Taiwan). Briefly, 1 mL of sample buffer was added to flexible plastic test tubes supplied with the kit, using the supplied dropper top. A swab with vaginal fluid was placed in the tubes containing the sample buffer, mixed vigorously, and allowed to sit for 1 minute, then the swab was removed and discarded. A test stick was placed in each tube containing the buffer-sample mixture, and results were read after 15 minutes. A positive result was indicated by the presence of a red line along with a red control line, whereas in a negative result, only a red control line was visible. If no red control line was visible, the test result was considered invalid.
Real-time PCR assay
Vaginal swab specimens were placed in 3-mL UTM Transport medium (Copan Italia S.p.A., Brescia, Italy). The collection tubes were equilibrated to room temperature and mixed by vortexing, and 1-mL mixed specimens were transferred to the sample cartridge. The DNA was extracted from the specimens using the instrument NucliSense easyMag (bioMérieux, Marcy-l’Etoile, France), in accordance with the manufacturer’s instructions, and stored frozen at -20°C until testing. Detection of T. vaginalis was performed by means of the AnyplexTM II STI-5 Kit (Seegene, Seoul, Korea), a multiplex real-time PCR relying on a newly developed TOCETM (Tagging Oligonucleotide Cleavage and Extension) technology which allows to detect simultaneously five microorganisms (Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis, Mycoplasma genitalium, T. vaginalis). Tests were performed according to the manufacturer’s protocol. Briefly, the amplification was performed in a CFX96 real-time thermocycler (Bio-Rad, Hercules, CA, USA). Each PCR was performed in 5-µL of eluted DNA and 15-µL of Anyplex PCR Mix in a 20-µL final volume per reaction. The thermal cycle conditions consisted of an initial incubation at 50°C for 4 min to activate the UDG system and prevent contamination, pre-denaturation at 95°C for 15 min, followed by 50 cycles of alternating incubations: 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. The melting temperature was analysed by increasing the reaction temperature from 55°C to 85°C (5 s/0.5°C). The whole process was monitored adding to each sample 1 µL of internal control (provided by the manufacturer) before the DNA extraction, to confirm the DNA extraction and to exclude PCR inhibition. DNA quantification was performed using the default algorithm that calculates the amount of DNA denaturation by means of analysis of melting temperatures after 8, 14, and 20 cycles, and is expressed as number of DNA copies/reaction.

Statistical analysis
The analysis was performed by SPSS 13.0 version. Statistical significance was assumed if a null hypothesis could be rejected at a P value of ≤0.05. Categorical variables are expressed as counts and percentages. Continuous variables are expressed as median and interquartile range. Pearson chi-square test was used to compare proportions, employing Yates’s correction for continuity where appropriate.

RESULTS
A total of 1487 patients fulfilled the inclusion criteria and were included in the study, 1035 (69.6%) were outpatients from the Microbiology laboratory, 238 (16%) from Prenatal Medicine, and 214 (14.4%) from the Infertility Centre. Median age was 32 years (interquartile range 27-37), 19% were positive for yeasts, and 9.5% were suffering from bacterial vaginosis. Twenty patients were positive for T. vaginalis at the real-time PCR assay, giving a prevalence of 1.3%. On the other hand, true positive results for wet mount and immunochromatographic assay were 8/1487 (0.5%) and 12/1487 (0.8%), respectively. Among the 1467 PCR-negative samples, all wet mount tests were negative. On the contrary, there was one immunochromatographic false-positive result. Compared to PCR, the sensitivity, specificity and negative likelihood ratio of wet mount were 40% [95% confidence interval (CI) 19.1% to 63.9%], 100% (95% CI 99.7% to 100%) and 0.6 (95% CI 0.4 to 0.9) respectively. The sensitivity, specificity, and negative likelihood ratio of the immunochromatographic assay were 60% (95% CI 36% to 80%), 99.9% (95% CI 99.6% to 100%), and 0.40 (95% CI 0.2 to 0.7), respectively. Positive likelihood ratio for the immunochromatographic test was 880 (95% CI 120 to 6450). No significant difference was found in T. vaginalis detection rate

<table>
<thead>
<tr>
<th>Table 1 - Wet mount identification rate according to different number of Trichomonas DNA copies/5 µL of eluted DNA among the 20 PCR-positive vaginal samples; chi-square 6.1, P=0.016.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of T. vaginalis DNA copies/5 µL of eluted DNA</td>
</tr>
<tr>
<td>Positive wet mount</td>
</tr>
<tr>
<td>Negative wet mount</td>
</tr>
<tr>
<td>Total</td>
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between wet mount and the immunochromato-
graphic test (8/1487 vs 12/1487, P=0.06). The de-
tection rates of wet mount and the immunochro-
matographic test, according to different number of
Trichomonas DNA copies/5 µL of eluted DNA, are described in Tables 1 and 2 respectively. The threshold for T. vaginalis identification by means of both wet mount and the immunochromato-
graphic test used in this study was ≥100 Tricho-
monas DNA copies/5 µL of eluted DNA. Below this limit all vaginal samples were false-negative by both wet mount and immunochromatographic assays.

**DISCUSSION**

The main result of this study was the finding of a threshold of 100 T. vaginalis DNA copies each 5 µL of eluted DNA from vaginal samples as lower detection limit for both wet mount and immunochromatographic assay. To the best of our knowledge, this is the first study reporting this result. The fragility of T. vaginalis trophozoite (not able to produce encysted forms) outside the body, makes the rapidity of the sample transport before the microscopic examination, and the inoculum size, pivotal for the success of its identification by wet mount, and, the inoculum size only, by immunochromatographic assays [5]. Data in Tables 1 and 2 show how all vaginal samples with Trichomonas DNA copies from 50 to 99 each 5 µL of eluted DNA (5 out of 20 PCR-positive samples, 25%) gave false-negative results with both the wet mount and the immunochromatographic assays. Although T. vaginalis infection has been associated with vaginitis, cervicitis, urethritis, pelvic inflammatory disease, nevertheless many women are asymptomatic [7]. Since trichomoniasis, symptomatic or asymptomatic, has been also associated with adverse birth outcomes (low birth weight, premature rupture of mem-

<table>
<thead>
<tr>
<th>Number of T. vaginalis DNA copies/5 µL of eluted DNA</th>
<th>50-99</th>
<th>100-100.000</th>
<th>&gt;100.000</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive IC</td>
<td>0 (0%)</td>
<td>4 (33.3%)</td>
<td>8 (66.7%)</td>
<td>12</td>
</tr>
<tr>
<td>Negative IC</td>
<td>5 (62.5%)</td>
<td>2 (25%)</td>
<td>1 (12.5%)</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>6</td>
<td>9</td>
<td>20</td>
</tr>
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</table>

The prevalence of 1.3% found in this study by means of PCR substantially matches those found in other studies performed in population with similar characteristics [3, 10, 11]. This quite low value must be interpreted considering the population evaluated, not attending a sexually transmitted infections (STIs) clinic, therefore at low risk for T. vaginalis or other STIs.

Similarly, also the values of sensitivity and specificity found for wet mount and the immunochromatographic assay are in line with those observed in a previous study by Pillay et al. [12]. Due to the very low prevalence of T. vaginalis infection observed in our population, likelihood ratios, sensitivity, and specificity, not influenced by the prevalence, were calculated, while positive and negative predictive values, intrinsically related to prevalence, were not [13]. From that values it appears how both wet mount and the immunochromatographic assay can be good confirmatory tests, with good specificity, but poor screening tests, as showed by the low sensitivities and high negative likelihood ratios.

No statistically significant difference between the T. vaginalis detection rate of wet mount compared with the immunochromatographic assay was observed. Indeed, a higher prevalence of infection would be necessary to detect it. This is also sug-
gested by the more pronounced negative trend for immunochromatographic negative tests in relation to increasing concentration of the number of Trichomonas DNA copies/5 µL of eluted DNA (Table 2), in comparison to that of wet mount (Table 1). This fits with the notion that the inoculum...
size of *T. vaginalis* cells influences the results of the immunochromatographic test. The main limitation of this study is the lack of clinical data, precluding the possibility to evaluate if the molecular threshold found could be associated to a threshold of clinical significance (i.e., asymptomatic vs symptomatic patients). Further studies are needed to address this issue. In conclusion, this study showed how the threshold of 100 *Trichomonas* DNA copies/5 μL of eluted DNA can be considered as a lower limit of sensitivity for both wet mount and the JD’s *Trichomonas vaginalis* immunochromatographic assay for detection of *T. vaginalis* in vaginal fluids.

**Conflict of interest:** All authors declare they have no conflicts of interest in connection with this article.

**REFERENCES**


