

Original Research Article

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Validity of Different Laboratory Methods for Diagnosis of Infectious Vaginitis: Real-Time Polymerase Chain Reaction for Detection of Trichomoniasis

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ABSTRACT

Vaginitis syndrome is one of the most common reasons for women to seek medical attention that is mostly infectious. The aim of this study was to validate the different laboratory methods for diagnosis of infectious vaginitis and to implement the role of molecular method for detection of *T. vaginalis* in comparison to routine diagnostic techniques. Four vaginal swabs were collected from each woman and subjected to full microbiological laboratory studies involving direct microscopic examinations and isolation techniques. In addition, A 145 bp long fragment of the repeated DNA target from the genome of *Trichomonas vaginalis* is amplified with specific primers and detected with hybridization probes using real-time PCR. All specimens were negative for *Trichomonas vaginalis* in wet mount preparations and twenty eight percent of cases were positive for fungal elements and bacterial agents in Gram stained smears. Forty one cases yielded positive culture, sixteen bacterial isolates, thirteen fungal isolates and no isolates of *T.vaginalis* were detected. Mixed bacterial and *Candida* isolates were found in twelve cases. Nine specimens tested positive using real-time PCR resulting in 9.4% prevalence of trichomoniasis in the women studied. From this study we can conclude that the direct microscopic examination and culture methods used shows reasonable sensitivity for detection of BV and vaginal candidiasis but not for *T. vaginalis* among the studied cases. Candidal and bacterial vaginosis can coexist. The real-time PCR was found more sensitive and rapid method for detection of *T. vaginalis*.

Keywords

Bacterial,
candidal and
trichomonal
vaginitis;
wet mount;
Real-time PCR.

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Introduction

Vaginitis syndrome is one of the most common reasons for women to seek medical attention and is characterized by pruritus and vaginal discharge. Bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), or trichomonal vaginitis (TV) represents over

90% of infectious vulvovaginitis (Cartwright *et al.*, (2013). Different groups of microorganisms are the causes of these three infectious syndromes so accurate and reliable diagnosis is necessary for appropriate treatment (Van Der Pol, 2010; Granato, 2010).

The most frequent cause of vaginitis is the bacterial vaginosis that involves a change in vaginal ecology where the anaerobic bacteria replaces normal *Lactobacillus* spp. Wei *et al.*, (2012) reported that the incidence of BV ranged from 8 to 75%. More than 50% of women with BV are asymptomatic. However, serious disorders such as pelvic inflammatory disease, urinary tract infections and increased susceptibility to HIV, spontaneous and preterm birth and postpartum endometritis can be caused by BV (Hainer and Gibson, 2011).

Seventy five percent of all women have experience of an episode of VVC in their lifetimes that is a common illness due to an overgrowth of *Candida* species. *Candida albicans* accounts for 80-95% of all episodes of VVC worldwide (Guzel *et al.*, (2011). The incidence of VVC due to non-*C.albicans Candida* species ranged previously from 5 to 20% while, over the last two decades, the number of reported cases has increased particularly for cases of *C. glabrata* (Guzel *et al.*, 2013).

Generally it is thought that coexistence of VVC caused by *C. albicans* and BV are rare, but a recent study has indicated that this view is incorrect. So, it is important to clarify the relationship between *C. albicans* and BV that helps to establish the causation, diagnosis and therapy of vaginitis (McClelland *et al.*, 2009; Donders *et al.*, 2011).

Trichomonas vaginalis infections are not self-limiting and produce non-ulcerative inflammation of the genital epithelium that can progress to necrosis and hemorrhage. Detection of low parasite number or defective parasites for diagnosis of trichomoniasis by using the traditional methods such as direct microscopic examination of wet mount preparations or culture based systems are both limited

because of low sensitivity (Garber, 2005). However, the improvement of highly specific and sensitive diagnostic techniques based on amplification of *T. vaginalis* DNA (e.g., polymerase chain reaction (PCR) and transcription-mediated amplification (TMA) changed significantly the view on detection of trichomoniasis in such these cases (Hobbs and Seña, 2013).

The aim of this study was to validate the different laboratory methods for diagnosis of infectious vaginitis which may have synergistic pathological effects from symptomatic and asymptomatic patients and to implement the role of molecular method for detection of *T. vaginalis* in comparison to routine diagnostic techniques.

Subjects and Methods

The study was conducted on 100 women patients who were referred to the gynecologic clinics from October 2012 to March 2015. Their ages ranged from 20 to 55 years. Four vaginal swabs were collected from each woman and examined as following:

First vaginal swab was immersed in a 10 ml test tube containing 2 ml normal saline for bacterial and yeast cultures as well as Gram stained smears

Yeast cultures were performed by inoculating 50µl of liquid from swabs onto Sabouraud's dextrose agar with chloramphenicol, incubating at 37°C for 3 days. Also, bacterial cultures were performed aerobically and anaerobically by inoculating 50µl of liquid from swabs onto blood and chocolate agar, incubating at 37°C for 16-24 h. Further identification for isolates was done by Gram stain and fully automated Vitek 2 Compact System (bioMérieux, France).

The remaining liquid in the tube was shaken well using vortex, centrifuged and the sediment was used for smear preparation.

Second vaginal swab was immersed in a 10 ml test tube containing 0.5 ml normal saline for wet mount preparation

One drop of the fluid was put on glass slide, covered and examined under the microscope. Positive result was defined as presence of one or more trichomonads with characteristic morphology and jerky motility.

Third vaginal swab was immersed in a tube containing 10ml of the modified Diamond culture medium for isolation of *Trichomonas vaginalis*

The culture tubes were incubated at 37°C for 72 hours then examined daily for 3-5 days by taking samples from the sediment by wet mount for detection of the *Trichomonas* motility.

Fourth vaginal swab was immersed in a 10 ml test tube containing 2 ml normal saline for PCR assay

The swab was vortexed, pressed against the tube side then removed. The tube was closed and kept at -80°C for further PCR assay.

Trichomonas vaginalis DNA was extracted using the high pure PCR template preparation kit (Roche Diagnostics) following the manufacturer's recommendations.

A 145 bp long fragment of the repeated DNA target from the genome of *Trichomonas vaginalis* is amplified with specific primers and detected with hybridization probes labeled with the dye LC690. Amplification reactions were

performed in a total volume of 20 µl including 25mM MgCl₂ (1.6 µl), reagent mix containing primer and probe 20 pM for each (2µl), 2 µl Light Cycler Fast Start DNA master hybridization probe buffer, 9.4µl PCR-grade water and 5 µl of template DNA. The cycling conditions used include the following: 95°C for 10 min, followed by 50 cycles of 95°C for 5 s, 62°C for 5 s, and 72°C for 15 s. Data collection was performed during the annealing phase of each amplification cycle, and the fluorescent signal was monitored through the F2 channel (Riley *et al.*, 1992; Kengne *et al.*, 1994). The melting-curve analysis was used for identification of the PCR products derived from target DNA.

Results and Discussion

Eighteen out of 100 studied cases were asymptomatic, 55.6% (10/18) were positive either for culture or Gram stained smear while the remaining eight cases were negative.

Forty one cases yielded positive culture, sixteen bacterial isolates included the following species: *Escherichia coli* (5/41), *Staphylococcus aureus* (3/41), *Klebsiella pneumoniae* and *Enterococcus faecalis* (2/41 for each), *Staphylococcus haemolyticus* and *Streptococcus agalactiae* (1/41 for each) and anaerobic bacteroides (2/41). Thirteen fungal isolates speciated into: *Candida albicans* (9/41), *Candida kefyr* (2/41), *Candida tropicalis* and *Candida famata* (1/41 for each). Mixed bacterial and fungal isolates were found in twelve cases. No isolates of *Trichomonas vaginalis* were detected, table 1.

Wet mount preparation showed negative results in all specimens for *Trichomonas vaginalis*. Gram stained smears were positive in 28% of cases for fungal elements

such as *Candida* blastospores and pseudohyphae and bacterial agents.

A positive result was accepted in the Light Cycler PCR when a sample showed amplification fluorescence and a crossing point (calculated by the Light Cycler software). The melting-curve analysis was used to confirm positive results. A 145 bp long fragment of the repeated DNA target is contained a single peak at approximately 67.5 °C. The melting-curve analysis was consistent with all positive results obtained in the study (Fig. 1 a and b). Out of 96 specimens, all (100%) were negative by wet mount microscopy and culture, whereas nine specimens tested positive using real-time PCR resulting in 9.4% prevalence of trichomoniasis in the population studied, table 2.

Vaginitis is one of the most common diseases mostly infectious that affect women's health, with over 50% of women have experience of at least one episode of vaginal infection in their lifetime. Infectious vaginitis is classified into three major categories of disease: bacterial vaginosis, vaginal candidiasis, and vaginal trichomoniasis. However, over 20% of infectious vaginitis cases may be mixed (Ferris *et al.*, 1995; Lowe *et al.*, 2009). The laboratory testing is essential for the appropriate management of patients due to clinically nonspecific nature of the vaginitis syndrome (Cartwright *et al.*, 2013).

In the current study, all specimens were negative for *Trichomonas vaginalis* in wet mount preparations. Twenty eight percent of cases were positive for fungal elements and bacterial agents in Gram stained smears. So the using of Gram stain for detection of BV as well as vaginal candidiasis showed reasonable sensitivity. Establishment of the microbial etiology of infectious vaginitis can

sometimes be obtained by microscopic examination of vaginal secretions, either by wet mount or Gram stain that is a reliable and inexpensive method. However, the sensitivity of the wet-mount examination for *Trichomoniasis* only 60% to 70%, and immediate evaluation of the wet mount is required for optimal results (Centers for Disease Control and Prevention, (2006). A 10-minute delay in examining the wet mount may reduce the sensitivity to 20% (Kingston *et al.*, (2003) or even may lack sensitivity, so a negative smear result does not rule out the presence of disease.

In the present study, aerobic and anaerobic bacteria were more prevalent organisms that isolated in 16% of cases followed by *Candida* species isolates in 13% and mixed bacterial and *Candida* isolates in 12% from all studied cases. No isolates of *Trichomonas vaginalis* were detected.

Villaseca *et al.*, (2015) found that the bacterial vaginosis were the most frequent infections and detected in (16.8%) followed by vulvovaginal candidiasis in (11.9%) and co-infections in (6.9%) with 3% of trichomoniasis. These findings were in agreement to our results for BV and VVC.

However, Nenadić and Pavlović, (2015) reported that cervicovaginal swab cultures yielded growth of several aerobes and facultative anaerobes in 6% of patients, whereas *Candida albicans* was isolated in 11% of women.

In this study, zero% of trichomoniasis was detected using the culture method and wet mount preparation. In the United States, among those who were referred to the STD clinics, the trichomoniasis was detected in 25% of cases but it was found to be higher (38%) in certain population groups such as in African American women (World Health

Organization, (2001). However, the other countries shows variables that ranges from 1.2% in Libya and Jordan to 3.2% in Turkey to 28.1% in Saudi Arabia (Kassem and Majoud, (2006); Selvitopu *et al.*, (2006);

Mahafzah *et al.*, (2008). Selection of different population groups and different isolation media used may be the reasons for the differences in results.

Table.1 Frequency of isolated microorganisms from the studied cases (n=100).

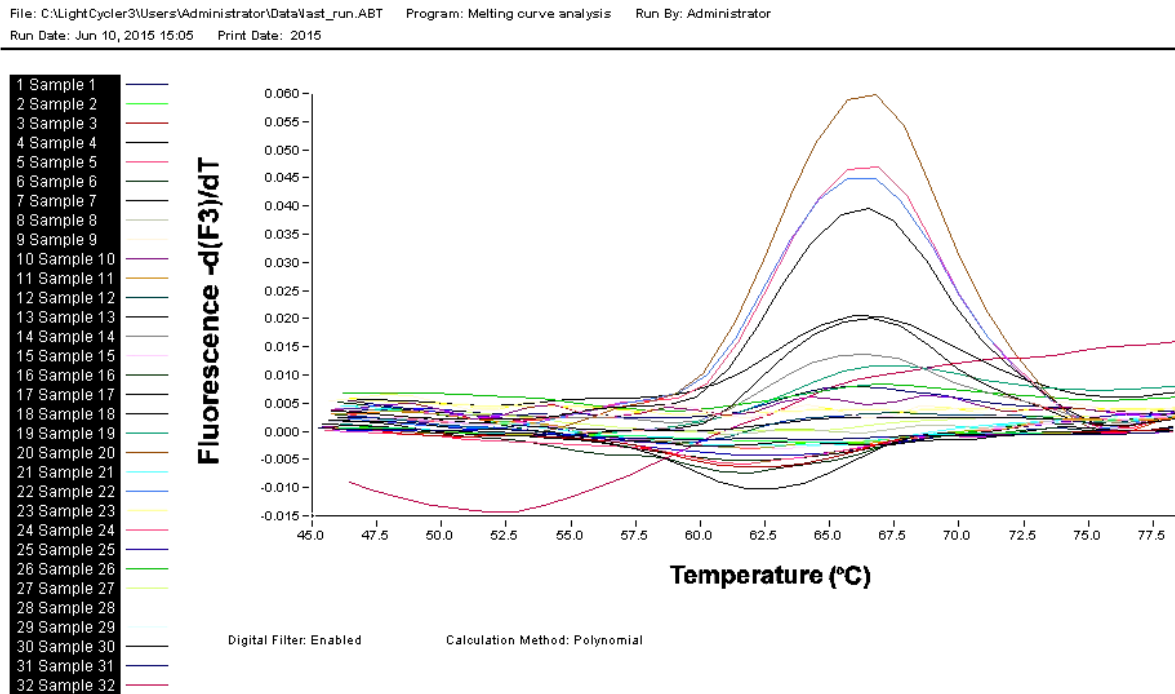
Microorganisms	Frequency	%
Bacterial agents (No=16)		
<i>Escherichia coli</i>	5	12.2
<i>Staphylococcus aureus</i>	3	7.3
<i>Klebsiella pneumoniae</i>	2	4.9
<i>Enterococcus faecalis</i>	2	4.9
<i>Staphylococcus haemolyticus</i>	1	2.4
<i>Streptococcus agalactiae</i>	1	2.4
<i>Anaerobic Bacteroides</i>	2	4.9
Fungal agents (No=13)		
<i>Candida albicans</i>	9	22
<i>Candida kefyr</i>	2	4.9
<i>Candida tropicalis</i>	1	2.4
<i>Candida famata</i>	1	2.4
Mixed bacterial and fungal agents	12	29.3
<i>Trichomonas vaginalis</i>	none	0.0
Total	41	100.0%

Table.2 *Trichomonas vaginalis* –positive cases by PCR and the results of their testing by wet mount microscopy and culture

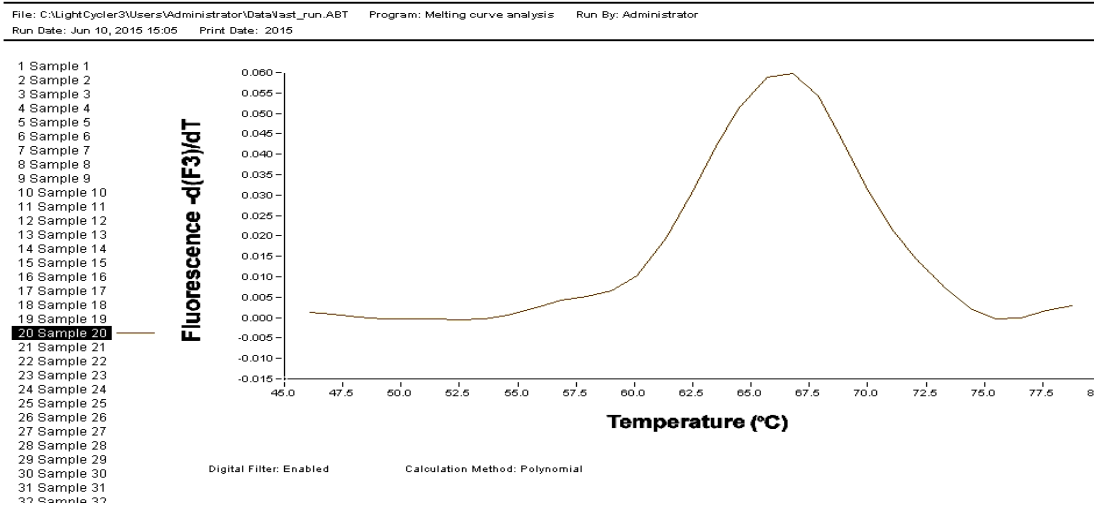
Cases no	Wet mount microscopy	Culture	Real-time PCR
1	-ve	-ve	+ve
2	-ve	-ve	+ve
4	-ve	-ve	+ve
5	-ve	-ve	+ve
6	-ve	-ve	+ve
20	-ve	-ve	+ve
39	-ve	-ve	+ve
71	-ve	-ve	+ve
86	-ve	-ve	+ve
97,98,99,100	-ve	-ve	Not tested

Fig.1 Melting-curve analysis of 145 bp long fragment of the repeated DNA target is contained a single peak at approximately 67.5°C(a)One running real-time PCR (total 32 cases) revealed positive cases with single peak and straight linear negative cases (b) Melting-curve analysis revealed single peak of one single case no 20 at 67.5°C

(a)



(b)



In the present study neither of the two methods wet mount preparation and culture method detected infections with *T. vaginalis* but the PCR detected reasonable rate of infection resulting in 9.4% prevalence of trichomoniasis in the studied cases. As has

been shown previously, the main drawback of wet preparation and culture tests is that they leave a large portion of infections undetected. However, for the diagnosis of trichomoniasis, the limitations of direct microscopic and culture methods prompted

the development of molecular methods for the detection of specific DNA in the vaginal samples (Wendel *et al.*, 2002; Pillay *et al.*, 2007; Nye *et al.*, 2009; Patil *et al.*, 2012; Nathan *et al.*, 2015) and several PCR protocols have been proposed so far for the identification of *T. vaginalis* (Garber, 2005). The results of our study are in agreement with previous findings which established that the sensitivity of real-time PCR is superior to that of culture and wet-mount microscopy. Furthermore, its use for diagnosing vaginitis should be considered.

In conclusion, the direct microscopic examination and culture methods used for vaginal secretions shows reasonable sensitivity for detection of BV and vaginal candidiasis but not for *T. vaginalis* among the studied cases. Candidal and bacterial vaginosis can coexist. The real-time PCR was found more sensitive and rapid method for detection of *T. vaginalis*. In addition, the implementation of molecular techniques may aid patient management and improve our understanding of the epidemiology of *T. vaginalis* disease by capturing accurate data.

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