Original Article

Correlation of *Trichomonas vaginalis* to bacterial vaginosis: a laboratory-based study

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Abstract

Background: This study aimed to define the occurrence of different organisms causing vulvovaginitis; to evaluate different laboratory methods used for diagnosis of *Trichomonas vaginalis* (*T. vaginalis*); and to evaluate the direct score system and clue cell method compared with culture for diagnosis of bacterial and *T. vaginalis* vaginosis.

Methodology: Clinical and laboratory evaluations were performed for 110 patients. Laboratory methods used for bacteriological diagnosis were direct Gram staining for clue cells and scoring by Nugent score system and bacterial culture. *T. vaginalis* was identified by wet mount microscopic examination, culture, direct Gram, Giemsa staining and acridine orange (AO).

Results: The Nugent score method revealed that the sensitivity and specificity for diagnosis of vaginal discharge by direct rapid microscopic methods were 30% and 80% and for clue cells sensitivity and specificity were 37% and 75% respectively for diagnosis of bacterial vaginosis compared to culture. For diagnosis of *T. vaginalis*, the Nugent score method revealed that the sensitivity and specificity were 60% and 90% respectively, and for clue cells 75% and 80% respectively. For microcopic methods used for *T. vaginalis* only, the Gram stain and Giemsa stain sensitivities were poor (15.2% and 48.5%, respectively). Wet mount showed reasonable sensitivity of 75.8%. Acridine orange sensitivity was 93.9% and specificity was 97.5%,

Conclusion: Prevalent pathogens associated with vaginitis were (*Gardnerella vaginalis*) G. *vaginalis*, *T. vaginalis* and *Mycoplasma hominis* (*M. hominis*). Wet mount microscopic examination, acridine orange, and high Nugent score were found as rapid and sensitive methods for diagnosis of *T. vaginalis*.

Introduction

Bacterial vaginosis (BV) was previously defined as a poly-microbial syndrome characterized by a shift in vaginal flora from a predominant population of lactobacilli to their gradual or total replacement with anaerobes such as *Gardnerella vaginalis* (*G. vaginalis*), *Prevotella*, Bacteroides and Mobiluncus species (spp), and with other bacteria including Mycoplasma and Ureaplasma species [1]. BV is one of the most frequent conditions encountered in sexually transmitted diseases (STD), genitourinary medicine (GUM) or other reproductive health clinics throughout the world. BV has been strongly associated with poor pregnancy outcomes such as preterm delivery of low-birth-weight infants [2] and several studies have now established associations between BV and HIV [3-5]. BV appears to be particularly common in sub-Saharan Africa where

several studies have reported high prevalence rates [6-8]. These are very much higher than the rates reported from industrialised countries [9-11].

The reasons for these disparities are not entirely clear, but may arise in part through the use of different case definitions for BV, and because the pattern of vaginal micro-flora associated with this condition may differ in different populations. There are few reports about this condition in our locality in Egypt. In Egypt, some community-based studies of the prevalence of reproductive tract infections (RTIs) among married women of childbearing age have been conducted. They documented the importance of Candida species as one of the major etiologies of RTIs [12,13].

Studies for diagnosis of BV have relied on Amsel's clinical definition of BV [14], while more recent studies have adopted the microbiological

Nugent's scoring technique [15]. The latter method relies on the identification of categories of vaginal micro-flora based on quantitative assessment of a vaginal Gram-stained smear. This method scores the smears in a standardized manner by quantification of some of the cell types present, designated as Lactobacillus, *G. vaginalis*, Bacteroides and Mobiluncus morphotypes. Nugent's method has been extensively validated in industrialised countries where numerous vaginal flora studies have been conducted [1], but little is known of the pattern of vaginal micro-flora associated with BV in Africa.

Another important pathogen causing vaginitis is *Trichomonas vaginalis* (*T. vaginalis*). The protozoon *T. vaginalis* causes the sexually transmitted disease trichomoniasis, with an annual incidence of more than 170 million cases (World Health Organization, 1995) [16]. It is known that trichomoniasis can lead to inflammatory small pelvic diseases, reproductive dysfunction and increased risk of premature rupture of fetal membranes and low birth weight [17]. Therefore, in order to accurately diagnose this disease, microbiological investigation is necessary.

The presence of *T. vaginalis* also increases susceptibility to bacterial vaginosis, and coinfection with both entities may promote acquisition of HIV [18]. Studies conducted with large groups of women suffering from vaginitis showed that *T. vaginalis* is clinically associated with *M. hominis* [19,20], a bacterium that, like the protozoon, resides exclusively in the human genital tract. Interestingly, both *M. hominis* infections and trichomoniasis are associated with several pregnancy and postpartum complications, including preterm delivery and low-birth-weight infants [21,22].

Women who are identified as infected on the day of their clinic visit are more likely to receive and to comply with treatment. A point-of-service assay conveys screening and management advantages, particularly if it performs well among asymptomatic women. For possible use as a screening test, it is encouraging that the use of gram staining would mirror concomitant trichomoniasis and bacterial vaginosis.

The aims of the present study were to define the occurrence of different organisms causing vulvovaginitis such as *T. vaginalis*, *G. vaginalis* and *M. hominis*; to evaluate different laboratory methods used for diagnosis of *T. vaginalis*; and to evaluate the direct score and clue cell system compared with culture for diagnosis of bacterial and *T. vaginalis* vaginosis.

Materials and Methods

The present study was conducted on 110 patients complaining of vaginitis with discharge. Their ages ranged from 25 to 55 years. They were attending gynecology and obstetric outpatients of the Mansoura Faculty of Medicine, Egypt. The patients signed written consents and the study was approved by the ethical committee of the faculty of medicine, Mansoura University.

From each patient examined, five samples of vaginal discharge from the posterior fornix were collected.

Vaginal smear slides were heat fixed, Gram-stained and examined for vaginal flora categories using the Nugent's method [15]. At least 20 fields of the wet mount were examined by one observer at 400x magnification in a phase-contrast microscope (Leica DM LB) and the presence or absence of clue cells was recorded to complete the ACC method. The method involves assigning a score between 0 and 10 based on quantitative assessment of the Gram-stain for three different bacterial morphotypes: (i) large Gram-positive rods (indicative of Lactobacillus spp); (ii) small Gram-negative or variable rods (indicative of Gardnerella, Bacteroides and other anaerobic bacteria); and (iii) curved, Gram-variable rods (indicative of Mobiluncus spp). Scores between 0 and 3 represent normal vaginal flora, between 4 and 6 intermediate vaginal flora, and scores between 7 and 10 are considered diagnostic for BV.

Vaginal swabs were directly inoculated at the clinic onto: (i) Columbia blood agar plates, which were incubated aerobically at 37°C for 24 to 48 hours to isolate aerobic bacteria, including lactobacilli; (ii) Columbia human blood bi-layer agar plates, which were incubated micro-aerophilically at 36°C and read after 48 to 72 hours for *Gardnerella vaginalis* isolation; (iii) Columbia-base lake horse blood kanamycin agar plates, which were incubated anaerobically at 36°C for 48 to 72 hours to isolate anaerobic bacteria; (iv) Mycoplasma broths incubated for 48 hours then sub-cultured onto Mycoplasma agar A7 agar (Becton Dickinson, Cockeysville, Md. 21030) and incubated at 37°C in 5% CO_2 for 5 days. Cultures were examined microscopically daily for 5 days for the appearance of typical mycoplasma colonies; (v) Mann Rogosa Sharpe (MRS) medium, which was used for the isolation of Lactobacillus spp after incubation in CO_2 at 37°C for 48 hours; and (vi) Sabouraud's agar plates, which were incubated micro-aerophilically at 36°C 24 to 48 hour to isolate Candida spp.

Growth of bacterial isolates was graded as confluent (heavy growth), semi-confluent (moderate pure to mixed growth of bacteria with visible separate single colonies); and scanty (occasional single isolated colonies).

Presumptive identification procedures

Lactobacilli and other aerobic flora: Lactobacilli were presumptively identified by their ability to grow well on MRS, Gram stain microscopy and catalase reaction. Isolates were further tested for their ability to produce hydrogen peroxide (H_2O_2) using a 2, 3 tetramethyl benzedine method [23], and classified as positive when they produced blue coloration. The level of H_2O_2 production was determined by visually grading the intensity of the blue colour produced into low, moderate and high categories.

Coliform spp were identified as Gram-negative lactose fermenting rods; Staphylococcus spp were identified by their characteristic colony and Gram stain morphology, then tested for coagulase production (slide test); Gram-positive beta-haemolytic Streptococcus spp isolates were further typed using a rapid latex test according to the manufacturer's instructions (Streptex, Murex Biotech Ltd, Darford Kent, UK).

Candida spp were identified as colonies with typical yeast-like morphology and by characteristic morphology on a wet preparation examination (presence of budding cells and/or pseudo-hyphae).

BV-associated bacteria: *G. vaginalis* was identified by beta-haemolytic appearance of the colonies on human blood bilayer agar plate but not on sheep blood agars, Gram stain morphology (Gram-variable pleiomorphic coccobacilli mostly forming clumps) and negative catalase and oxidase reactions.

Suspected anaerobic isolates were sub-cultured onto Columbia blood agar without antibiotics and incubated in aerobic and anaerobic conditions. Strict anaerobes were further identified by Gram staining and antibiotic susceptibility to erythromycin, rifampicin, colistin, penicillin, kanamycin and vancomycin (Oxoid Discs, Unipath, Basinstoke, Hampshire UK). Gram-negative anaerobic bacilli were tested. The isolates that grew on bile medium and hydrolysed aesculin were identified as Bacteroides spp. Isolates that failed to grow on bile or hydrolyse aesculin were identified as Prevotella spp [24].

Mycoplasma spp were identified as typical "fried egg" colonies and stained with Diene's stain. They were further presumptively identified as *M. hominis*

by colonial appearance and staining characteristics with a permanent diffused light blue periphery and a dark blue centre [25].

Laboratory diagnosis of Trichomonas vaginalis

The first swab was placed in 1 ml of sterile saline solution and then used for wet mount, Giemsa and AO staining. The second swab was used for culture in Diamond's medium. For bacteriological examinations, one swab was directly cultured in prewarmed blood agar and another swab was cultured on chocolate agar; both were incubated at 37°C for 24 to 48 hours at 5% CO_2. One swab was used for direct Gram stain.

The whiff test and vaginal pH were not performed. The wet mount method was conducted by placing one drop of the sample onto a microscope slide covered by a cover slip. Preparations were examined at 400x by light microscope to detect the motile trophozoites of *T. vaginalis*. For Giemsa staining, the smears were air-dried, fixed by methanol for 5 minutes, and stained for 20 minutes at a dilution of 1:20. The preparations were scanned at 1000x to detect violet, pear-shaped trophozoites with characteristic morphologic features.

During AO staining, smears of vaginal exudates were air dried, heat fixed, and stained for 20 seconds. The preparations were stored in pH 7.2 holding buffer at room temperature in the dark until microscopic examination. The slides were examined while wet, by placing them on the cover slip and scanning them at 400x by fluorescent microscope (Leica DMLB). *T. vaginalis* trophozoites stained brick red with a yellowish-green banana-shaped nucleus. Epithelial cells and leucocytes fluoresced light-green with a bright green nucleus.

Yeasts and bacteria stained red, but were significantly smaller and morphologically different, so they were easily distinguishable from trichomonads.

Immediately after specimen collection the second swab was placed in 14 ml of Diamond's medium, which was prewarmed at 37°C and had been kept in screw-capped tubes.

Cultures were incubated at 37°C for 7 days and examined using the wet mount method every day to detect motile trophozoites of *T. vaginalis*.

Results

The present study was conducted on 110 patients ranging in age from 25 to 45 years. There were 77 females with infections that manifested as vaginal

Table 1. Frequency of isolated pathogens from patients (n=110)

Isolated Pathogen	No.	%
Garbdenella vaginalis	43	39.1
Mycoplasma hominis	16	14.5
Trichomonas vaginalis	33	30
Staphylococcus aureus	15	13.6
Candida albicans	1	0.01

discharge and 33 patients with vaginal discharge associated with infertility. The pathogenic organisms were isolated by culture from 67.3% of patients (data not shown).

As shown in Table 1, the most prevalent pathogen was *G. vaginalis* (39.1%), followed by *T. vaginalis* (30%) and *M. hominis* (14.5 %).

Table 2 shows the statistically significant correlation between *M. hominis* and *T. vaginalis* infections (P = 0 .013). Moreover, there was significant correlation between *T. vaginalis* infection either alone (P = 0.03) or combined with *M. hominis* and patients with infertility (P = .013), as illustrated in Table 3.

Table 2. Correlation between *Gardenella vaginalis*, *Trichomonas vaginalis* and *Mycoplasma hominis* in studied patients (n = 110).

Pathogens	r	P
Mycoplasma hominis and *Trichomonas vaginalis*	0.24	0.013
Gardenella vaginalis and *Trichomonas vaginalis*	0.045	0.64
Gardenella vaginalis and *Mycoplasma hominis*	-0.013	0.89

Regarding direct rapid microscopic methods used for diagnosis of vaginal discharge, the Nuget score method revealed that the sensitivity and specificity were 30% and 80%, and for clue cells sensitivity and specificity were 37% and 75% respectively for diagnosis of bacterial vaginosis compared to culture (Figure 1). For diagnosis of *T. vaginalis*, the Nugent score method revealed that the sensitivity and specificity were 60% and 90% respectively and for clue cells 75% and 80% respectively, (Figure 2).

For microcopic methods used for *T. vaginalis* only, the Gram stain and Giemsa stain sensitivities were poor (15.2% and 48.5%, respectively),l while wet mount showed reasonable sensitivity of 75.8%. For acridine orange, sensitivity was 93.9% and specificity was 97.5% (Figure 3).

Table 3. Correlation between *Gardenella vaginalis*, *Trichomonas vaginalis* and *Mycoplasma hominis* in studied infertile patients (n = 33).

Pathogens	R	P
Trichomonas correlation with infertility Combined Trichomonas and Mycoplasma infections and infertility	0.21 0.263	0.03 0.013
Mycoplasma hominis with infertility	-0.12	0.2
Gardenella vaginalis and infertility	0.45	0.6

Discussion

In the present study, the commonest isolated pathogen was *G. vaginalis*, followed by *T. vaginalis* and *M. hominis*. Reports differ in pathogens associated with vaginal discharge and their prevalence.

Our findings should be interpreted in light of the various limitations of our study. First, someone may consider the number of analyzed results of vaginal fluid cultures to be relatively small. Second, it should be mentioned that our study population differs from that of other studies conducted in Western societies, a fact that might be affected by the differences in personal hygiene and sexual behavior, which may potentially influence the incidence and etiology of vaginitis [25,26].

The epidemiological and clinical similarities between bacterial vaginosis and trichomoniasis suggest a similar pathogenetic process; i.e., primary causation by a specific agent, with secondary anaerobic activation and resultant amine production [27]. Another organism isolated with high frequency was *M. hominis*. The causative role of genital mycoplasmas in the pathogenesis of some female upper genital system infections is clearly known; however, their roles in lower genital system infections are highly controversial, except for bacterial vaginosis (BV). Despite opposing issues [28,29] there is a strong consideration of an association between BV and *M. hominis* alone or together with Ureaplasma urealyticum.

In the present study there was significant correlation between the presence of *T. vaginalis* and *M. hominis*.

Previousely it was reported that there was experimental evidence of the localization and multiplication of *M. hominis* within *T. vaginalis* cells.

Figure 1. Operating characteristic curve analysis of sensitivity and specificity of score and clue cells for diagnosis of bacterial vaginosis compared with culture.

ROC Curve

1 - Specificity

Diagonal segments are produced by ties.
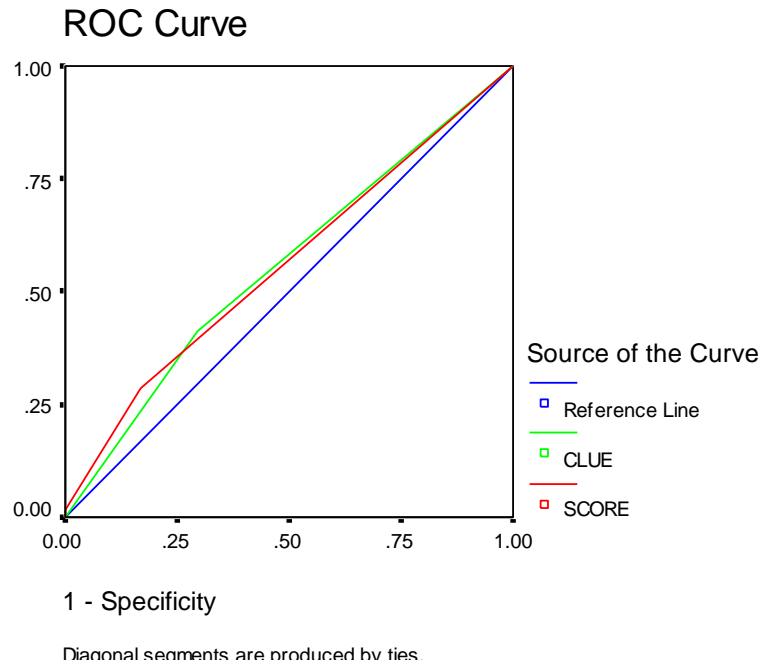
Figure 2. Receiver operating characteristic curve analysis of sensitivity and specificity of Gram score and clue cells versus *T. vaginalis* positive culture.

ROC Curve

1 - Specificity

Diagonal segments are produced by ties.
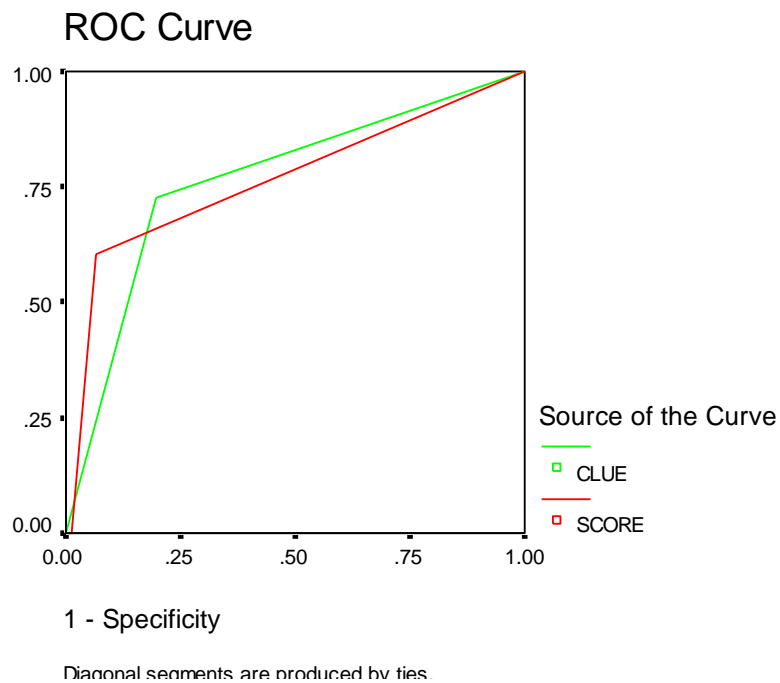
Figure 3. Receiver operating characteristic curve analysis of sensitivity and specificity of the methods used: (a) modified Diamond's medium; (b) acridine orange fluorescent staining; (c) wet mount; (d) Giemsa staining; (e) Gram staining.

1 - Specificity
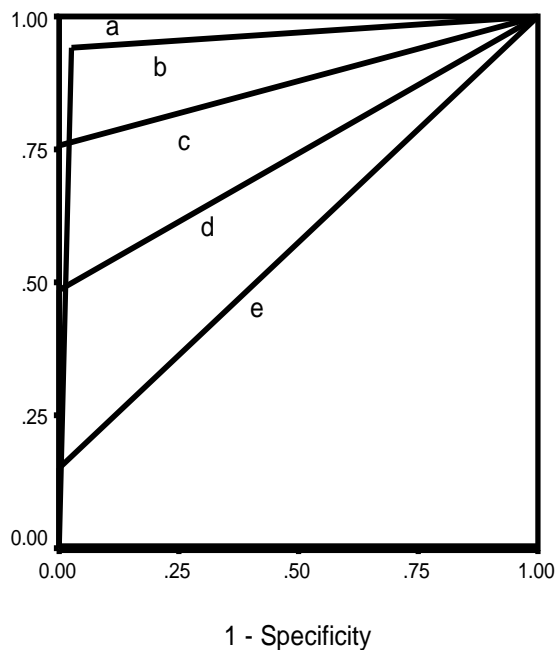
The presence of mycoplasmas within *T. vaginalis* cells was demonstrated by several experimental approaches [29].

The persistence of mycoplasmas in the *T. vaginalis* cytoplasm as well indicates that the bacteria are able to evolve specific strategies to resist killing mechanisms and to adapt to intracellular environments.

Rarely pathogenic microorganisms have been described as symbionts, and the role of protozoa as vectors for the transmission of human diseases has received little attention. Interest increased after the finding that symbiotic relationships between protozoa and bacteria could exert a strong influence on the pathogenesis of one or both microorganisms [30].

Interestingly, we had found an association between patients with infertility and both *T. vaginalis* and *M. hominis*. Although the association between *M. hominis* and *T. vaginalis* had been found in several reports, we found a new association between both organisms and infertility. This could be attributed to endomitritis [31] or tubal affection [32] and pelvic inflammatory diseases (PID) [33]. Further studies with extended samples are required to explore such relationships.

Vaginal culture is one of the most difficult cultures to be evaluated in a clinical microbiology practice. The necessity of some expensive and complicated processes for diagnosis of some specific agents, age related variability of normal vaginal flora, and failure to make a diagnosis caused by the temporary presence of some pathogens in normal flora can be listed among the probable causes of that problem [33].

In the present study an attempt was made to reach a proper diagnosis by using several laboratory methods. Both the Nugent score method and the presence of clue cells were used for diagnosis of bacterial vaginosis. Though the Nugent score method had a low level of sensitivity, it had good specificity and can therefore provide a useful tool for rapid laboratory diagnosis for initiation of treatment. Clue cells examination had similar value.

Our interesting findings that both tests had reasonable sensitivity and specificity for diagnosis of *T. vaginalis* compared with culture could lead us to conclude that *T. vaginalis* is also associated with disturbed bacterial flora of the vagina. To our knowledge this is the first report to describe this finding.

Laboratory methods used in the diagnosis of trichomoniasis have advantages and faults. Direct microscopic examination by wet mount was the first to be discovered and remains the method most commonly used. Wet mount showed reasonable sensitivity of 75.8%. Different reports indicate that the sensitivity of the wet mount method is between

35% and 80% and specificity up to 100% when compared with the culture method [34,35].

The sensitivity of Gram stain examination for detection of *T. vaginalis* was poor. Gram-stained smears of female genital tract specimens were frequently noted to have contained clue cells, indicative of *G. vaginalis*, and are increasingly being [15] used to diagnose bacterial vaginosis. Awareness that this finding is also associated with *T. vaginalis* infections can give a clue for its presence.

Another staining method with poor sensitivity was Giemsa. During the microscopic examination of Giemsa staining preparations, it is possible to overlook or not recognize trophozoites, because the preparations can be damaged during the process of staining. Overall, the Giemsa staining method is subjective and the result largely depends on the competence and qualification of the examiner [36].

In this study, the acridine orange sensitivity was 93.9% and specificity 97.5%. Similar results were reported previously [37,38] which encourage the use of AO as a simple and rapid method for diagnosis of *T. vaginalis*.

The prevalent pathogens associated with vaginitis in the present study were *G. vaginalis*, *T. vaginalis* and *M. hominis*. *G. vaginalis* was isolated from most of patients as monopathogen. *T. vaginalis* was associated with disturbed bacterial flora of the vagina with association with high Nugent score and clue cells. Wet mount microscopic examination, acridine orange, and high Nugent score were found to be rapid and sensitive methods for diagnosis of *T. vaginalis*.

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