

# *Gardnerella, Trichomonas vaginalis, Candida, Chlamydia trachomatis, Mycoplasma hominis and Ureaplasma urealyticum* in the genital discharge of symptomatic fertile and asymptomatic infertile women

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## SUMMARY

This study aimed to establish the different prevalence of the microorganisms investigated in the two groups considered: fertile women with symptoms and asymptomatic women with infertility problems.

The data from women (n=952) investigated for two years for quality of genital discharge and the presence of *Gardnerella vaginalis*, *Trichomonas vaginalis*, *Candida species*, *Streptococcus agalactiae*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Chlamydia trachomatis* were retrospectively analyzed.

In the population of fertile women with symptoms the microorganisms most frequently involved are *Gardnerella vaginalis* (26.6%), *Candida species* (12.1%) and *Streptococcus agalactiae* (9.2%).

The genital discharges of asymptomatic women with infertility problems are characterized by a prevalence of *Gardnerella vaginalis* (19.7%), Enterobacteriaceae or Enterococci (12.1%) and *Streptococcus agalactiae* (8.6%).

The reduction of vaginal lactobacilli flora and the presence of an elevated number of polymorphonucleates in the vaginal discharge are important parameters to consider for the evaluation of the health status of the human female urogenital tract. Our results indicate that is important to culture the vaginal discharge for *Streptococcus agalactiae* and for prevalence of Enterobacteriaceae and Enterococci. Lastly, the reasons for the prevalence of some microorganisms (*Gardnerella vaginalis*, Enterobacteriaceae and Enterococci, *Streptococcus agalactiae*) in the population of infertile asymptomatic women need to be better analyzed especially after the recent studies correlating idiopathic infertility with the presence of cervical cytokines in women with an abnormal vaginal flora.

**KEY WORDS:** *Gardnerella vaginalis*, *Candida species*, *Trichomonas vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Chlamydia trachomatis*, Vaginal discharge, Infertility

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## INTRODUCTION

The vagina is an environment where, from puberty to the menopause, the usual microbiota are dominated by lactobacilli called Döderlein's bacilli, comprising glycogen-fermenting *Lactobacillus aci-*

*dophilus* and related species. Despite the control over the vaginal micro-environment exerted by the lactobacilli, many other microorganisms can be cultivated from the vaginal samples of healthy women. These organisms alone do not trigger a pathological state, but when one class of them dominates, the resulting imbalance is the prelude to vaginitis/vaginosis. There are also sexually transmitted diseases such as chlamydiosis, syphilis, gonorrhoea and herpes simplex virus infections, as well as trichomoniasis (Murray *et al.*, 1995).

The primary sites of *Chlamydia trachomatis* infections are the endocervix of women and the ure-

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thra of both genders. Symptomatic female patients will experience either cervicitis or urethritis, but the majority of patients will be symptom-free. If untreated, the bacteria can ascend the female genital tract, causing endometritis, salpingitis, pelvic inflammatory disease, ectopic pregnancy or tubal factor infertility (Stamm, 1999). Factors predisposing to candida vulvovaginitis are antibiotic treatment and diabetes mellitus (De Leon *et al.*, 2002). *Trichomonas vaginalis* infection is due to anaerobic flagellated protozoa; it may cause serious health consequences, but is readily treatable (Kurth *et al.*, 2004).

Bacterial vaginosis, widely believed to be the most common vaginal disorder affecting women of reproductive age, is defined as an alteration of the vaginal bacterial morphotypes characterized by overgrowth of several anaerobic and microaerophilic bacteria, gram-positive cocci, and decreased prevalence of lactobacillus species (Schenbach *et al.*, 1993; Hillier *et al.*, 1993).

The role of *Streptococcus agalactiae* as the most common cause of neonatal sepsis is well recognized, but vaginal colonization by this organism and its connection with vaginal symptoms is still controversial. In the view of some authors (Maniatis *et al.*, 1996), *Streptococcus agalactiae* in symptomatic women with microscopic evidence of inflammation should be considered a causative agent of vaginitis. Other authors have recommended that patients should not be treated with antibiotics to isolate *Streptococcus agalactiae* from routinely collected genital swabs in female attendees of a genitourinary medicine clinic (Shaw *et al.*, 2003).

Moreover, some studies have associated *Escherichia coli* with symptomatic vaginal infections (Gonzales Pedraza *et al.*, 2004): the relatively low carriage rate indicates that this organism should not be considered part of the normal indigenous vaginal flora.

Recently, due to the high incidence of infertility, vaginosis/vaginitis has been investigated not only in symptomatic women, but also for screening before assisted reproduction. A recent study (Wilson *et al.*, 2002) concluded that women with tubal infertility were three times more likely to have bacterial vaginosis than women with endometriosis, male factor and unexplained infertility, supporting the association between bacterial vaginosis, pelvic inflammatory disease and

tubal damage. Another factor supporting this hypothesis is that women with anovulation are three times more likely to have bacterial vaginosis than women with endometriosis or male factor infertility, suggesting a hormonal influence on the vaginal flora. However, several studies concern the relationships between tubal infertility and bacterial vaginosis or prior bacterial infections (Rivero *et al.*, 2002; Rodriguez *et al.*, 2001; Okonofua *et al.*, 1995; Andreeva *et al.*, 2002; Uzlova *et al.*, 2000).

*Ureaplasma urealyticum* and *Mycoplasma hominis* have also been involved in female fertility (Fenkci *et al.*, 2002; Rosemond *et al.*, 2006). The role of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection in pelvic inflammatory disease (PID), and hence tubal infertility, is well recognized (Gaudoin *et al.*, 1999; Cates Jr *et al.*, 1990).

The aim of this study was to verify our local epidemiology and in particular the most common bacterial species in a group of fertile women with symptoms and in asymptomatic infertile women and to establish the different prevalence in the two groups considered.

To determine the proportional rate of colonization, a retrospective study was conducted based on microbiological investigations of the vaginal flora performed on fertile women with symptoms (WWS) attending our ambulatory and on asymptomatic women with infertility problems (WWI) enrolled on screening program two weeks before *in vitro* fertilisation. In particular *Gardnerella vaginalis*, *Trichomonas vaginalis*, *Candida species*, *Streptococcus agalactiae*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Chlamydia trachomatis* were investigated. A study of the microorganism eventually prevalent in the vaginal discharge was conducted with a semiquantitative assessment of lactobacilli and polymorphonucleate numbers.

## MATERIALS AND METHODS

### Patient population

Between December 2005 and December 2007 women (n=952), attending our outpatient clinic (situated in the south of Milan) undergoing vaginal swabs to test for *Gardnerella vaginalis* (Gv), *Candida* (C), *Trichomonas vaginalis* (Tv) and *Streptococcus agalactiae* (Sa) and endocervical

swabs to test for *Mycoplasma hominis* (*Mh*), *Ureaplasma urealyticum* (*Uu*) and *Chlamydia trachomatis* (*Ch*) were classified, after providing informed consent, on the basis of the reason for the test: screening program before *in vitro* fertilisation or symptomatic status. Women who had been treated with antibiotics or antifungals within the past week were excluded, as were women undergoing vaginal douches and postmenopausal women.

### Specimen collection

For every patient, one vaginal swab was obtained with the Affirm VPIII sample collection set (Becton Dickinson, Milan, Italy), and one was collected with Amies-Stuart medium (Becton Dickinson, Milan, Italy). A vaginal smear was then performed by rolling a third vaginal swab over a glass slide.

Two endocervical specimens were also collected with a cytobrush; one was inserted into a transport medium of universal transport medium UTM kit (Copan, Brescia, Italy), and the other was inoculated in 2 mL urea-arginine broth (UMM) using the Mycoplasma IST 2 commercial kit (Elitech, salon de Provence, France) for *Mh* and *Uu* investigation.

### Specimen processing

#### Tests for lactobacilli and polymorphonucleates

The vaginal smears were Gram stained and examined with an optical microscope.

#### Tests for Gardnerella vaginalis, Trichomonas vaginalis and Candida

300 µL of lysis solution was added to the vaginal swab in the Affirm collection vial (Becton Dickinson, Milan, Italy). The swab was mixed with the lysis solution to allow complete destruction of the prokaryotic cell wall by twisting the swab with an up-and-down motion for 10 s. With the swab remaining in the tube, the suspension was incubated for 10 min at 85°C in a heating block. After incubation, 450 µL of Affirm buffer solution was added to the lysis suspension to maintain the pH solution between 7.0 and 8.0. The contents of the swab were expressed along the wall of the tube, and the swab was discarded. The solution obtained was then filtered into the first well of the seven-well processing cassette caddy. The Affirm card containing the embedded

beads to which the specific oligonucleotide probes for *Gv*, *Tv* and *C* (capture beads) were fixed was placed in well 1, and the automated processing was begun.

#### Test for the presence of Streptococcus agalactiae

The vaginal swab was rolled out onto blood agar with colistin-nalidixic acid (Becton Dickinson, Milan, Italy). The plate was incubated at 37°C in a CO<sub>2</sub> thermostat. The test was considered positive in presence of colonies of *Sa* at the third seeding dial plate.

#### Test for the valuation of Enterobacteriaceae or Enterococci prevalence

The vaginal swab was rolled out onto a 5% sheep blood agar plate (Becton Dickinson, Milan, Italy). The plate was incubated at 37°C in a CO<sub>2</sub> thermostat. The test was considered positive in presence of Enterobacteriaceae or Enterococci colonies at the third seeding dial plate.

#### Test for Mycoplasma hominis and Ureaplasma urealyticum

The adhesive film was removed from the screening tray of Mycoplasma IST 2 commercial kit (Elitech, salon de Provence, France) and 100 µL of inoculated UMM medium was dispensed into one of the wells of the screening tray.

Two drops of paraffin oil were added to the wells. The tray was incubated at 37°C for 24 hours. Urogenital mycoplasma growth is indicated when the medium turns red.

The enumeration of mycoplasma was based on the rate of urea or arginine hydrolysis which is proportional to the number of germs contained in the sample. The test was considered positive when the enumeration of urogenital mycoplasma was up to 10<sup>5</sup> CCU/mL.

#### Test for Chlamydia trachomatis

A 50 µL sample of processed specimen was added to 50 µL of Master Mix containing internal control and amplified with the thermal cycler of the COBAS Amplicor system (Roche, Milan, Italy). The thermal cycling conditions were performed automatically. Upon completion of amplification the COBAS Amplicor system automatically denatured the amplified DNA, hybridized the amplicon to the target-specific oligonucleotides bound to magnetic microparticles, and colori-

metrically detected the captured amplicon with an avidin-horseradish peroxidase complex.

#### Statistical analysis

The data are described as number and percentage, or mean and standard deviation, when appropriate. The correlations between variables were explored with the Mann-Whitney test and the  $\chi^2$  test, using the Fisher correction when appropriate.  $p < 0.05$  was considered significant. All calculations were performed with Stata 10 (www.stata.com).

## RESULTS

Full data were available for 952 women. The median age was 35 years (range 20-50).

The women were divided into two groups on the basis of the reason for the laboratory tests: fertile women with different symptoms (WWS) like pruritus and leukorrhoea (n=556) and symptom-free women with infertility problems (WWI) waiting for an assisted reproduction procedure (n=396).

TABLE 1 - Results of statistical processing on the presence of lactobacilli.

Microorganism	Lactobacilli		p
	0/+ (n=479)	++/+++ (n=473)	
<i>Chlamydia trachomatis</i>	1.67%	1.27%	0.789
<i>Ureaplasma urealyticum</i>	6.68%	2.11%	0.001
<i>Mycoplasma hominis</i>	0.42%	0.00%	0.499
<i>Streptococcus agalactiae</i>	14.20%	3.59%	<0.001
<i>Enterobacteriaceae/Enterococci</i>	13.57%	5.07%	<0.001
<i>Trichomonas vaginalis</i>	0.84%	0.00%	0.124
<i>Candida species</i>	9.39%	8.88%	0.822
<i>Gardnerella vaginalis</i>	41.96%	5.29%	<0.001

TABLE 2 - Results of statistical processing.

Microorganism	Polymorphonucleates		p
	0/+ (n=626)	++/+++ (n=326)	
<i>Chlamydia trachomatis</i>	1.92%	0.61%	0.157
<i>Ureaplasma urealyticum</i>	4.63%	3.99%	0.742
<i>Mycoplasma hominis</i>	0.16%	0.31%	1.000
<i>Streptococcus agalactiae</i>	7.83%	11.04%	0.099
<i>Enterobacteriaceae/Enterococci</i>	8.15%	11.66%	0.078
<i>Trichomonas vaginalis</i>	0.32%	0.61%	0.610
<i>Candida species</i>	7.35%	12.58%	0.008
<i>Gardnerella vaginalis</i>	27.64%	16.26%	<0.001

TABLE 3 - Microorganisms in genital discharge in WWS and WWI.

Microorganism	WWS (n=556)	WWI (n=396)	p
<i>Chlamydia trachomatis</i>	2.16%	0.51%	0.053
<i>Ureaplasma urealyticum</i>	4.86%	3.79%	0.552
<i>Mycoplasma hominis</i>	0.18%	0.25%	1.000
<i>Streptococcus agalactiae</i>	9.17%	8.59%	0.754
Enterobacteriaceae/Enterococci	7.37%	12.12%	0.013
<i>Trichomonas vaginalis</i>	0.54%	0.25%	0.645
<i>Candida species</i>	12.05%	5.05%	<0.001
<i>Gardnerella vaginalis</i>	26.62%	19.70%	0.013

#### Statistical data on lactobacilli

Statistical analysis showed a highly significant ( $p \leq 0.001$ ) association between the presence of fewer lactobacilli and *Uu*, *Sa*, *Gv* and a prevalence of Enterobacteriaceae or Enterococci in the vaginal flora (Table 1) considering all the patients together. There was no significant association with *Ch*, *Mh*, *C* or *Tv*.

#### Statistical data on polymorphonucleates

Statistical analysis showed a significant ( $p < 0.05$ ) association between the presence of ++/+++ polymorphonucleates and *C* in the vaginal flora considering all the patients together (Table 2).

#### Statistical data on the prevalence of the different microorganisms in the two populations

In the population of WWS the microorganisms involved were distributed as follows: *Gv* (26.7%), *C* (12.1%), *Sa* (9.2%), prevalence of Enterobacteriaceae or Enterococci (7.4%), *Uu* (4.9%), *Ct* (2.2%), *Tv* (0.54%) and *Mh* (0.18%). WWI were characterized by overgrowth of several aerobic bacteria, especially a prevalence of *Gv* (19.7%), Enterobacteriaceae or Enterococci (12.12%) and *Sa* (8.59%) (Table 3).

## DISCUSSION

The health status of the human female urogenital tract largely depends on the vaginal microflo-

ra (Larsen *et al.*, 2001). Our data demonstrate that the presence of *Uu* in the vaginal discharge at a concentration of up to 100000 CCU/mL is correlated with a reduction of vaginal lactobacilli flora, as previously described by other authors (Christopoulos *et al.*, 2007; Zdrodowska-Stefanov *et al.*, 2006).

The causal role of *Sa* in vulvovaginal symptoms has not been well researched, since *Sa* are known to colonise the genital tract in 4-18% of healthy women (Rowen, 1993).

Some authors, evaluating samples from women with vaginal symptoms, concluded that *Sa* in symptomatic women with evidence of microscopic inflammation should be considered a causative agent of vaginitis, but a retrospective study conducted in the UK demonstrated that the isolation of *Sa* from routinely collected genital swabs in females attending a GUM clinic is not causally related to vulvovaginal symptoms.

These findings are confirmed by a study conducted to determine whether *Sa* infection is sexually transmitted and related to vaginal symptoms or signs (Honig *et al.*, 2002).

In our study, females with a high *Sa* concentration in the vaginal discharge (in fact our culture procedure did not include a broth enrichment passage, as with Todd Hewitt broth) demonstrated a low lactobacilli load and a high polymorphonucleate number in the vaginal smear. All these findings supported our conviction that *Sa* is a causative agent of vulvovaginal symptoms.

The correlation between the absence of lactobacilli and *Gardnerella vaginalis* is well known (Hillier *et al.*, 1993; Mazzulli *et al.*, 1990; Tokyol *et al.*, 2004; Verhelst *et al.*, 2004; Tamrakar *et al.*, 2007).

In particular, bacterial vaginosis is characterized by the replacement of lactobacillus-predominant vaginal flora with *Gv*, *Bacteroides* species, *Mobilunculus* species and genital mycoplasmas. We obtained the same findings in our two populations.

Recent studies associated *Escherichia coli* with symptomatic infections at vaginal level, mainly due to changes in the normal flora. Culturing the vaginal discharge on sheep blood agar plate, we demonstrated in some cases a prevalence (growth at third quadrant) of gram-negative bacilli or gram-positive cocci, and the simultaneous absence of lactobacillus flora on microscopic examination.

In view of the relatively low carriage rate in normal flora, these findings suggest that the prevalence of Enterobacteriaceae or Enterococci has a role in vaginosis.

Our observations regarding the presence of a large number of polymorphonucleates in the vaginal discharge of women with a prevalence of Enterobacteriaceae or Enterococci further corroborate the role played by the prevalence of aerobic microorganisms in causing vaginosis.

From an epidemiological point of view, the microorganisms most frequently involved in our symptomatic population were *Gardnerella vaginalis* (26.7%), *Candida* species (12.1%), *Streptococcus agalactiae* (9.2%) and the Enterobacteriaceae or the Enterococci as prevalent microorganisms (7.4%). *Ureaplasma urealyticum* (4.9%) was more frequent than *Mycoplasma hominis* (0.18%) and *Chlamydia trachomatis* (2.2%) and *Trichomonas vaginalis* (0.54%) accounted for only 3% of the microorganisms involved.

The influence of bacterial vaginosis on conception and miscarriage has been studied (Kurki *et al.*, 1992; Hay PE *et al.*, 1994; Mc Gregor *et al.*, 1995; Hillier SI *et al.*, 1995), and a predisposition to preterm labour, postpartum endometritis and low birthweight infants was demonstrated. However, the influence of bacterial vaginosis on in vitro fertilization and embryo implantation during assisted reproduction treatment is con-

troversial (Gaudoin *et al.*, 1999; Liversedge *et al.*, 1999; Wittemer *et al.*, 2004; Burrello *et al.*, 2004). There is no doubt about the role of chlamydial infection in the genesis of tubal-peritoneal infertility (Paavonen *et al.*, 1999; Cates *et al.*, 1990; Akande *et al.*, 2003), but the role of other intracellular microorganisms such as *Uu* and *Mh* remains unclear (Imudia *et al.*, 2008; Grzesko *et al.*, 2007; Samra *et al.*, 1994).

Other microorganisms which are causative agents of bacterial vaginosis have been blamed for infertility.

In practice, the disturbed proportion of microorganisms in the vagina led to a predisposition to a disturbance of the natural defence mechanism and the spread of infectious agents towards the upper female genital tract leading to a predisposition for the development of tubal patency and motility disorders, ovarian function disorders and endometritis.

In particular, an abnormal vaginal flora is associated with elevated cervical levels of IL-1 beta and IL-8 and this can be the previously unrecognized cause of idiopathic infertility (Spandorfer *et al.*, 2001; Reddy *et al.*, 2004). In effect we found that WWI were characterized by overgrowth of several aerobic bacteria, especially a prevalence of *Gv* (19.7%), Enterobacteriaceae or Enterococci (12.12%) and *Sa* (8.59%).

In conclusion, the reduction of vaginal lactobacilli flora and the presence of an elevated number of polymorphonucleates in the vaginal discharge are important parameters to consider for the evaluation of the health status of the human female urogenital tract.

Our results indicate that it is important to culture the vaginal discharge for *Sa* and for prevalence of Enterobacteriaceae and Enterococci. The microorganism most frequent involved in the fertile symptomatic women population was *Gv* and *C*. Finally, the reasons for the prevalence of some microorganisms (*Gv*, Enterobacteriaceae and Enterococci, *Sa*) in the population of asymptomatic women with infertility problems need to be better analyzed especially after recent studies on the correlation of idiopathic infertility and the presence of cytokines in cervical levels in women with an abnormal vaginal flora.

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