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Articoli originali

Original articles

Microbial and vaginal determinants influencing *Mycoplasma hominis* and *Ureaplasma urealyticum* genital colonization in a population of female patients

Determinanti microbici e biochimici di colonizzazione genitale da Mycoplasma hominis *e* Ureaplasma urealyticum *in una popolazione di pazienti di sesso femminile*

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INTRODUCTION

Ureaplasma urealyticum and *Mycoplasma hominis* are frequently isolated from the genital tract of sexually active healthy women. Their colonization rates are around 40% and 1.5%-11%, respectively [1, 2]. Colonization is associated to young age, lower socioeconomic status, sexual activity with multiple partners, black race, and oral contraceptive use [3].

The pathogenic role of these two genital mycoplasmas is still not unanimously accepted, although *M. hominis* has been isolated from amniotic fluid samples of women with severe chorioamnionitis and subsequent premature labor, and from the lower genital tracts of women who had preterm delivery [4, 5]. Antibodies to *M. hominis* have been found three times more often in infertile women who suffered from pelvic inflammatory disease, than in controls [6].

Likewise, *U. urealyticum* also has been isolated more frequently from spontaneously aborted fetuses and from premature infants, than from healthy full-term infants, and with a higher prevalence in infertile women [7, 8].

Bacterial vaginosis (BV) is a disorder of normal vaginal ecosystem, characterized by replacement of normal lactobacilli by large numbers of *Gardnerella vaginalis* and anaerobic bacteria along with vaginal discharge and pH above 4.5 [9].

There is still debate about the rate of occurrence of *U. urealyticum* and *M. hominis* in different biochemical and microbial vaginal conditions. Indeed, if *M. hominis* has been found to be more frequently associated to BV compared to healthy controls, some authors found no difference in the recovery of *U. urealyticum* in women with or without BV, while others found a twofold vaginal colonization by *U. urealyticum* in women suffering from BV [10-12].

The aim of this study was to compare demographic parameters, vaginal pH and microbiota of women colonized by *U. urealyticum* or *M. hominis* with the purpose of evaluating possible differences, and therefore possible risk factors of co-colonization by *M. hominis* in patients already colonized by *U. urealyticum*, or vice versa. To this end, only women positive for *U. urealyticum* or *M. hominis* were included in the study, while co-colonized women were excluded.

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PATIENTS AND METHODS

Patient population

A total of 452 patients positive for *U. urealyticum* or *M. hominis* were analyzed during a 14 month period between August 2011 and September 2012. All females with an age between 18 and 60 years admitted to our Hospital in Obstetrics or outpatients from Prenatal Medicine, Fertility Center, or outpatients visiting the laboratory for routine genital examination, independently of pregnancy or presence/absence of symptoms of vulvovaginitis were included in the study. Exclusion criteria were: detection of *U. urealyticum* and *M. hominis* co-colonization, antibiotic therapy or vaginal medication one month prior to sample collection, sexual intercourse or vaginal procedure in the 72 hours preceding the examination, estrogen replacement therapy, structural abnormality of the urogenital system, language barrier, psychiatric disorders and physical disability.

The study was performed according to good clinical practice and the Declaration of Helsinki, and consent was obtained from each patient.

Specimen collection, culture and identification

Test for Ureaplasma urealyticum and Mycoplasma hominis

Cervical samples were collected from the endocervical region after inserting a sterile speculum into the vagina. Mucus had been cleaned with a sterile cotton swab without causing any bleeding or using antiseptics. All the samples were taken twice. The first was plated on modified Thayer-Martin agar immediately after sampling and the plate was incubated in an atmosphere containing approximately 5% CO_2 at 37°C, and analyzed after 24, 48 and 72 hours. The second sample was processed for *U. urealyticum* and/or *M. hominis* detection, and their antimicrobial susceptibilities, by means of the commercially available MYCOFAST® Screening EvolutioN 3 Kit (ELITech MICROBIO, Signes, France). Clinical samples were placed in R1 transport medium which inhibits the growing of Gram-positive and Gram-negative bacteria. The inoculated R1 medium was vortexed and 3 ml added to the growth R2 medium, containing lyophilized urea/arginine broth. After reconstitution, 100 µl was inoculated into each of the 20 wells of MYCOFAST EvolutioN3 tray and overlaid with paraffin oil. During growth, *U. urealyticum* and *M. hominis* metabolize urea and arginine respectively, resulting in a colour change of the medium, which contains phenol red indicator, from yellow to red. This colour change is due to liberation of ammonia resulting in an alkaline pH of the medium. The strips provided an estimate of the bacterial load, based upon enzyme kinetics (colour-changing units [CCU]/ml): for *U. urealyticum* 10^3, 10^4, $\geq 10^5$ CCU/ml, for *M. hominis* $\geq 10^4$ CCU/ml [13].

Test for lactobacilli, Gardnerella vaginalis, Trichomonas vaginalis, Candida spp., Streptococcus agalactiae, and Enterobacteriacee

Vaginal specimens were collected with the aid of a disposable vaginal speculum and by sterile swabs: one sample was taken for wet mount examination, one for Gram-staining, and one for culture of most prevalent vaginal pathogens, such as *Candida* spp., *Gardnerella vaginalis*, *Streptococcus agalactiae* (GBS) and Enterobacteriacee. Vaginal pH was also determined. All swabs were plated within two hours from sampling.

Wet mounts were examined for the presence of *Trichomonas vaginalis*, blastospores and hyphae of *Candida* spp. Gram stained smears were examined for large Gram-positive rods, small Gram-variable rods, curved Gram-variable rods, Gram-positive cocci, Gram-negative rods. All swabs were cultured on blood agar, colistin-nalidixic acid blood agar (CNA), *Gardnerella* selective agar, MacConkey II Agar, Saboraud Dextrose Agar with 40 mg/ml of chloramphenicol (all media from Becton Dickinson, Erembodegem, Belgium). Blood agar, CNA, and *Gardnerella* selective agar were incubated in an atmosphere containing approximately 5% CO_2 at 37°C, and analyzed after 24 hours. MacConkey agar and Saboraud Dextrose Agar with chloramphenicol were incubated at 37°C and analyzed after 48 hours. The recovered microorganisms underwent definitive identification according to standard procedures [14].

Diagnosis of candidiasis was done by microscopic examination and culture of *Candida* spp. on selective media. For *C. albicans* identification, yeast colonies underwent the Germ tube test. For Germ tube test negative isolates, identification was performed by the API *Candida* systems (bioMerieux, Marcy-l'Etoile, France).

Statistical analysis

Statistical analysis was performed by SPSS 13.0 version. Data are expressed as counts and percentages. The chi-square test was used to compare categorical data, with Yates correction for continuity, when applicable, because of small

numbers. When a cell value of <5 was encountered, a 2-tailed p value was obtained by means of the Fisher's exact test. A p value ≤0.05 was considered significant. The effect of the variables studied on colonization by *U. urealyticum* or *M. hominis* was evaluated using a logistic regression analysis performed including all the variables resulted significant in the chi-square analysis.

RESULTS

A total of 452 patients were included in the study. Of these, 277 (61.3%) were Microbiology laboratory outpatients, 106 (23.5%) from Prenatal Medicine, 40 (8.8%) hospitalized in Obstetrics, 29 (6.4%) from the Fertility Center. Two hundred eighty-three patients (62.6%) were Italian, and median age was 29 (interquartile range 23-35), with 338 (74.8%) of the patients <35 years. Two hundred twenty-two (49.1%) were pregnant.

Four hundred twenty-one patients (93.1%) were positive for *U. urealyticum* and 31 (6.9%) for *M. hominis*. Two hundred three patients (45%) had BV and vaginal pH was ≥4.5 in 270 (59.7%). Lactobacilli have been recovered in 264 (58.4%) patients, *G. vaginalis* in 100 (22.1%), *Candida* spp. in 99 (21.9%), GBS in 38 (8.4%), and *T. vaginalis* in 5 (1.1%). All patients resulted negative for *Neisseria gonorrhoeae*. Patients characteristics and laboratory findings in relation to colonization by *U. urealyticum* or *M. hominis* are summarized in Table 1. Patients positive for *M. hominis* compared to patients positive for *U. urealyticum* resulted more frequently colonized by *G. vaginalis* (71% vs 18.5%; p<0.0001), less frequently colonized by lactobacilli (16.1% vs 61.5%; p<0.0001) and more frequently had a pH value ≥4.5 (96.8% vs 57%; p<0.0001).

No differences were found between patients colonized by *M. hominis* compared to patients colonized by *U. urealyticum* in regard to nationality (p=0.354), age <35 or ≥35 years (p=0.234), pregnancy (p=0.160), *Candida* spp. colonization

Table 1 - Patients characteristics and laboratory findings in patients colonized by *Ureaplasma urealyticum* or *Mycoplasma hominis*.

Variable	*Ureaplasma urealyticum (n=421)*	*Mycoplasma hominis (n=31)*	χ^2	*P-value*
Age <35 years	316 (75.1)	22 (71.0)	0.26	NS
Immigrant	155 (36.8)	14 (45.2)	0.86	NS
Pregnancy	203 (48.2)	19 (61.3)	1.97	NS
pH ≥4.5	240 (57.0)	30 (96.8)	18.9	<0.0001
Presence of lactobacilli	259 (61.5)	5 (16.1)	24.5	<0.0001
Candida spp.	93 (22.1)	6 (19.4)	0.13	NS
GBS	38 (9.0)	0 (0.0)	3.10	NS
Gardnerella vaginalis	78 (18.5)	22 (71.0)	46.1	<0.0001
Trichomonas vaginalis	1 (1.0)	1 (3.2)	1.36	NS

Data are count. Values in parentheses represent percentages of isolates of each column category. NS = not significant.

Table 2 - Variables predictive of *Mycoplasma hominis* colonization.

	B	*Wald*	*Sig.*	*OR*	*95% C.I. for OR*	
					Lower	*Upper*
Gardnerella vaginalis	1.937	20.89	<0.0001	6.939	3.023	15.924
pH ≥4.5	2.504	5.845	0.016	12.236	1.607	93.191
Constant	-5.546	1.016	<0.0001	0.004		

Presence of lactobacilli variable failed to enter the final model.

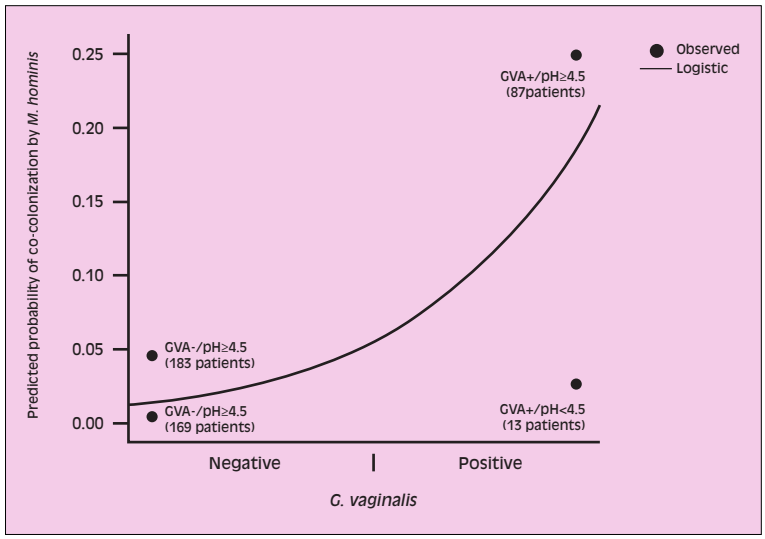

Figure 1 - Predicted probabilities of *Mycoplasma hominis* co-colonization in relation to *Gardnerella vaginalis* colonization and pH ≥4.5.

(p=0.722), GBS colonization (p=0.080), or presence of *T. vaginalis* (p=0.242). Likewise, no differences were found between patients colonized by *M. hominis* compared to patients colonized by *U. urealyticum* in regard to ward of hospitalization. Logistic regression analysis showed that only *G. vaginalis* colonization (p<0.0001) and pH ≥4.5 (p=0.016) were independently related to *M. hominis* colonization (Table 2). The predicted probabilities of *M. hominis* co-colonization in relation to *G. vaginalis* colonization and pH ≥4.5 are illustrated in Figure 1, showing how the probability of being co-colonized by *M. hominis* in women with both *G. vaginalis* and pH ≥4.5 is around 25%. The goodness-of-fit of the logistic regression model used is illustrated in Figure 2, with a value of the area under the curve of 0.826 (Confidence Interval: 0.757-0.895; p<0.0001).

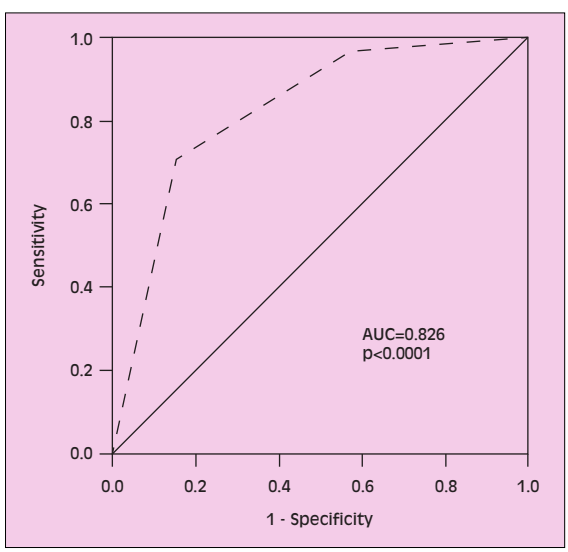

Figure 2 - The goodness-of-fit of the logistic regression model used (AUC=0.826; Confidence Interval: 0.757-0.895; p<0.0001).

DISCUSSION

The main finding of this study was the statistically significant greater colonization rate of *G. vaginalis* and the greater prevalence of pH values ≥4.5 in patients colonized by *M. hominis* in comparison to patients colonized by *U. urealyticum*. These two variables resulted independently associated with a greater risk of *M. hominis* colonization in patients already colonized by *U. urealyticum*. This finding matches with a previous study conducted in a cohort of women with or without BV, in which *M. hominis* was the only mycoplasma detected significantly more often in women with BV, while no difference was found in colonization rate by *U. urealyticum* in women with or without BV [9].

Similarly, also another study conducted on 174 pregnant women, found how *G. vaginalis* and *M. hominis* were isolated from a much larger number of women with BV [8].

It is noteworthy how in the population evaluated, the 43% of the women colonized by *U. urealyticum* had a vaginal pH <4.5, and the 61.5% resulted colonized by lactobacilli, suggesting how this bacterium could survive and share the vaginal milieu with lactobacilli much easier than *M. hominis*. As stated in the introduction, the purpose of this study was to find possible dif-

ferences between patients colonized by *U. urealyticum* or *M. hominis*, and the latter has already been found to be associated to BV, therefore a control group of patients not colonized was not necessary [8]. Recent evidence shows how in co-colonization sustained by both microorganisms, the resistance rates of both isolates to nine antimicrobials were significantly higher than that of *U. urealyticum* single isolates [15]. In this study we found that in women colonized by *U. urealyticum*, BV is an independent risk factor for *M. hominis* colonization. This finding suggests the importance of treatment of BV to prevent therapeutic challenges sustained by co-colonization by *U. urealyticum* and *M. hominis*.

Indeed, preliminary results from our laboratory showed that among the 421 patients colonized by *U. urealyticum*, the 14.2% of the isolates were susceptible to at least one of the fluroquinolones tested, while in a group of 95 patients co-colonized by *U. urealyticum* and *M. hominis*, excluded from this study, no isolate was susceptible to these antimicrobials (data not shown). Interestingly, an higher colonization by *G. vaginalis*, and pH ≥4.5 was observed also in the latter group (data not shown).

Limitations of this study are the lack of evaluation of the role of anaerobes or well-known behavioural variables, and of signs and symptoms of BV on vaginal colonization by *U. urealyticum* or by *M. hominis.* These issues need to be addressed in further studies. In conclusion, the results of this study suggest that, in women positive for *U. urealyticum*, the recovery of *G. vaginalis* and values of pH ≥4.5 are independent risk factors for *M. hominis* colonization, so that the diagnosis and treatment of BV can be critical to prevent *M. hominis* co-colonization.

Keywords: *Mycoplasma hominis*, *Ureaplasma urealyticum*, vulvovaginal/microbiology, vagina/microbiology.

Conflict of interest: none.

SUMMARY

Mycoplasma hominis and *Ureaplasma urealyticum* are associated with chorioamnionitis, preterm delivery and pelvic inflammatory disease. The aim of this study was to evaluate the possible risk factors of co-colonization by *M. hominis* in patients already colonized by *U. urealyticum* and compare demographic parameters, vaginal pH and microbiota of women colonized by *U. urealyticum* or *M. hominis*. A total of 452 patients positive for *U. urealyticum* or *M. hominis* were analysed, 421 (93.1%) of whom were positive for *U. urealyticum* and 31 (6.9%) for *M. hominis*. Patients positive for *M. hominis* compared to patients positive for *U. urealyticum* were more frequently colonized by *Gardnerella vaginalis* (71% vs 18.5%; p<0.0001), less frequently by lactobacilli (16.1% vs 61.5%; p<0.0001), and more frequently had a pH value ≥4.5 (96.8% vs 57%; p<0.0001), all conditions associated to bacterial vaginosis (BV). Logistic regression analysis showed that only *G. vaginalis* colonization and pH ≥4.5 were independently related to *M. hominis* colonization (respectively p<0.0001 and p=0.016). Thus, in women colonized by *U. urealyticum*, BV is an independent risk factor for *M. hominis* co-colonization.

RIASSUNTO

Mycoplasma hominis *e* Ureaplasma urealyticum *sono stati associati a condizioni come corioamnionite, parto pretermine e malattia infiammatoria pelvica. Lo scopo di questo studio è stato quello di confrontare parametri demografici, pH vaginale e microbiota di donne colonizzate da* U. urealyticum *o* M. hominis *con lo scopo di valutare i possibili fattori di rischio di co-colonizzazione da* M. hominis *in pazienti già colonizzate da* U. urealyticum. *Un totale di 452 pazienti positive per* U. urealyticum *o* M. hominis *sono state analizzate, 421 (93,1%) sono risultate positive per* U. urealyticum *e 31 (6,9%) per* M. hominis. *Le pazienti positive per* M. hominis *rispetto alle pazienti positive per* U. urealyticum *sono risultate più frequentemente colonizzate da* Gardnerella vaginalis *(71% vs 18,5%, p<0,0001), meno frequentemente da lattobacilli (16,1% vs 61,5%, p<0,0001), più spesso mostravano valori di pH ≥4,5 (96,8% vs 57%, p<0,0001), tutte condizioni associate a vaginosi batterica (BV). Una analisi di regressione logistica ha mostrato che solo la colonizzazione da* G. vaginalis *e pH ≥4,5 sono risultati indipendentemente correlati alla colonizzazione da* M. hominis *(rispettivamente p<0,0001 e p=0,016). Pertanto, nelle donne colonizzate da* U. urealyticum, *BV è un fattore di rischio indipendente per la co-colonizzazione da* M. hominis.

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