New Microbiologica, 41, 3, 225-229, 2018, ISSN 1121-7138

FULL PAPER

Distribution of genital Mollicutes in the vaginal ecosystem of women with different clinical conditions

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SUMMARY

Ureaplasma urealyticum (UU), *Ureaplasma parvum* (UP), *Mycoplasma hominis* (MH) and *Mycoplasma genitalium* (MG) are the most common Mollicutes of the female genital tract. Although many studies have addressed their possible role in the vaginal ecosystem, many aspects remain to be elucidated. The aim of this study was to evaluate the vaginal presence of ureaplasmas/mycoplasmas in women with different clinical conditions.

By means of quantitative PCR assays, the prevalence and load of each Mollicute were assessed in different groups of pre-menopausal women: 'healthy' (n=29), women with bacterial vaginosis (BV) (n=21), patients with *Chlamydia trachomatis* (CT) infection (n=25) and subjects with vulvo-vaginal candidiasis (VVC) (n=23).

Globally, UP was the most prevalent Mollicutes in the vagina (67.3%), followed by MH (14.3%), UU (9.2%) and MG (3.1%). The presence of UU and UP was almost never associated. MH showed a significantly higher prevalence and higher bacterial loads in BV-positive women ($P<0.05$), whereas patients with CT and VVC were characterized by a Mollicutes pattern similar to healthy women.

Mollicutes can be frequently found in the vaginal ecosystem, even in asymptomatic 'healthy' women. Although its presence is not a strict requirement, MH displays a significant role in the pathogenesis of BV.

Received August 7, 2017 *Accepted* February 27, 2018

INTRODUCTION

The human vaginal microbiome contains a multitude of bacterial species, including members of the class Mollicutes, order Mycoplasmatales (mycoplasmas and ureaplasmas) (Ravel *et al.,* 2011). The most important Mollicutes found in the female genital tract are *Ureaplasma urealyticum* (UU), *Ureaplasma parvum* (UP), *Mycoplasma hominis* (MH) and *Mycoplasma genitalium* (MG) (Taylor-Robinson, 2017).

Their presence in the vaginal ecosystem is influenced by age, hormones, sexual activity and pregnancy (McCormack *et al.,* 1972; Taylor-Robinson *et al.,* 1980; Taylor-Robinson, 2017). Typically, women are colonized more frequently by ureaplasmas than by mycoplasmas. Epidemiological data show that ureaplasmas can be detected in the cervix or vagina of 40-80% of sexually mature asymptomatic females, whereas MH and MG in only 20-50% and 0-5% of them, respectively (Taylor-Robinson *et al.,* 2010).

Recently, much attention has been paid to MG as a sexually transmitted pathogen in relation to urogenital tract inflammation in women (Taylor-Robinson *et al.,* 2011; McGowin *et al.,* 2011). Specifically, several clinical investigations indicate that MG should be considered an independent risk factor for cervicitis (McGowin *et al.,* 2011). For that reason, MG can be found in the vagina as a consequence of cervical discharge in women with cervicitis and can be detected in association with other sexually transmitted pathogens like *Chlamydia trachomatis* (CT) (Spence *et al.,* 2007; Oakeshott *et al.,* 2010; Lis *et al.,* 2015). Although many studies have addressed the possible pathogenic role of Mollicutes in the vaginal ecosystem, numerous aspects remain to be elucidated and their association with different clinical conditions is not yet understood.

Vginal MH has been positively associated with bacterial vaginosis (BV), a clinical condition characterized by the depletion of vaginal lactobacilli, together with an increase in different species of facultative or strictly anaerobes (Shipitsyna *et al.,* 2013; Cox *et al.,* 2016). It has been shown that women with BV not only have MH in the vagina more often, but also in much larger numbers than BV-negative women (Cox *et al.,* 2016; Taylor-Robinson, 2017). Besides MH, the role of other Mollicutes in the pathogenesis of BV is less consistent and regarded as controversial (Taylor-Robinson, 2017).

Since the significance of Mollicutes detection is often unclear, the aim of this study was to evaluate the presence of ureaplasmas and mycoplasmas in the vaginal ecosystem of women with various clinical conditions of the genital

Key words:
Mollicutes, Ureaplasmas, Mycoplasmas, vaginal microbiota, bacterial vaginosis, *Chlamydia trachomatis*.

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tract. By means of quantitative PCR assays, the bacterial loads and presence of the principal Mollicutes were assessed in different groups of pre-menopausal women: 'healthy' (asymptomatic subjects, negatives for microbiological investigations), BV-positive women, patients with CT genital infections and women suffering from vulvo-vaginal candidiasis (VVC). Thus, the possible associations between Mollicutes and other pathogens, their patterns of synergy and their role in the vaginal microbiome was investigated.

MATERIALS AND METHODS

Study group and sample collection

From January to July 2016, all pre-menopausal sexually active non-pregnant Caucasian women attending the STI Outpatients Clinic of Sant'Orsola-Malpighi Hospital in Bologna (Italy) and meeting one of the following criteria were enrolled: urogenital symptoms (i.e. vaginal discharge, dysuria, abnormal bleeding, dyspareunia, itching) and/or STI risk factors (age <25 years, new or multiple sexual partners, unsafe intercourse). Exclusion criteria comprised the use of any antibiotics or vaginal medications in the past month, the use of estro-progestinic and probiotic products, chronic diseases, HIV positivity and a state of grade II obesity (BMI>35). Moreover, patients with aerobic vaginitis, trichomoniasis, gonorrhea or lymphogranuloma venereum infection (Foschi *et al.,* 2016a) were further excluded from the study, when their microbiologic results were available.

All the patients' personal data and information on urogenital symptoms were recorded. After a clinical examination, a vaginal swab (E-swab, Copan, Brescia, Italy) was performed for *C. trachomatis* and *Neisseria gonorrhoeae* detection by a commercial duplex real-time PCR (Versant CT/GC DNA 1.0 Assay; Siemens Healthineers, Tarrytown, NY, USA) and for the diagnosis of other genital tract conditions (Marangoni *et al.,* 2015).

Trichomoniasis was assessed by a qualitative nucleic acid amplification test (Aptima *Trichomonas vaginalis* assay, Hologic, Marlborough, MA, USA) (Keating *et al.,* 2015), whereas aerobic vaginitis was diagnosed by means of a microscopic score, adapted from that of Donders and colleagues (Donders *et al.,* 2002). VVC diagnosis was based on suggestive symptoms together with microscopic and/or culture-based detection of *Candida* spp., whereas BV was assessed by the presence of at least 3 of 4 Amsel criteria (vaginal pH >4.5, homogeneous grayish-whitish vaginal discharge, clue cells on wet mount examination, amine 'fishy' odour at whiff test), together with a Nugent score >7 (Hilbert *et al.,* 2016).

Eligible women were allocated to one of the four following groups: 'healthy' (absence of symptoms and negative microbiological investigations), 'BV' (positivity for 3/4 Amsel criteria and Nugent score>7), 'CT' (detection of CT by NAAT) or 'VVC' (presence of suggestive symptoms and microscopic/culture-based positivity for *Candida* spp.).

The Ethical Committee of St. Orsola-Malpighi Hospital approved the study protocol (7/2016/U/Tess) and all the subjects gave written informed consent to the work.

CT genotyping

In case of a CT positive result, the corresponding remaining eluate was recovered from the Versant PCR plate and used for CT molecular genotyping. Molecular genotyping was performed by an *omp1* gene semi-nested PCR followed by RFLP analysis, as previously described (Foschi *et al.,* 2014; Foschi *et al.,* 2016b).

Mollicutes real-time qPCR

Starting from the remaining DNA eluate of the Versant PCR plate, each sample was tested against UU, UP, MG and MH with quantitative PCR (qPCR) assays. After the production of a standard curve using serial dilutions of individual plasmids each containing qPCR amplicons, singleplex TaqMan real-time qPCR assays were used, as described elsewhere (Cox *et al.,* 2016). Primers and probes are listed in *Table 1*. Briefly, all qPCR assays were carried out using the following reaction mix composition: 1 × Platinum Quantitative PCR SuperMix-UDG (Thermo Fisher Scientific, Waltham, MA, USA), and final working concentrations of reagents of 0.4 mM forward and reverse primer, 0.1 mM TaqMan probe, 0.2 mg BSA ml^{-1} (Promega) and 4 mM $MgCl_2$ (Thermo Fisher Scientific). Final reaction volumes of 10 µl, comprising 2 µl of nucleic acid extract and 8 µl of reaction mix, were used. All qPCR thermal cycling reactions were performed with the following cycling conditions: 95°C for 5 min and 45 cycles of 95°C for 15 s and 59°C for 45 s. Results were expressed as DNA copies/reaction for a single sample and as mean DNA load ± Standard Error of the Mean (SEM) when considering a group of patients.

Statistical analysis.

All statistical analyses were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software). Chi-square analysis was used to compare Mollicute prevalence between the groups of women. Mollicute loads were log_{10} transformed to fit a normal distribution and a one-way analysis of variance (ANOVA) was performed to compare the mean load between the groups. A P value <0.05 was considered statistically significant.

Table 1 - *List of primers and probes used for Mollicutes quantitative real-time PCR.*

Target	*Primers*	*Probes*
UU	F: 5'-CATTGATGTTGCACAAGGAG-3' R: 5'-CGTGATTTTAATGTATCGGCTTTC-3'	FAM-TTGTCCGCCTTTACGAG-Q
UP	F: 5'-CATTGATGTTGCACAAGGAG-3' R: 5'-CGTGATTTTAATGTATCGGCTTTC-3'	FAM-TTGACCACCCTTACGAG-Q
MH	F: 5'-TTTGGTCAAGTCCTGCAACGA-3' R: 5'-CCCCACCTTCCTCCCAGTTA-3'	FAM-TACTAACATTAAGTTGAGGACTCTA-Q
MG	F: 5'-CATAGTTCATTATGCGCACCAGTTACTTG-3' R: 5'-CTCTTTAACAACAGGGGTTGGGATTAG-3'	FAM-GGTGTGGATCGAGCGGC-Q

RESULTS

Study group

During the study period a total of 177 women were enrolled and, out of these, 98 patients were considered eligible for the study. Specifically, 29 (29.9%) women were considered 'healthy', 21 (21.6%) received a diagnosis of BV, 25 (25.5%) were positive for CT infection, whereas VVC was found in 23 patients (23.7%).

The remaining 79 patients were excluded from the study because of other clinical conditions, such as trichomoniasis, gonococcal infections, aerobic vaginitis or mixed infections. Although CT-positive women were younger (mean age: 24.5 years) than healthy, BV and VVC groups (27.7, 28.2 and 28.6 years, respectively), no significant differences were found (P=0.12).

Almost half (11/25; 44%) of CT-infected women were completely asymptomatic: in case they showed symptoms, the patients complained especially about vaginal discharge and abnormal bleeding, and less frequently about dysuria and dyspareunia.

C. albicans was the most common *Candida* species found by culture in VVC patients (86.9%). In this group, the most common symptoms were vaginal discharge and pruritus.

CT genotyping

Out of the 25 CT-positive cases, 23 (92%) were available for typing. The most common serovar in our population was E (52.2%) followed by F (17.4%), G (13%), D (8.7%) and K (8.7%). Symptomatic CT cases were significantly associated with serovar E (P=0.006).

Mollicutes prevalence

Using individual qPCRs, the respective detection rates for UP, MH, UU and MG were as follows: 67.3%, 14.3%, 9.2%, and 3.1%.

Globally, 24 of the 98 (24.5%) patients were negative for all the Mollicutes tested, with no significant differences between groups (P=0.26). Considering the entire population, UU and UP dual positivity was never found, except for one case. Women with a vaginal positivity for MH showed a concomitant detection of UP in almost all cases (92.8%).

The prevalence of each Mollicute in the four groups of women analyzed is outlined in *Table 2*. We found a significantly higher prevalence of *M. hominis* in women with BV compared to the other groups (P<0.05). Generally, BV-positive subjects were characterized by the deepest changes in Mollicutes pattern when compared to 'healthy' women: a complete negativity for UU, together with the highest detection rate for UP and MH were noted in this group.

In the group of CT-positive women, no differences in the distribution of Mollicutes were found between the different CT serovars (data not shown).

Mollicute bacterial loads

As shown in *Table 3*, the bacterial load of MH was significantly higher in BV-positive women (P<0.05). UU, UP and MG loads did not display a significant difference between the four groups of women.

Even though CT symptomatic women showed a higher UP bacterial load than asymptomatic ones, no statistically significant differences were found (mean ± SEM: 214589±115682 vs 12362±8483; P=0.40). Similarly, no dissimilarities were noted in UP load between women infected with different CT serovars (data not shown).

DISCUSSION

The role of Mollicutes in the vaginal ecosystem is controversial and their association with different clinical conditions affecting the female genital tract is still poorly understood (Taylor-Robinson, 2017).

Table 2 - *Comparison of Mollicutes prevalence between the four groups of women enrolled: 'healthy' women, women with genital* Chlamydia trachomatis *infection (CT), women with bacterial vaginosis (BV) and women suffering from vulvo-vaginal candidiasis (VVC).*

Target organism	*Total number of positive women (%)*				*P value*
	'Healthy' N=29	*CT N=25*	*BV N=21*	*VVC N=23*	
U. urealyticum	3 (10.3%)	4 (16%)	0 (0%)	2 (8.7%)	0.29
U. parvum	21 (72.4%)	13 (52%)	17 (80.9%)	15 (65.2%)	0.18
M. hominis	1 (3.4%)	2 (8%)	9 (42.9%)	2 (8.7%)	0.0004*
M. genitalium	1 (3.4%)	1 (4%)	1 (4.7%)	0 (0%)	0.7

*Indicates statistically significant differences

Table 3 - *Comparison of Mollicutes mean load (log10 transformed) between 'healthy' women, women with* C. trachomatis *infection (CT), women with bacterial vaginosis (BV), and women with vulvo-vaginal candidiasis (VVC).*

Target organism	*Mean log10 gene copy number/reaction ± SEM*				*P value*
	'Healthy' N=29	*CT N=25*	*BV N=21*	*VVC N=23*	
U. urealyticum	0.46±0.27	0.78±0.35	0.0±0.0	0.54±0.37	0.39
U. parvum	3.47±0.44	2.30±0.47	3.88±0.48	3.68±0.58	0.12
M. hominis	0.10±0.10	0.19±0.16	1.84±0.49	0.36±0.25	<0.0001*
M. genitalium	0.04±0.04	0.01±0.01	0.12±0.12	0.0±0.0	0.52

*Indicates statistically significant differences

The aim of this study was to analyze the distribution of the most common Mollicutes found in the vagina in different groups of pre-menopausal women: 'healthy', women with CT genital infection, BV-positive women and patients suffering from VVC.

To prevent possible biases, we excluded from the study all the women with conditions able to disrupt the homeostatis of the vaginal microbiota, such as pregnancy or the use of antibiotic and estro-progestinic products.

Globally, we found that UP was the most prevalent Mollicutes in the vaginal niche (67.3%), followed by MH (14.3%), UU (9.2%) and MG (3.1%), and we detected all the Mollicutes tested even in the group of asymptomatic 'healthy' women. This distribution reflects the one described by Cox and colleagues (Cox *et al.,* 2016) and is in agreement with previous reports underlining the high rates of detection of ureaplasmas and MH in asymptomatic sexually mature females (Taylor-Robinson *et al.,* 2010; Taylor-Robinson, 2017).

Although our population consisted of pre-menopausal sexually active women, we found a very low prevalence of MG detection, equally distributed in the different groups. In accordance with previous reports, even though MG has been recognized as an emerging sexually transmitted pathogen (Manhart *et al.,* 2007; Lis *et al.,* 2015), its worldwide prevalence in the general population is quite low (Andersen *et al.,* 2007; Hamasuna *et al.,* 2008; Olsen *et al.,* 2009).

When comparing Mollicutes prevalence and loads in the four groups, we first found that women with BV showed significant differences in Mollicutes pattern both qualitatively and quantitatively, compared to the other subjects. In particular, we noted that women with BV were characterized by the highest MH detection rate and loads, thus corroborating a potential link between MH and BV. Effectively, as suggested in previous reports, MH can be part of the BV-associated microbiota, replacing the *Lactobacillus*-dominated vaginal ecosystem of healthy women (Vitali *et al.,* 2015; Bautista *et al.,* 2016). On the other hand, there was no significant difference in the prevalence or loads of UU, UP and MG between women with and without BV. According to these findings, the role played by ureaplasmas in the development of BV is less well-defined (Cox *et al.,* 2016). Moreover, our results emphasize that the contribution of each Mollicute to the development of BV is difficult to judge and that BV may develop even in the absence of genital mycoplamas/ureaplasmas.

When considering women with VVC, we noticed the absence of specific signatures in Mollicutes profile compared to 'healthy' women. In line with these findings, it has been shown that the composition of the vaginal microbiota of women with VVC is more similar to 'healthy' subjects than BV-positive women. Vitali showed that the vaginal microbiota of women with VVC is dominated by lactobacilli, with no particular perturbations in the composition of other bacterial species (Vitali *et al.,* 2007).

Likewise, we found no particular modifications in Mollicutes composition in women with CT infection compared to healthy subjects. In this context, it has been shown that the cervico-vaginal microbiota of CT-positive women can harbor *Lactobacillus iners* and diverse anaerobic bacteria more frequently than CT-negative subjects (van der Veer *et al.,* 2017; Filardo *et al.,* 2017). Nevertheless, we recently demonstrated that the vaginal microbiome of CT-positive women resembled that of healthy subjects, being characterized by a high colonization of lactobacilli and relatively low occurrences of BV-related bacteria (unpublished data).

In conclusion, the major findings of this study are the following:

1) Mollicutes, especially UP, are frequently found in the vaginal niche even in asymptomatic 'healthy' women;
2) BV has a strong qualitative and quantitative association with MH;
3) women with VVC and CT genital infections have a Mollicutes pattern analogous to that of 'healthy' women.

Further studies enrolling a larger number of women are needed to better understand the potential role of Mollicutes in different clinical conditions of the female genital tract and to shed light on their interaction with other endogenous/exogenous microorganisms.

Acknowledgements

We thank Chiara Urbinati, Camilla Valentino and Riccardo Melloni for providing excellent technical support during this study, and Marco Rizzi for editing.

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