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FULL PAPER

Prevalence and antimicrobial resistance of genital Mollicutes in Italy over a two-year period

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SUMMARY

Knowledge of the prevalence and antimicrobial susceptibility of genital Mollicutes is crucial to offer guidelines for empirical treatments. The aim of this study was to investigate the prevalence and the resistance profile of *Mycoplasma hominis* (MH) and *Ureaplasma urealyticum*/*Ureaplasma parvum* (UU/UP) in genital samples over a two-year period in Bologna, Italy.

From January 2015 to December 2016, data on all the subjects providing uro-genital specimens for Mollicutes detection by culture were analyzed.

A total of 4660 subjects (84.4% females) were enrolled and an overall Mollicutes prevalence of 30.9% was found. Women turned positive for Mollicutes infection twice as often as men (33.3% vs 17.8%) and the detection rate progressively decreased with increasing age. Ureaplasmas represented the commonest species isolated (overall prevalence: 24.2%), whereas mixed infections (6.5%) and MH single infections (3.9%) were far less common. *Ureaplasma* species showed significant levels of quinolone resistance, especially to ciprofloxacin (77%), whereas MH strains were non-susceptible to azithromycin and roxithromycin in about 90% of cases. Mollicutes co-infections showed a more severe resistance pattern than single infections. Over time, the resistance rate for azithromycin and roxithromycin increased significantly.

Globally, our results revealed that minocycline and doxycycline can still be first-line drugs for Mollicutes treatment.

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INTRODUCTION

Mycoplasma hominis (MH) and *Ureaplasma* species, including *U. parvum* (UP) and *U. urealyticum* (UU) are members of the class Mollicutes, order Mycoplasmatales (Taylor-Robinson, 2017). They can frequently be found in the uro-genital tract of healthy people, as common commensal microorganisms. Effectively, up to 40-80% of healthy adult women may harbor ureaplasmas in their cervix or vaginal tract, and more than half may be colonized by MH (Waites *et al.,* 2005; Song *et al.,* 2014). By contrast, the detection rate of *Ureaplasma* species in men is lower, reaching approximately 20-30% (Paralanov *et al.,* 2012; Song *et al.,* 2014). In this context, it is well known that both the quantity and quality of Mollicutes are influenced by age, gender, menstrual cycle, pregnancy and the use of vaginal contraceptives (He *et al.,* 2016).

The possible pathogenic role of ureaplasmas and MH is controversial and many aspects of their pathobiology remain to be elucidated. Previous papers associated ureaplasmas and MH with several clinical conditions of the uro-genital tract, including urethritis, cervicitis, postpartum endometritis, chorioamnionitis, spontaneous abortion and premature birth, as well as infertility (Waites *et al.,* 2005; Larsen and Hwang, 2010; Ondondo *et al.,* 2010; Murtha *et al.,* 2014; Michou *et al.,* 2014; Shimada *et al.,* 2014). Moreover, MH in the vaginal niche has been linked to the onset of bacterial vaginosis (BV), a condition characterized by the depletion of vaginal lactobacilli and the overgrowth of different Gram-negative coccobacilli (Bautista *et al.,* 2016; Cox *et al.,* 2016; Taylor-Robinson, 2017).

Mollicutes have an intrinsic natural resistance to all the β-lactam drugs (i.e. penicillins, cephalosporins) because of the lack of the cell wall in their structure. For that reason, antimicrobials affecting DNA replication (quinolones) and ribosomal protein synthesis (macrolides and tetracyclines) are commonly used for the treatment of clinically relevant Mollicutes infections (He *et al.,* 2016).

However, because of the overuse of drugs and incomplete or erroneous treatments, Mollicutes antimicrobial resistance is becoming more and more significant in different countries worldwide (Song *et al.,* 2014; He *et al.,* 2016). The significant global spread of bacterial resistance has led to the development of antimicrobial stewardship programs, including the aware use of empirical antimicrobials on the basis of local epidemiology (Dik *et al.,* 2017; Klepser *et al.,* 2017).

To our knowledge, only few limited data on Mollicutes prevalence and antimicrobial resistance have been pub-

Key words:
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lished to date in Italy (Leli *et al.,* 2012; De Francesco *et al.,* 2013; Pignanelli *et al.,* 2015). The aim of this study was therefore to investigate the prevalence and the resistance profile of MH and *Ureaplasma* species in a large number of clinical genital isolates over a two-year period in a high-density urban area in the North of Italy.
This epidemiological report represents an important contribution to our knowledge of Mollicutes distribution and can offer guidelines for clinical prescriptions in case on empirical treatments for Mollicutes infection.

MATERIALS AND METHODS

Study group

In the period from January 2015 to December 2016, data on all consecutive patients providing uro-genital samples submitted to the Microbiology Laboratory of St. Orsola Hospital in Bologna (Italy) for ureaplasma/mycoplasma detection by culture were collected and analyzed. Specimens were obtained from different groups of patients: symptomatic subjects complaining of several uro-genital disorders (i.e. burning sensation or pain during urination, vaginal/cervical discharge, etc), pregnant women undergoing microbiological screening and couples seeking infertility counseling and support. Globally, most subjects turned to general practitioners, gynecological clinics and family counseling, whereas only occasionally were specimens collected from hospitalized patients. Only samples collected at the first visit were taken into account, excluding specimens obtained during follow-up from the same patient.
This study was conducted according to the regulations of the St. Orsola-Malpighi University Hospital Ethical Committee and the 1964 Helsinki declaration and subsequent amendments.

Specimen collections, Mollicutes culture and antimicrobial susceptibility testing

Clinical specimens, collected according to the principles of aseptic manipulation, consisted of uro-genital samples of various origin. In particular, urethral, endocervical and vaginal secretions were collected with Dacron sterile swabs and immediately placed in a 2 ml specific transport medium (T-broth). First-catch urine and seminal fluids were collected in sterile tubes and a 200 µl aliquot was transferred to the transport medium immediately on arrival at the laboratory. For the urethral site, swabs were turned to obtain as many cells as possible after cleaning the external meatus, whereas for cervical sampling, specimens were taken after the exocervical mucus had been cleaned.
The culture, detection and antimicrobial susceptibility testing (AST) of genital Mollicutes (*Ureaplasma* species and MH) were performed with a commercially available kit (Mycoview, Zeakon Diagnostics) according to the manufacturer's instructions. Briefly, the mixture containing clinical specimens placed in the transport medium (T-broth) was added to a lyophilized growth medium. The solution was vortexed until the lyophilized pellet had completely dissolved and afterwards, starting from the rehydrated growth medium, a Mycoview strip consisting of 12 wells was inoculated. The remainder of the broth and the inoculated strip were then incubated at 37°C for 48 h and any color change from orange-yellow to red-violet, indicating Mollicutes growth, was noted.
The strips include 1 well for growth control, 4 wells for the differential identification of *Ureaplasma* species (UU/UP) from MH, as well as 7 wells for antimicrobial susceptibility testing. In particular the antimicrobials tested and the relative concentrations were as follows: azithromycin (AZM, 4 µg/ml), roxithromycin (ROX, 4 µg/ml), josamycin (JM, 4 µg/ml), minocycline (MNO, 8 µg/ml), doxycycline (DO, 8 µg/ml), ofloxacin (OFX, 4 µg/ml) and ciprofloxacin (CIP, 2 µg/ml).
For a positive cost-benefit ratio, Mollicutes differential identification and AST are performed only in selected cases in our setting:
1) pregnant women;
2) couples seeking infertility counseling;
3) physician's specific request (e.g. in case of relapsed or complicated infections).

For all the remaining cases, only a screening culture in growth medium (positive/negative), without the use of the Mycoview strip, are usually performed and no information on Mollicutes species identification and AST is available.

Data analysis and statistics

First, a descriptive analysis was performed to assess the main characteristics of all the subjects enrolled in the study (number of subjects, proportion of males and females, mean age, number and type of specimens submitted). Second, the prevalence of Mollicutes infection was evaluated and stratified by sex, age, specimen and year. Finally, Mollicutes identification and resistance profiles were analyzed, evaluating the different distribution related to the available variables (age, gender, type of specimens, study year).
In order to evaluate statistically significant differences, ANOVA test was used to compare quantitative data and categorical data were analyzed with chi-square test. Data were analyzed with GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). A $P<0.05$ was considered statistically significant.

RESULTS

Study population and specimens submitted

During the study period, 4660 subjects, mainly women (84.4%), underwent uro-genital sampling for Mollicutes detection by culture. Primary demographic characteristics and specimens provided by the study population are detailed in *Table 1*.

Mollicutes infection prevalence

Considering the entire population, 1440 subjects (30.9%) were found positive for Mollicutes detection by culture. *Table 2* shows Mollicutes infection prevalence stratified by age, gender, type of clinical sample and study year.
The mean age of positive patients was significantly lower than that of negative subjects (34.3 years vs 36.8; $P<0.0001$) and Mollicutes positivity peaked between 30 and 39 years (40.3% of all positive subjects). Considering the sample size of each age range, a significant difference in the distribution of Mollicutes detection was found: in particular, the positive rates tended to decrease with increasing age (43% of positivity in 0-19 age range; 22.9% of positivity in subjects ≥50 years) ($P<0.00001$).
Overall, the male-to-female ratio (M:F) of the Mollicutes

positive patients was 0.09, and the positive rates in the male group were almost half those in females (17.8% vs 33.3%) (P<0.00001).

Considering Mollicutes positivity by type of specimen provided, the highest positive rates were found for vaginal swabs (38.2%), followed by endocervical and urethral swabs (32.1% and 23.2%, respectively), whereas the lowest detection rate was found for urine samples (14.3%) (P<0.00001).

Globally, Mollicutes infection prevalence was stable throughout the study period with no significant differences between the two years (P=0.245).

Table 1 - *Primary demographic characteristics and specimen provided by the subjects enrolled.*

	Entire population	*Males*	*Females*
Total patients	4660	725 (15.6%)	3935 (84.4%)
Mean age (years ± SD)	36.0 ± 10.6	38.9 ± 11.2	35.5 ± 10.4
Samples submitted			
Seminal fluid	327 (7%)	327	-
Urines	202 (4.3%)	166	36
Vaginal swabs	767 (16.5%)	-	767
Endocervical swabs	3119 (66.9%)	-	3119
Urethral swabs	245 (5.2%)	223	22

Table 2 - *Mollicutes infection prevalence stratified by age, gender, type of clinical sample and study year. The percentages in round brackets represent the positive rates of each sub-group considered, whereas the percentages in square brackets represent the constituent rates, calculated on the entire group of positive subjects.*

	Prevalence of Mollicutes infection	*P value*
By age		
Mean age (years ± SD)	34.3 ± 9.9	
0-19 year range	58/135 (43%) [4%]	
20-29 year range	419/1082 (38.7%) [29.1%]	P < 0.00001*
30-39 year range	581/1961 (29.6%) [40.3%]	
40-49 year range	282/1045 (27%) [19.6%]	
≥50 years	100/437 (22.9%) [6.9%]	
By gender		
M	129/725 (17.8%) [9%]	
F	1311/3935 (33.3%) [91%]	P < 0.00001*
By samples		
Seminal fluids	59/327 (18%) [4.1%]	
Urines	29/202 (14.3%) [2.0%]	
Vaginal swabs	293/767 (38.2%) [20.3%]	P < 0.00001*
Endocervical swabs	1002/3119 (32.1%) [69.6%]	
Urethral swabs	57/245 (23.2%) [4.0%]	
By study year		
2015	678/2135 (31.7%) [47.1%]	
2016	762/2525 (30.2%) [52.9%]	P = 0.245

*Statistically significant

Table 3 - *Identification of Mollicutes species. The distribution of Ureaplasma species (UU/UP), Mycoplasma hominis (MH) and Mollicutes co-infection (UU/UP + MH) is reported and stratified by age, gender and study year. Percentages in square brackets are referred to the total number of positive subjects (1440), whereas percentages in round brackets are referred to the cases where the Mollicutes identification was available (500).*

	UU/UP	*MH*	*UU/UP + MH*
Total	349/500 (69.8%) [24.2%]	57/500 (11.4%) [3.9%]	94/500 (18.8%) [6.5%]
By age			
0-19 year range	13/18 (72.2%)	0/18 (0%)	5/18 (27.8%)
20-29 year range	103/158 (65.2%)	19/158 (12%)	36/158 (22.8%)
30-39 year range	163/222 (73.4%)	24/222 (10.8%)	35/222 (15.8%)
40-49 year range	61/86 (71%)	9/86 (10.4%)	16/86 (18.6%)
≥50 years	9/16 (56.2%)	5/16 (31.2%)	2/16 (12.5%)
By gender			
M	11/13 (84.6%)	1/13 (7.7%)	1/13 (7.7%)
F	338/487 (69.4%)	56/487 (11.5%)	93/487 (19.1%)
By study year			
2015	192/255 (75.3%)*	17/255 (6.6%)*	46/255 (18.0%)
2016	157/245 (64.1%)	40/245 (16.3%)	48/245 (19.6%)

* Statistically significant (P < 0.05)

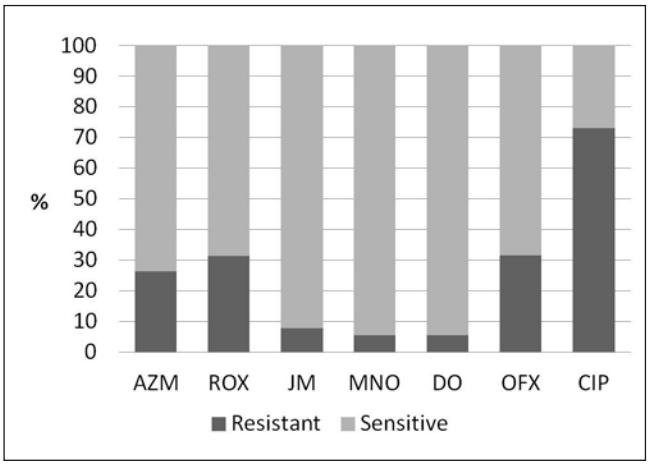

Figure 1 - *Overall antimicrobial susceptibility of Mollicutes strains tested in the study period. Percentages are calculated on the number of cases where antimicrobial susceptibility testing was available (500).*

Although the Mycoview strips were performed for 694 patients, Mollicutes species identification (UU/UP and MH) was available for about a third of positive patients (34.7%; 500/1440). In the remaining cases, the low bacterial load (<10^3 CFU/ml) did not allow a correct species identification. Overall, *Ureaplasma* species (UU/UP) were much more frequently identified than *Mycoplasma hominis* (69.8% and 11.4% among tested isolates, respectively), and a genital Mollicutes co-infection was detected in 18.8% of tested cases. No statistically significant differences were found in the distribution of Mollicutes species when considering the age and the gender of subjects. On the contrary, a different distribution of Mollicutes species was found between the two years of the study: going from 2015 to 2016, *Ureaplasma* species prevalence significantly decreased, while *Mycoplasma hominis* isolates were more frequently detected. Detailed results on Mollicutes species identification are presented in *Table 3*.

Mollicutes antimicrobial susceptibility

As for the species identification, information on the antimicrobial susceptibility of strains was available for 500 of the 1440 positive cases (34.7%). The overall antimicrobial susceptibility of Mollicutes strains tested is reported in *Figure 1*, whereas *Table 4* shows the differences in resistance rates between the two years analyzed. Globally, the highest levels of resistance were found for ciprofloxacin (73% of resistant strains), followed by ofloxacin (31.6%) and roxithromycin (31.4%). Conversely, tetracyclines (minocycline and doxycycline) showed the lowest rate of resistance (5.6% each).

A significant increase in the resistance rate of azithromycin and roxithromycin was found between 2015 and 2016 (P<0.05). The increased resistance to azithromycin and roxithromycin was associated with the increase in MH detection with a concomitant reduction in UU/UP prevalence (P=0.01 for azithromycin and P=0.003 for roxithromycin). For the other antimicrobial drugs, except for an almost significant decrease in the quinolone resistance rate, the susceptibility levels were quite stable throughout the study.

When the antimicrobial resistance of Mollicutes was stratified by the different species (*Table 5*), the major findings were the following:

1) MH isolates showed extremely high levels of resistance for azithromycin and roxithromycin (about 90% of resistant strains);
2) ureaplasma (UU/UP) strains were characterized by a significant reduction in quinolone susceptibility

Table 4 - *Antimicrobial resistance rates of Mollicutes, stratified by year of study.*

Antimicrobial drugs	*2015*	*2016*	*P value*
AZM	57/255 (22.3%)	75/245 (30.6%)	0.03*
ROX	68/255 (26.6%)	89/245 (36.3%)	0.01*
JM	18/255 (7.1%)	21/245 (8.6%)	0.5
MNO	13/255 (5.1%)	15/245 (6.1%)	0.6
DO	15/255 (5.9%)	13/245 (5.3%)	0.6
OFX	90/255 (35.3%)	68/245 (27.8%)	0.06
CIP	195/255 (76.5%)	170/245 (69.4%)	0.07

*Statistically significant

Table 5 - *Antimicrobial resistance stratified by Mollicutes species.*

Antimicrobial drugs	*UU/UP*	*MH*	*UU/UP + MH*	*P value*
AZM	11/349 (3.2%)	50/57 (87.7%)	71/94 (75.5%)	<0.00001*
ROX	16/349 (4.6%)	53/57 (93%)	88/94 (93.6%)	<0.00001*
JM	1/349 (0.3%)	7/57 (12.3%)	31/94 (33%)	<0.00001*
MNO	3/349 (0.9%)	4/57 (7%)	21/94 (22.3%)	<0.00001*
DO	3/349 (0.9%)	3/57 (5.3%)	22/94 (23.4%)	<0.00001*
OFX	92/349 (26.3%)	10/57 (17.5%)	56/94 (59.6%)	<0.00001*
CIP	269/349 (77.1%)	15/57 (26.3%)	81/94 (86.1%)	<0.00001*

* Statistically significant

(77.1% of strains resistant to ciprofloxacin and 26.3% to ofloxacin);
3) globally, tetracyclines were the most active drugs regardless of species identification;
4) when Mollicutes co-infection (UU/UP + MH) was detected, resistance patterns were often more severe than those in cases with a single infection.

For *Ureaplasma* species, significant cross-resistance was found between ciprofloxacin and ofloxacin (34% of ciprofloxacin-resistant strains were also resistant to ofloxacin), whereas for MH between roxithromycin and azithromycin and between roxithromycin and ciprofloxacin (94.3% and 24.5% of roxithromycin-resistant strains were also resistant to azithromycin and ciprofloxacin, respectively).

Although a slight increase in macrolide and tetracycline resistance was noticed with the increasing age of subjects, no statistically significant differences were found. Likewise, antimicrobial resistance was not influenced by the gender of subjects (data not shown).

DISCUSSION

Knowledge of the prevalence and antimicrobial susceptibility of genital Mollicutes infection within a population is fundamental to monitor the local drug resistance rate and to provide guidance for the rational use of antibiotics. Therefore the aim of this study was to assess the prevalence and resistance profile of MH and *Ureaplasma* species (UU/UP), analyzing data from 4660 subjects who provided uro-genital specimens for Mollicutes detection by culture over a two-year period in a high-density urban area in the North of Italy.

First, we confirmed that ureaplasma/mycoplasma are commonly isolated from uro-genital tract specimens, regardless of population characteristics. Effectively, we found an overall Mollicutes prevalence of 30.9%, in line with previous reports, showing positivity ranges between 20 and 50%, depending on the subjects enrolled, the geographical area and the clinical setting (De Francesco *et al.,* 2013; Verteramo *et al.,* 2013; Song *et al.,* 2014; He *et al.,* 2016; Wang *et al.,* 2016; Zeng *et al.,* 2016). Moreover, as previously reported, we noticed that women were found positive for Mollicutes infection twice as often as men, and that the detection rate reached the highest level in young people and progressively decreased with increasing age (Zdrodowska-Stefanow *et al.,* 2006; Ponyai *et al.,* 2013; Song *et al.,* 2014; He *et al.,* 2016). On the one hand, these aspects probably reflect male and female differences in anatomical and endocrine factors that make the female genital tract more suitable for Mollicutes colonization and infection. On the other, the relatively active sexual behavior of young people can explain the highest infection rate in subjects aged <29 years.

Interestingly, we noticed that the Mollicutes detection rate was influenced by the type of sample analyzed: in particular, samples collected with a swab (vaginal, urethral and endocervical swabs) allowed a higher rate of Mollicutes isolation compared to urine and seminal fluids. Considering that Mollicutes tend to display an intimate adherence and surface colonization of the epithelial host cells, genital swabs can recover a higher number of cells and are more suitable for ureaplasma/mycoplama detection than other specimens (Ueno *et al.,* 2008; Marovt *et al.,* 2015).

Second, when data on Mollicutes species identification were available (34.7% of positive subjects), we found a general distribution of *Ureaplasma* species (UU/UP) and MH comparable to many other reports (De Francesco *et al.,* 2013; Verteramo *et al.,* 2013; Song *et al.,* 2014; Zeng *et al.,* 2016). In particular, ureaplasma represented the commonest species isolated with an overall prevalence of 24.2%, whereas ureaplasma/MH mixed infection (6.5%) and MH single infection (3.9%) were far less common.

Evaluating the trend of Mollicutes infection during the two-year period (2015-2016), significant changes were noticed in the distribution of species, despite a stable overall prevalence. Effectively, ureaplasma detection significantly decreased from 2015 to 2016, together with a major increase in MH prevalence. If these results emphasize that Mollicutes infection is 'endemic' in our setting, it is clear that significant changes in species epidemiology can occur, probably due to differences in the use of antimicrobial drugs, the pathobiology of circulating strains and the social/sexual behavior of subjects.

Finally, regarding data on the antimicrobial susceptibility testing of isolates, several aspects are merit discussion. In agreement with other reports, *Ureaplasma* species showed significant levels of quinolone-resistance (77% of strains resistant to ciprofloxacin and 26.3% to ofloxacin), whereas in case of MH single infection, high rates of resistance for azithromycin and roxithromycin were found (87.7% and 93%, respectively) (Kechagia *et al.,* 2008; Leli *et al.,* 2012; Song *et al.,* 2014; Vargović *et al.,* 2014; Pignanelli *et al.,* 2015; Zeng *et al.,* 2016). Moreover, as previously reported, the resistance rates of Mollicutes mixed infection isolates to the majority of antimicrobials were higher than those of single infection isolates, indicating that co-infections will be more difficult to treat (Wang *et al.,* 2016; Zeng *et al.,* 2016).

Globally, as already shown, tetracyclines and macrolides were the most active antimicrobial drugs regardless of Mollicutes species identification (De Francesco *et al.,* 2013; Song *et al.,* 2014; He *et al.,* 2016). In particular, in case of *Ureaplasma* species, josamycin, minocycline and doxycycline were highly and equally effective (resistance rate <1%), whereas azithromycin and roxithromycin could represent good alternatives, considering that the resistance rate did not exceed 5%.

In case of MH infections or Mollicutes co-infections, tetracyclines showed a better pattern of sensitivity than josamycin. All together, these results revealed that minocycline and doxycycline can still be first-line drugs for Mollicutes therapy, followed by josamycin. For the other antimicrobial drugs, local *in vitro* susceptibility testing is recommended to avoid treatment failure. Although significant discrepancies in antimicrobial susceptibilities from various countries are present because of differences in antimicrobial use policies, our results are in line with several reports indicating doxycycline, minocycline and josamycin as options for initial empirical treatment (De Francesco *et al.,* 2013; He *et al.,* 2016; Wang *et al.,* 2016; Zeng *et al.,* 2016). In addition, in our setting and unlike other areas (De Francesco *et al.,* 2013; Zeng *et al.,* 2016) the susceptibility profiles showed major changes over the study period. In particular, we found a significant increase in the resistance rate for azithromycin and roxithromycin, together with an almost significant decrease of quinolone resistance levels. This might be related to the antimicrobial drugs usage strategy in various regions and periods. For example, the antimicrobial stewardship policy in our area led in recent years to a significant reduction in fluoroquinolones use and this may explain the concomitant

reduction in quinolone resistance in Mollicutes isolates (Tedeschi *et al.,* 2017).

In conclusion, our data demonstrate the high and constant prevalence of genital Mollicutes, especially for *Ureaplasma* species in women aged <29 years. In case of empirical anti-Mollicutes treatment, doxycycline, minocycline and josamycin may be the antimicrobial drugs of choice. This aspect is particularly crucial in our setting where antimicrobial susceptibility testing is not universally performed but restricted to subjects for whom a positive cost-benefit ratio is recognized.

We are fully aware that major limitations could have affected our results:

1) the lack of clinical information on the subjects enrolled did not distinguish actual Mollicutes genital infection from common commensal colonizations;
2) the lack of Mollicutes species identification and antimicrobial resistance profile for all the positive subjects could have led us to miss significant epidemiological associations. For these reasons, further studies are needed to devise strategies for infection control policies and critical antimicrobial stewardship.

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