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PII: S0732-8893(18)30441-3
DOI: doi:10.1016/j.diagmicrobio.2018.10.001
Reference: DMB 14686

To appear in: *Diagnostic Microbiology & Infectious Disease*

Received date: 26 July 2018
Revised date: 25 September 2018
Accepted date: 1 October 2018

Please cite this article as: Tatiana Rumyantseva, Guzel Khayrullina, Alexander Guschin, Gilbert Donders , Prevalence of Ureaplasma spp. and Mycoplasma hominis in healthy women and patients with flora alterations. Dmb (2018), doi:10.1016/j.diagmicrobio.2018.10.001

Prevalence of *Ureaplasma spp.* and *Mycoplasma hominis* in healthy women and patients with flora alterations.

Running title. Mycoplasmas in normal and altered flora.

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Abstract

Objective. To estimate the prevalence of *Mycoplasma hominis, Ureaplasma parvum, Ureaplasma urealyticum* in healthy women and patients with altered vaginal microflora.

Methods. Vaginal samples from 2594 unselected female patients were divided into Normal, BV and AV groups and tested for *U. parvum, U. urealyticum* and *M. hominis.*

Results. Normal flora was detected in 1773 patients (68.4%), BV - in 754 patients (29.1%), and AV - in 67 patients (2.6%). In the control group 771 (43.5%) patients were *U. parvum* positive, 104 (5.9%) - *U. urealyticum* positive, 158 (8.9%) - *M. hominis* positive. In the BV group those bacteria were detected in 452 (59.9%), 102 (13.5%) and 202 (26.8%) patients, respectively ($p<0.001$); in AV group – in 16 (23.9%), 3 (4.5%) and 4 (6.0%) patients, respectively ($p<0.001$; 0.63 and 0.40, respectively).

Conclusions. This study demonstrated that mycoplasmas may be a marker or a symbiont of the BV flora, but not AV flora.

Keywords: bacterial vaginosis, aerobic vaginitis, *Mycoplasma hominis, Ureaplasma parvum, Ureaplasma urealyticum,* polymerase chain reaction.

1. Introduction

Genital mycoplasmas are a diverse group of bacteria variable in virulence and pathogenicity. *Mycoplasma genitalium* is a sexually transmitted bacteria and is a cause of urethritis in males[1] and cervicitis, PID, preterm birth and spontaneous abortion[2]. *Mycoplasma hominis* and Ureaplasma *spp. (Ureaplasma parvum* and *Ureaplasma urealyticum*) are opportunistic bacteria found in both healthy women and severe diseases and complications during or outside pregnancy[3, 4]. Prevalence of mycoplasmas varies in many studies (4.5-40%, 28.8-63.8%, 1.3-51% for *U.urealyticum, U.parvum* and *M.hominis,* respectively[3, 5-8] reaching very high numbers even for healthy individuals in some studies: 85.2% (summarized prevalence for *U.urealyticum, U.parvum* and *M.hominis*[9]) and 95.5% for *U.parvum*[10].

M. hominis is associated with a very common flora alteration - bacterial vaginosis (BV)[3, 11], whereas the role or association of *Ureaplasma* spp. and BV is still a topic for discussion[10]. Apart from flora alterations, *M. hominis* and *Ureaplasma* spp. have been linked to a number of morbidities: pyelonephritis, PID, chorioamnionitis, endometritis, postpartum fever, infertility, low birth weight, spontaneous abortion, stillbirth, preterm delivery, perinatal mortality[4, 8, 12-15].

As *M. hominis* and *Ureaplasma* spp. are found both in healthy women as well as in patients with BV and other morbidities, the need for the detection of those species in female patients is being argued. At the moment it is suggested that detection of *Mycoplasma hominis* and *Ureaplasma* spp. alone without flora evaluation seems useless in women[3].

Flora alterations include not only BV but also symptomatic *Trichomonas vaginalis* infection, *Candida* vaginitis and aerobic vaginitis (AV)[16]. *M. hominis* and *Ureaplasma* spp. are frequently found in BV-positive patients, whereas their prevalence in AV-positive patients has not been thoroughly investigated yet. Investigation of the association between mycoplasmas and two types of flora alteration (BV and AV) may give a deeper insight if mycoplasmas are associated with elevated pH in BV or AV - positive women, are a specific constituent of BV

only, or whether they are pathogenic without that lactobacillus deficient climate found in BV/AV – positive patients. Despite the focus on the prevalence of mycoplasmas in women with healthy or BV or AV type vaginal flora, some loads of *M. hominis* and *Ureaplasma* spp. may play a role in the balance of health and disease as well. Thus the aim of the present study was to evaluate the prevalence and loads of *U.parvum, U.urealyticum* and *M.hominis* in healthy, BV-positive and AV-positive women.

2. Methods

2.1.Samples.

A total of 2705 vaginal samples from reproductive-aged female patients were included in this study. Samples were collected from both asymptomatic women (planning pregnancy; after unprotected intercourse or requesting STI testing for any other reasons) and patients with vaginal complaints attending gynecologists in a variety of clinics in Moscow, Russia (clinics sending samples for laboratory testing to Federal Budget Institute of Science "Central Research Institute for Epidemiology"), from August to September 2015. All participants signed the informed consent form.

2.2.Testing.

Vaginal samples from all participants were tested by real-time PCR. DNA-sorb-AM kit (InterLabService, Russia) was used for DNA extraction. The AmpliSens® N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT kit was used to detect the DNA of four STIs (*Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium, Trichomonas vaginalis*)[17]. Samples positive for one or more of these STIs were excluded from the further analysis. Flora was further assessed using AmpliSens® Florocenosis/Aerobes-FRT (detection and quantification of *Enterobacteriaceae spp., Staphylococcus spp., Streptococcus spp.)*[18] and AmpliSens® Florocenosis/Bacterial vaginosis-FRT (detection and quantification of total bacterial DNA, *Lactobacillus spp, G.vaginalis, A.vaginae)*[19] kits (InterLabService, Russia). Those

kits allow discrimination between normal flora, BV and AV-like flora by making the distinction between 1) normal flora with dominance of *Lactobacilli*, 2) BV with dominance of *G.vaginalis/ A.vaginae* in the presence of low *Lactobacilli* counts and 3) AV with dominance *Enterobacteriaceae spp./ Staphylococcus spp./ Streptococcus spp.* with low *Lactobacilli* counts.

Calculations and interpretation were performed based on the DNA loads of the detected bacteria, according to the manufacturer's instructions. In brief, the ratio coefficients (RC) for AmpliSens® Florocenosis/Bacterial vaginosis-FRT were calculated as follows: RC1 = lg [*Lactobacillus* spp.] – lg [*G. vaginalis* + *A. vaginae*], RC2 = lg [Bacteria] – lg [*Lactobacillus* spp.], and RC3 = lg [Bacteria] – lg [*G. vaginalis* + *A. vaginae*].

In summary, the result of the test was interpreted as normal vaginal microbiota (Control group), if *G. vaginalis* and/or *A. vaginae* as well as *Enterobacteriaceae spp.*+ *Staphylococcus spp.*+ *Streptococcus spp*. were absent or their cumulative load was less than the *Lactobacillus* spp. load. A sample was categorized as BV (BV group), if the *G. vaginalis* and/or *A. vaginae* loads were equal to or exceeded the *Lactobacillus* spp. load (RC1<0.5). Finally, a sample was categorized as AV-like flora (AV group), if the *Lactobacillus* spp. load was decreased and the *G. vaginalis* and/or *A. vaginae* load was substantially lower than the load of total bacteria (RC2>1 and RC3>2) with summarized load of *Enterobacteriaceae spp.*+ *Staphylococcus spp.*+ *Streptococcus spp*. dominating over both *Lactobacillus* spp. and *G.vaginalis/ A.vaginae.* This analysis allowed us to divide the sample into BV, AV and Control groups.

U. parvum, *U. urealyticum* and *M. hominis* DNA were detected and quantified in all samples using AmpliSens® Florocenosis/Mycoplasma-FRT kit. Testing was performed according to manufacturer's instructions.

2.3. Data analysis.

Descriptive statistics used for summarizing quantitative PCR results included median values. For testing differences between the groups of patients, chi-square statistics were used for categorical variables (presence of bacteria), and the Mann-Whitney U test was used for loads of bacteria. All tests for significance were two-sided, and statistically significant differences were assumed when $p<0.05$.

2.4. Funding.

This work was supported by the Ministry of Education and Science of Russian Federation, project no. 03.G25.31.0226, March 3, 2017.

3. Results.

3.1. Prevalence

After exclusion of STI-positive samples, 2594 samples (mean age 31± 5y.o.) were included in the analysis. Normal lactobacillary-dominated flora was detected in 1773 patients (68.4%), BV - in 754 patients (29.1%), and AV - in 67 (2.6%) patients (**Figure 1**).

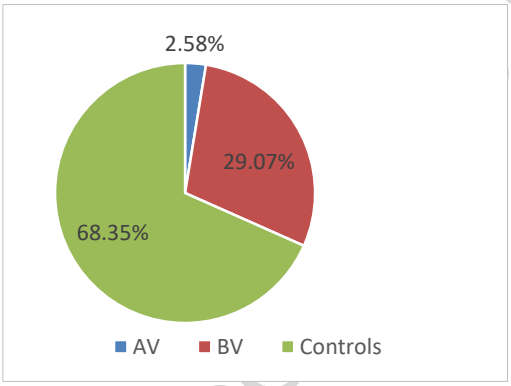

Figure 1. Results of flora assessment in 2594 participants.
AV – aerobic vaginitis; BV – bacterial vaginosis.

DNA of one or more Mycoplasmas was discovered in 1475 (56.9%) women. *U. parvum* DNA was detected in 1239 (47.8%) patients, *U. urealyticum* – in 209 (8.1%) patients, *M. hominis* – in 364 (14.0%) patients. In the control group 771 (43.5%) patients were *U. parvum* positive, 104 (5.9%) patients were *U. urealyticum* positive and 158 (8.9%) patients were *M. hominis* positive. In the BV group those bacteria were detected in 452 (59.9%), 102

(13.5%) and 202 (26.8%) patients, respectively (p<0.001 versus Controls); in the AV group – in 16 (23.9%), 3 (4.5%) and 4 (6.0%) patients, respectively (p-values versus Controls <0.001; 0.63 and 0.40, respectively; p-values versus BV group <0.001; 0.034; <0.001, respectively). *U. parvum*, *U. urealyticum* and *M. hominis* were significantly more prevalent in the BV group (p<0.001) compared to Control and AV groups. *U. parvum* was significantly less prevalent (p<0.001) in the AV group compared to the Control group (**Figure 2**).

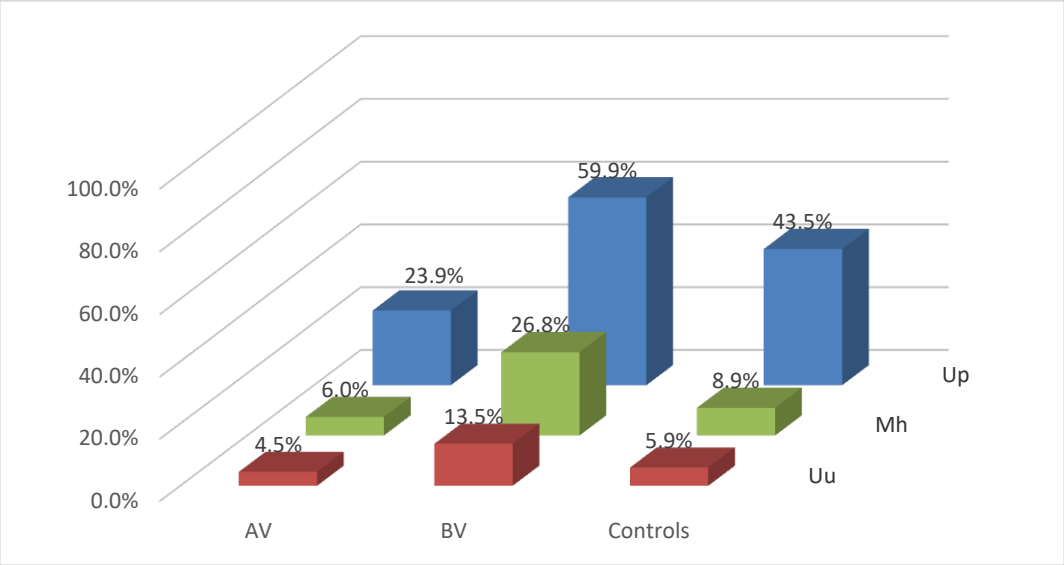

Figure 2. Prevalence of *U. parvum*, *U. urealyticum* and *M. hominis* in the BV, AV and Control groups.

AV – aerobic vaginitis; BV – bacterial vaginosis.

Uu - *U. urealyticum*, Mh - *M. hominis*, Up - *U. parvum*

3.2. Bacterial loads

Median loads of *U. parvum* and *M. hominis* were significantly higher in BV group compared to AV and Control groups (p<0.001). Median load of *U. urealyticum* did not vary significantly in three groups (p=0.21)(Table 1, Figure 3)

Table 1.

Median loads of *U. parvum*, *U. urealyticum* and *M. hominis* in BV, AV and Control groups (Geq/ml).

	AV	**BV**	**Control**	**p-value**
U.parvum	$1*10^5$	$1*10^6$	$2*10^5$	AV vs Control p=0.30 BV vs Control p<0.001
U.urealyticum	$1*10^5$	$8*10^4$	$1*10^4$	p=0.21
M.hominis	$5*10^2$	$1.5*10^6$	$2*10^2$	AV vs Control p=0.50 BV vs Control p<0.001

AV – aerobic vaginitis; BV – bacterial vaginosis.

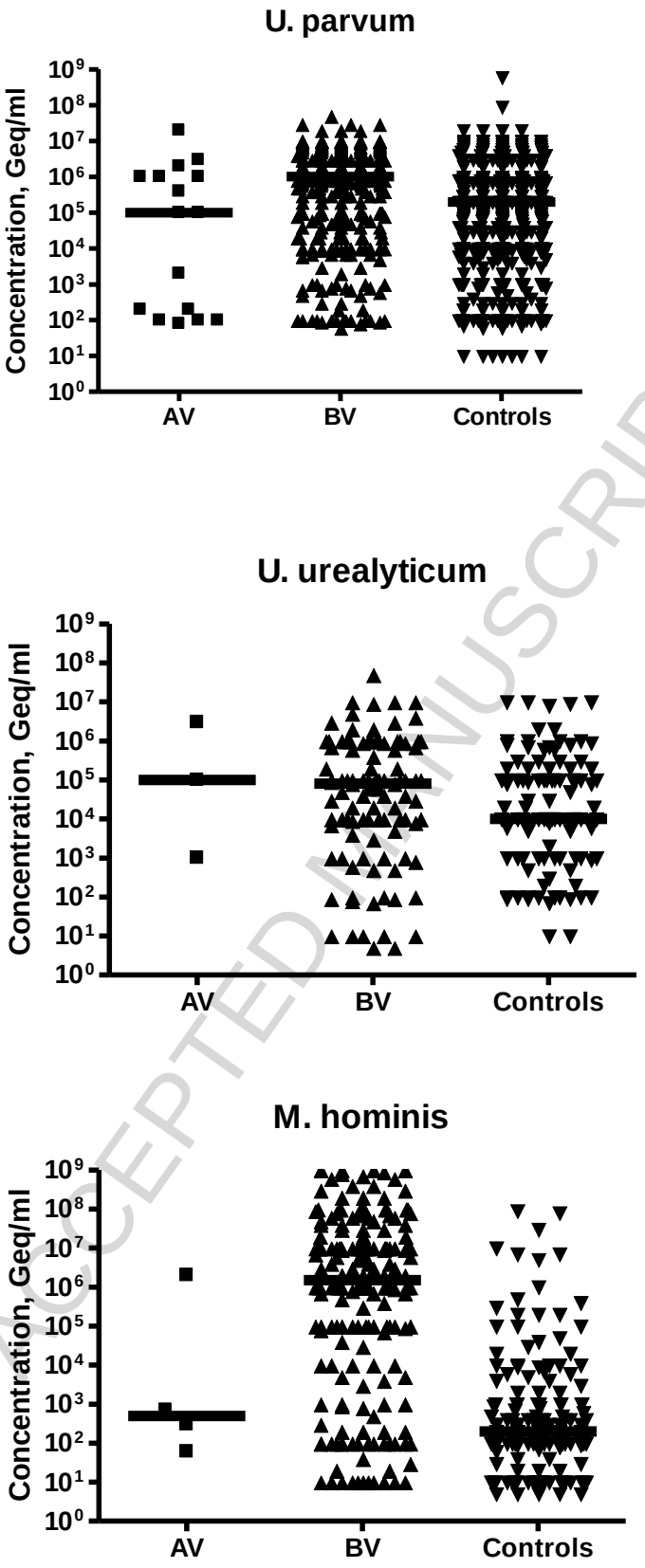

Figure 3. U. parvum, U. urealyticum and M. hominis load distribution in BV, AV and Control groups (Geq/ml). Horizontal bar = median load of mycoplasmas in each group. AV – aerobic vaginitis; BV – bacterial vaginosis.

Overall *U. parvum* was the most prevalent mycoplasma in all groups, whereas *M. hominis* was most strongly associated with BV (demonstrated a 3-fold increase in prevalence and a 7500 - fold increase in load compared to the Control group, $p<0.001$).

4.Discussion.

The role of mycoplasmas (*U. parvum, U. urealyticum* and *M. hominis*) in the reproductive health and disease is still being evaluated. Conflicting results are being reported, and one of the reasons for that may be the insufficient evaluation of vaginal microflora in some studies devoted to mycoplasmas only. Thus in this study we investigated the prevalence of mycoplasmas in healthy, BV-positive and AV-positive women.

4.1. Main Findings.

All mycoplasmas were less prevalent in the AV group as compared to the BV group; *U. parvum* was less prevalent in the AV group compared to Controls; loads of mycoplasmas did not vary significantly in AV and Control groups, whereas *U. parvum* and *M. hominis* were detected in BV-positive patients in significantly higher loads.

4.2. Interpretation.

In our setting of 2594 female patients BV was detected in 29.1%, AV in 2.6% and normal flora in 68.4% of women. These data correlate well with known prevalence, BV being the most prevalent lower genital tract bacterial infection in women of reproductive age worldwide[20]. In most settings the real prevalence of BV may be lower than described before, due to the underestimation of AV, e.g. Donders et al. found BV in 25% and AV in 11% of women in Uganda, whereas most authors claim that 35 to 50% prevalence of BV in central African populations[21]. Prevalence of AV in our study is lower then the prevalence demonstrated in other studies, as AV was encountered in 8.3%-10.8% of pregnant women[22, 23] and in 5%-23.7% of women reporting vaginal complaints[21, 24-26]. Besides accepting real differences between specific

populations, this may also be due to the variations of diagnostic tools used or to the specific population studied and the proportion of symptomatic patients.

The prevalence of mycoplasmas in this study was lower than in previous studies. Cox et al. detected *U. parvum* in 78.6% of BV-positive patients and in 61,3% of healthy controls[27]. In our study *U. parvum* was detected in 59.9% of BV-positive patients and 43.5% of healthy controls. *U. urealyticum* was previously detected in 65% of BV-positive patients and in 48% of healthy controls by Keane et al.[28] and in 17.6% of BV-positive patients and in 22.5% of healthy controls by Cox et al[27]. We report the prevalence of *U. urealyticum* being 13.5% and 5.9% in BV and healthy women, respectively. For *M. hominis*, a prevalence of 60.7% and 11.3%; 81% and 31% in BV-positive and healthy women was reported previously[27, 29], whereas in our study *M.hominis* was detected in only 26.8% and 8.9% of participants, respectively. In one study, *M.hominis* was not detected in healthy controls[28], demonstrating even lower prevalence than in our study, but in the BV group they still found a prevalence of 53%, which is much higher compared to our results. In one study a 17% prevalence of *M. hominis* was demonstrated in the "AV-like group"[30], compared to 6% in our study. Further comparison of the prevalence of mycoplasmas in AV-positive patients is impossible due to the lack of data.

According to our data, prevalence of all mycoplasmas was significantly higher in the BV-group than in both the AV group and healthy controls. This leads us to the conclusion that mycoplasmas need more to thrive than a favorable pH in the environment, but may survive better in the symbiotic relationships with anaerobic BV-associated bacteria, e.g. *Gardnerella vaginalis, Atopobium vaginae, Mobiluncus spp.* and other anaerobes. The most prominent difference was observed by the 3-fold increase in prevalence for *M. hominis* between healthy and BV-positive women, which is in line with previous findings and supports the suggestion made by Cox et al. that *M. hominis* and *G.vaginalis* have a symbiotic relationship[27]. Some previous studies did not find any increase in the prevalence of *Ureaplasma spp.* in BV-positive women versus healthy controls[27, 28], whereas in our study both Ureaplasmas were more prevalent in the BV group.

Ethnical characteristics of the groups, different diagnostic tools used, various principles of groups' formation (e.g. selection of AV-positive patients in our study) could have accounted for that difference.

In our study, quantitative parameters (DNA load) showed no significant variation of *U. urealyticum* in the normal, BV or AV groups, similar to their bacterial loads found in BV-positive and healthy women in Cox et al's study[27]. Compared to both healthy and AV-positive participants the load of *U. parvum* in BV-positive women was significantly higher in our study, whereas no significant difference was observed by Cox et al.[27] This disagreement may be explained by varying quantification techniques and sample size and variations in populations studied. Quantitative data for *M. hominis* load, on the other hand, correlate well with earlier studies and demonstrated a 7500-fold increase in BV-positive participants versus controls, which is in agreement with findings by Rosenstein et al.[30]

4.3.Strengths and Limitations.

Discussion of the features of the present study requires special attention to the laboratory methods used. We used molecular-based techniques for the detection and quantification of mycoplasmas as well as for the flora assessment. Although this technique does not include clinical data or microscopy findings, it demonstrated good ability for BV, AV and normal flora discrimination in previous studies17-19. In the majority of previous studies of other authors, on the other hand, culture-based tools were used for the detection and quantification of mycoplasmas, and/or non-molecular methods, like microscopy were used to diagnose microflora abnormalities. The PCR-based assay used in the current study, however, has two major advantages: standardized quantification of the microorganisms, validated testing of microflora alterations, and reliable discrimination between Ureaplasma species (U.parvum and U.urealyticum).

The present study has three major limitations: lack of clinical data, relatively small amount of AV-positive patients and no opportunity for follow-up (to determine prevalence and load after BV or AV treatment).

5. **Conclusion.**

This study demonstrates that mycoplasmas, in special M. *hominis*, are associated with BV, but not with AV. Vaginal mycoplasmas even seem to be slightly less prevalent in women with AV, but larger numbers are needed to prove this is statistically significant. Further studies are needed to demonstrate if flora normalization or treatment leads to the elimination or decrease of the DNA load of *U.parvum*, *U.urealyticum* and/or *M.hominis* in the vaginal fluid.

6. **Disclosure of Interests**

No conflict of interests declared.

7. **Contribution to Authorship**

TR performed data analysis, produced the draft of the paper, worked on the paper text after corrections and comments of other authors, approved the final version of the manuscript. GK performed data collection, data analysis, produced the draft of the paper, approved the final version of the manuscript. AG planned the project, read and revised the manuscript, and approved the final version. GD read and revised the manuscript, and approved the final version. All authors accept responsibility for the paper as published.

8. **Details of ethics approval**

This study was approved by the Ethical Committee of the Federal Budget Institute of Science "Central Research Institute for Epidemiology" of Rospotrebnadzor on June, 26, 2015 (Protocol #34).

9. **Funding:**

This work was supported by the Ministry of Education and Science of Russian Federation, project no. 03.G25.31.0226, March 3, 2017

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Figure 1. Results of flora assessment in 2594 participants.
AV – aerobic vaginitis; BV – bacterial vaginosis.

Figure 2. Prevalence of *U. parvum*, *U. urealyticum* and *M. hominis* in the BV, AV and Control groups.
AV – aerobic vaginitis; BV – bacterial vaginosis.
Uu - *U. urealyticum*, Mh - *M. hominis*, Up - *U. parvum*

Figure 3. U. parvum, U. urealyticum and M. hominis load distribution in BV, AV and Control groups (Geq/ml). Horizontal bar = median load of mycoplasmas in each group. AV – aerobic vaginitis; BV – bacterial vaginosis.

Highlights

- Genital mycoplasmas (*U. parvum, U. urealyticum* and *M. hominis*) are positively associated with bacterial vaginosis (BV), not aerobic vaginitis (AV).
- *U. parvum* and *M. hominis* are detected in higher loads among patients with BV compared to healthy individuals and patients with AV.
- Genital mycoplasmas may have symbiotic relationship with BV-associated microflora rather then be attracted by elevated pH in BV-positive patients.