


RESEARCH ARTICLE

Detection of ureaplasmas and bacterial vaginosis associated bacteria and their association with non-gonococcal urethritis in men

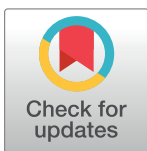
Maria Frølund¹ , Lars Falk² , Peter Ahrens¹ , Jørgen Skov Jensen¹ *

1 Research Unit for Reproductive Microbiology, Statens Serum Institut, Copenhagen S, Denmark,

2 Department of Dermatovenereology, Linköping University Hospital and Linköping University, Linköping, Sweden

 These authors contributed equally to this work.

* jsj@ssi.dk



OPEN ACCESS

Citation: Frølund M, Falk L, Ahrens P, Jensen JS (2019) Detection of ureaplasmas and bacterial vaginosis associated bacteria and their association with non-gonococcal urethritis in men. PLoS ONE 14(4): e0214425. <https://doi.org/10.1371/journal.pone.0214425>

Editor: David N. Fredricks, Fred Hutchinson Cancer Research Center, UNITED STATES

Received: January 15, 2019

Accepted: March 12, 2019

Published: April 4, 2019

Copyright: © 2019 Frølund et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This study was supported by ALF-Grant LIO-60221; ALF-Grant LIO-60311; ALF-Grant LIO-205751, to LF, all from County Council of Östergötland, Sweden, <https://www.researchweb.org/is/lio/>. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

No aetiology is found in up to 40% of men with symptomatic urethritis. Male partners of women with bacterial vaginosis (BV) may be at higher risk of non-gonococcal urethritis (NGU). The aim of this study was to examine the role of BV associated bacteria in first-void urine (FVU) in 97 asymptomatic men without urethritis (controls) and 44 men (cases) with NGU including 20 men with idiopathic urethritis (IU) attending a Swedish STD-clinic between January and October 2010. BV-associated bacteria and ureaplasmas were detected by quantitative PCR assays. All BV associated bacteria, except *Megasphaera*-like type 1, were strongly positively correlated with *U. urealyticum* $p < 0.005$ and even stronger with the combined *U. urealyticum* and *U. parvum* load ($p < 0.0005$) suggesting that ureaplasma induced elevated pH may stimulate the growth of BV associated bacteria. No statistically significant differences were found between IU cases and controls in the prevalence or load of BV associated bacteria or ureaplasmas. In multiple logistic regression, *Megasphaera*-like type 1 was associated with IU ($p = 0.03$), but most positive FVU samples contained very few bacteria and the finding may not be clinically relevant.

Introduction

Symptoms of urethritis is a common reason for attendance at sexually transmitted diseases (STD) clinics and in most cases, urethritis is caused by sexually transmitted pathogens [1]. In Scandinavia, non-gonococcal urethritis (NGU) is much more common than urethritis caused by *Neisseria gonorrhoeae*, although there has been a steady increase of reported cases of *N. gonorrhoeae* in the last decade from 7 to 25 cases per 100,000 inhabitants in Sweden [2, 3]. NGU is characterised by inflammation of the male urethra and symptoms such as urethral discharge and dysuria are common. However, asymptomatic urethritis with microscopically documented inflammation on stained urethral smears is frequent and well documented for

Competing interests: MF, LF, and PA have no conflicts; JSJ has received speaker's fee from Hologic, BD and Cepheid and serves scientific advisory boards of Roche Molecular Systems, Abbott Molecular, and Cepheid. SSI has received remuneration for contract work from SpeedX, Hologic, NYTor, Diagenode, Abbott, Nabriva, GSK, and Angelini. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Chlamydia trachomatis, *Mycoplasma genitalium* and *Ureaplasma urealyticum* [4–6]. If left untreated, *C. trachomatis* infection and probably *M. genitalium* infection may be transmitted to female partners and cause pelvic inflammatory disease and infertility. No aetiology is found in the up to 40% of male urethritis cases where known pathogens such as *C. trachomatis*, *M. genitalium*, *N. gonorrhoeae*, *Trichomonas vaginalis*, *U. urealyticum*, herpes simplex virus type 1 and 2, and adenovirus have been excluded [7, 8].

It has been suggested that male partners to women with bacterial vaginosis (BV) are at a higher risk of NGU [1, 9, 10], but this has not been widely studied. BV, present in 10–20% of Scandinavian women [11, 12], is the most common vaginal infection in women with symptoms such as abnormal vaginal discharge, foul odour, itching and burning, although many women have no symptoms or have been accustomed to the condition [13]. It has been associated with a higher risk of preterm birth, HIV transmission, acquisition of other STDs and pelvic inflammatory disease [14, 15]. BV is characterized as an imbalance of the normal bacterial microbiota in the vagina where lactobacilli are reduced and replaced by anaerobic bacteria, the most frequently *Gardnerella vaginalis*, *Atopobium vaginae*, *Prevotella* spp., *Eggerthella*-like uncultured bacteria, *Megasphaera*-like type 1, *Sneathia* spp. and Bacterial Vaginosis Associated Bacterium–2 (BVAB-2) [16].

However, little is known about the presence of BV associated bacteria in men and their association with NGU. One study found that male partners to women with BV were more likely to suffer from NGU [17] and a more recent study showed one of the key BV associated species *Sneathia amnii* (previously *Leptotrichia amnionii*) or *S. sanguinegens* to be associated with male NGU [10]. In men, first void urine has been shown to be an excellent proxy for the urethral microbiota [18] and since this sample type is non-invasive, this is the preferred sample for studies of the urethral microbiota. Next generation sequencing methods and sequencing the 16S rRNA gene in urine have shown that BV associated bacteria such as *Prevotella* spp., *Gardnerella* spp., *Mobiluncus* spp., *Veillonella* spp., *Atopobium* spp. and *Megasphaera* spp. are found in both sexually experienced and inexperienced men [19, 20].

The aim of this study was to examine the role of selected BV associated bacteria: *G. vaginalis*, BVAB-2, *Eggerthella*-like uncultured bacteria, *Megasphaera*-like type 1, *S. amnii*, *A. vaginae*, *S. sanguinegens* and *Prevotella* spp as well as *U. parvum* in urine specimens from men with and without NGU attending an STD clinic. These species were selected based on their strong association with BV in a previous study [21, 22] and because *U. parvum* is found very frequently in the female lower genital tract; however, the role of this species is still disputed [23].

Materials and methods

Study design

The study was a cross-sectional case-control study in men attending a Swedish STD-clinic.

Patients and specimens

Men attending the STD clinic in Norrköping, Sweden were included in this study as they took part in a cross-sectional study of prevalence, signs and symptoms of various bacterial STIs among 614 men. Patients selected for the present study were enrolled between January 2010 and October 2010. Men without symptoms and signs of urethritis by microscopy serving as controls in this study, attended the clinic for a check-up for STIs (42%) or as part of contact tracing of patients with STIs (55%); 2% attended for erectile dysfunction. Cases and controls negative for *C. trachomatis* and *M. genitalium* were consecutively selected for the present study. In addition, the first nine *C. trachomatis* positive and the first nine *M. genitalium* cases,

all negative for *U. urealyticum* and *U. parvum* and still fulfilling the inclusion criteria were selected aiming to control for associations with BV associated bacteria in men with known causes of urethritis. All selected samples were analysed by polymerase chain reaction (PCR) for adenovirus [6, 24], HSV 1 and 2 and selected BV associated bacteria [22].

Data were collected on a standard questionnaire regarding the reasons for attendance, age, symptoms of urethritis (dysuria and discharge), number of sexual partners within the last year and life-time sexual partners, condom use at the first sexual intercourse with a new partner, sexual intercourse with men (MSM), previous STIs, antibiotic treatment within the last month, current partner (yes or no), whether the partner had a current STI and if so which STI. The patients' identity was coded and the variables noted in a database.

Urethral smears were obtained from all patients with a blunt curette and stained with methylene blue. All smears were examined with a Zeiss (standard 14) light microscope by the physician. Patients with dysuria and/or discharge and smears showing ≥ 5 polymorphonuclear leukocytes (PMNL) per high-power ($\times 1,000$) microscopic field (hpf) in more than 4 hpf were defined as having urethritis. Low grade urethritis was defined as 5–9 PMNL/hpf, Medium grade as 10–30 PMNL/hpf, and high grade as > 30 PMNL/hpf. The controls had no symptoms of urethritis and had urethral smears with < 5 PMNL/hpf. They had no other symptoms or signs of genital disease such as ulcers, itching, warts or rash. Neither cases nor controls had received antibiotics within the previous month prior to enrolment.

When *N. gonorrhoeae* was suspected by microscopy (intracellular diplococci) or due to high-risk behaviour, culture was performed, and if positive, the patient was excluded. The patients had not voided within at least one hour before sampling. After the clinical examination, the patient was asked to collect approximately 20 mL of first void urine; 7–13 mL was immediately decanted into 1.1 mL Genelock transport medium (Sierra Molecular Corp., Sonoma, CA, USA) and sent the same day by mail to Statens Serum Institut (SSI), Copenhagen, Denmark. Specimens were received and DNA extracted in the laboratory within a median of 5 days (mean 4.5 days; range 2–22 days) after they had been collected. From the remaining urine, 3 mL was collected in a Multi-collect tube (Abbott, IL, USA) and analysed at the Linköping University Hospital laboratory within two days for *C. trachomatis* using the Abbott m2000 system. DNA was extracted from 400 μ L according to the manufacturer's protocol. This assay employs two separate targets of the cryptic plasmid detecting also the new variant *C. trachomatis*, which is common in Sweden and described initially in 2007 [25]. *Trichomonas vaginalis* was not included in the screening panel as this organism is extremely rare in the population under study.

PCR assays

All other PCR assays were performed at SSI, Copenhagen. Urine samples in Genelock medium were concentrated by centrifugation of 1,900 μ L at 30,000 \times g for 15 min. The pellet was resuspended in 300 μ L of a 20% Chelex-100 slurry (BioRad, Hercules, CA) in TE-buffer (10 mM Tris-HCL [pH 8.0], 1 mM EDTA) and incubated at 95° for 10 minutes as previously described [26]. The prepared samples were stored at -20° C until PCR was performed.

M. genitalium was detected by real-time PCR targeting the MgPa-gene [26] and confirmed by conventional PCR targeting the 16S rRNA gene [27]. Macrolide resistance-mediating mutations were demonstrated by sequencing region V of the 23S rRNA gene in *M. genitalium* positive samples [28]. For detection of *U. urealyticum* and *U. parvum*, a multiplex PCR targeting the urease gene was used [29].

HSV 1 and 2 were detected with a multiplex real-time Taqman based PCR assay [6]. The primers produced a < 100 -basepair fragment of the Envelope Glycoprotein G and D in HSV 1

and 2, respectively. Target specific HSV 1 and 2 probes were labelled with FAM and VIC, respectively. Adenovirus was detected with real-time PCR amplifying the hexon gene using the primers AdF1 and AdR1 [24].

Detection of the BV associated bacteria *G. vaginalis*, BVAB-2, *Eggerthella*-like uncultured bacteria, *Megasphaera*-like type 1, *S. amnii*, *A. vaginae*, *S. sanguinegens* and *Prevotella* sp. was performed by qPCR as previously described [6, 22]. These species were selected based on their strong association with BV [21, 22]. All qPCR results were expressed as DNA copies per mL of the original urine sample.

Ethics statement

Men attending the STD clinic in Norrköping, Sweden were included in this study as they took part in a cross-sectional study of prevalence, signs and symptoms of various bacterial STIs among 614 men. Patients selected for the present study were enrolled between January 2010 and October 2010. The study was approved by the regional research ethical committee of Linköping (protocol # M 134–09). All men received written and oral information about the study and all provided written informed consent.

All samples were coded and analysed anonymously in the SSI research laboratory. Routine testing for STIs at Linköping University Hospital laboratory was performed without anonymization according to standard procedures.

Statistical analysis

Fisher's exact test and odds-ratios with confidence intervals were used for dichotomous variables, the Mann-Whitney test for continuous variables, Spearman's correlation coefficients and multiple logistic regression and other statistical analyses were calculated in StatsDirect (StatsDirect Ltd., Cheshire, UK).

Graphs were produced in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Significance level was 5%, and two-sided results were used throughout.

Results

Characteristics of included patients

A total of 141 patients were included of which 97 men were asymptomatic controls and 44 were symptomatic cases. In the latter group, 20 men were negative for *C. trachomatis*, *M. genitalium*, HSV, adenovirus and *U. urealyticum* and defined as having idiopathic urethritis (IU) (Fig 1).

Controls (n = 97), patients with IU (n = 20) and NGU patients with known aetiology (n = 24) did not differ with respect to median age, number of life-time sexual partners or partners within the last year, MSM or with respect to habits for condom use at first sexual intercourse with a new partner (Table 1).

The distribution of symptoms in the patients with IU was similar to that of the 24 patients with urethritis with known aetiology for dysuria, discharge and urethral itching 18 (90%) vs. 20 (83%), 10 (50%) vs. 13 (54%) and 3 (15%) vs. 0 (0%), respectively. Neither did the distribution of low, medium, or high-grade urethritis differ between cases with IU and patients with NGU of known aetiology (Table 1).

Distribution of NGU associated organisms

Of the 97 asymptomatic controls, 26 men (27%) were *U. urealyticum* positive. When excluding the nine *C. trachomatis* and nine *M. genitalium* positives, the remaining non-*M. genitalium*,

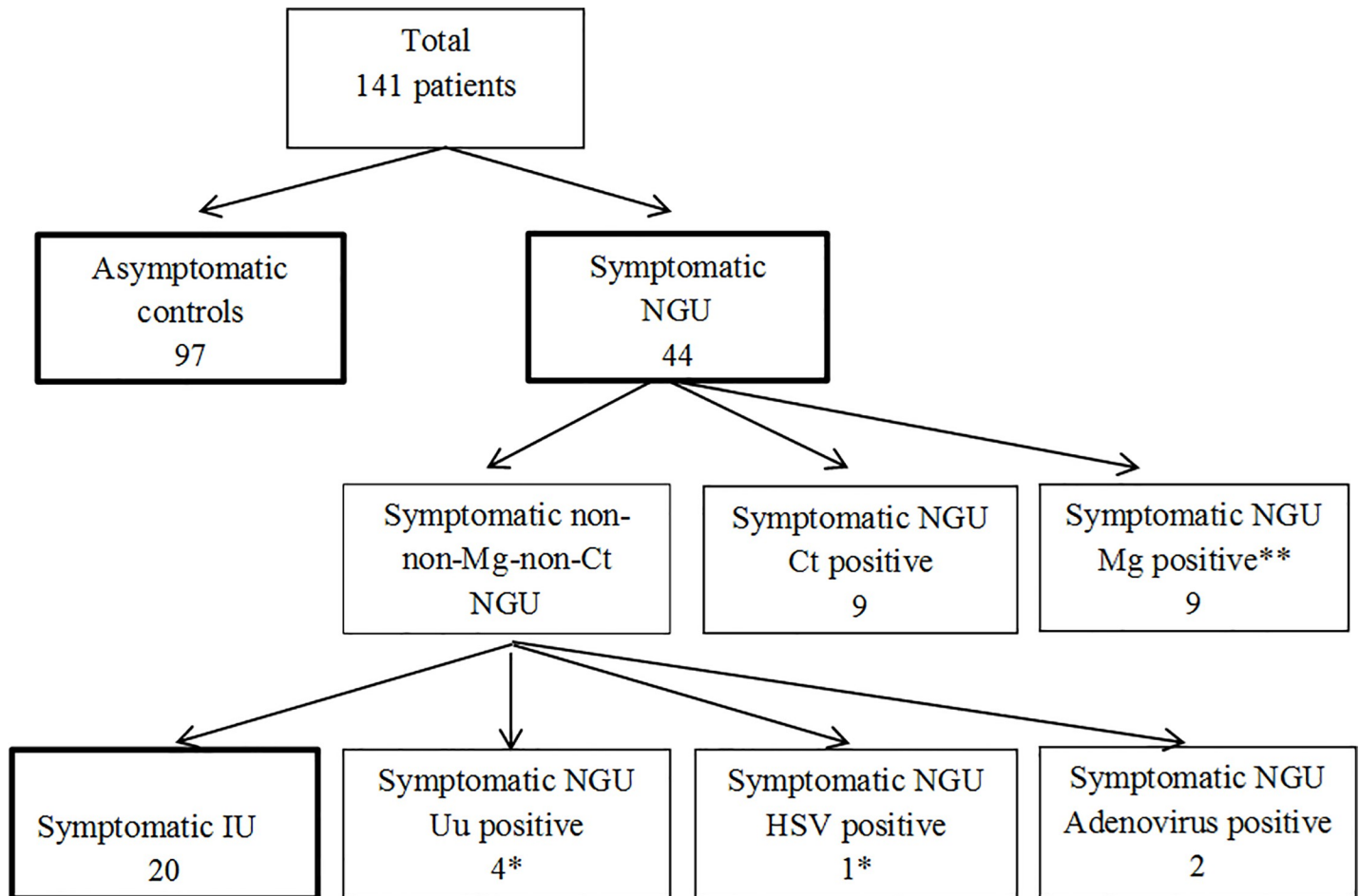


Fig 1. Flowchart showing included male patients (n = 141) and the different subgroups. *C. trachomatis* (Ct), *M. genitalium* (Mg), *U. urealyticum* (Uu), Idiopathic urethritis (IU). * 26 samples were *U. urealyticum* positive. † One sample was double-infected with HSV type 1 and *M. genitalium*. ‡ One sample was double-infected with HSV type 1 and *U. urealyticum*.

<https://doi.org/10.1371/journal.pone.0214425.g001>

non-*C. trachomatis* symptomatic NGU (NMGNCNGU) cases comprised 26 patients (cases) (Fig 1). In this group, four men (15%) were *U. urealyticum* positive. No difference in *U. urealyticum* prevalence (positive rate) between controls and cases ($p = 0.31$) or in bacterial load could be demonstrated ($p = 0.31$). Twenty-five (26%) controls were *U. parvum* positive compared with six men in the NMGNCNGU group (23%) (NS). No difference in bacterial load could be demonstrated for *U. parvum*.

None of the controls were HSV or adenovirus positive. One control was co-infected with *U. urealyticum* and *U. parvum*. Among 26 NMGNCNGU cases, one man (4%) was HSV-type 1 positive (1,250 copies/mL and co-infected with *U. urealyticum*) and two men were adenovirus positive (type D and type F, respectively). Adenovirus was found significantly more frequently among the NMGNCNGU cases ($p = 0.04$).

Association between *U. parvum* and BV associated bacteria in IU compared with controls

U. parvum was analysed together with the BV associated bacteria as it was not considered a cause of NMGNCNGU whereas *U. urealyticum* was excluded from the IU group. None of the

Table 1. Characteristics of patients (n = 141) in the groups “Asymptomatic controls”, “Symptomatic NGU with known aetiology” and “Symptomatic IU”.

Characteristic	Asymptomatic controls (n = 97)	Symptomatic NGU with known aetiology (n = 24)	Symptomatic IU (n = 20)
Age in years	25 (23–30)	25 (20–41)	24 (22–30)
No. sex partners, past year	3 (2–4)*	3 (1–20)	4 (3–6)
No. life-time sex partners	13 (8–23)	17 (7–100)	16 (12–25)^
MSM	2 (2.1%)^	0 (0.0%)	0 (0.0%)
Regular sex partner	55 (56.7%)	11 (45.8%)	11 (55%)
Condom use^^			
Usually	32 (33.0%)	6 (25.0%)	5 (25.0%)
Never or rarely	49 (50.5%)	15 (62.5%)	13 (65.0%)
Always first intercourse	11 (11.3%)	3 (12.5%)	2 (10.0%)
Symptoms			
Dysuria	-	20 (83.3%)	18 (90.0%)
Discharge	-	13 (54.2%)	10 (50.0%)
Urethral itching	-	0 (0.0%)	3 (15.0%)
PMNL/hpf			
<5	97	-	-
5–10	-	4 (16.7%)	6 (30.0%)
10–30	-	20 (83.3%)	13 (65.0%)
>30	-	0 (0.0%)	1 (5.0%)

N (%) for dichotomous data. Median (range) for continuous, non-normally distributed variables (all). None of the comparisons reached a 5% level of significance.

* No information for 3 persons

^ No information for 1 person

^^ Condom use at first sexual intercourse with a new partner (No information for 5 persons in the group of asymptomatic controls (5.2%))

<https://doi.org/10.1371/journal.pone.0214425.t001>

BV associated bacteria or *U. parvum* were found more frequently in the group of symptomatic IU cases (n = 20) than in controls (n = 97) in univariate analysis. The median DNA load for BV associated bacteria and *U. parvum* did not differ between the controls and the patients with IU (Table 2). There was a trend towards *Megasphaera*-like type 1 being present more often and in higher loads among men with IU than in controls (p = 0.09 and p = 0.1, respectively).

The presence of *U. parvum* and BV associated bacteria was analysed in a multivariate model adjusting for all tested pathogens (Table 3). *Megasphaera*-like type 1 was associated with urethritis with adjusted odds ratios (ORadj) 4.6 (95% CI 1.2–18.6) (p = 0.03).

G. vaginalis and *Prevotella* spp. were the BV associated bacteria most commonly found, and they were detected in both patient groups. Seventy-one percent of the controls and 55% of the IU cases were *G. vaginalis* positive, whereas 70% and 80% respectively were *Prevotella* spp. positive (NS) (Table 2).

Relationship between urethral inflammation and ureaplasmas and BV associated bacteria

The bacterial load of ureaplasmas and BV associated bacteria was analysed according to the stratified urethral inflammatory response (<5; 5–10; >10 PMNL/hpf) for all men included in the study regardless of the cause of urethritis. Only 10 men had low-grade urethritis (5–10 PMNL/hpf) but the combined *U. urealyticum* and *U. parvum* load was significantly lower (p = 0.009) in this group than in the asymptomatic controls. The only other significant difference was seen for *G. vaginalis* which was found to have a higher median load in controls than

Table 2. Bacterial vaginosis associated bacteria and *Ureaplasma parvum* in asymptomatic male controls (n = 97) and in men with symptomatic idiopathic urethritis (n = 20). Symptomatic IU patients are negative for *C. trachomatis*, *M. genitalium*, HSV, adenovirus and *U. urealyticum*.

Organism	Asymptomatic controls (n = 97)	Symptomatic IU (n = 20)	Crude OR (95% CI)	P
Bacterial load (DNA copies/mL)*				
<i>U. parvum</i>				
Positive n (%)	25 (26)	6 (30)	1.2 (0.35–3.89)	0.78
Median (range)	0 (0–170,625)	0 (0–10,050)	...	0.75
<i>G. vaginalis</i>				
Positive n (%)	69 (71)	11 (55)	0.50 (0.17–1.52)	0.19
Median (range)	250 (0–3,028,500)	69 (0–314,500)	...	0.1
BVAB2				
Positive n (%)	21 (22)	3 (15)	0.64 (0.11–2.54)	0.76
Median (range)	0 (0–643,000)	0 (0–964,125)	...	0.74
<i>Eggerthella</i>-like bacterium				
Positive n (%)	30 (31)	4 (20)	0.56 (0.13–1.94)	0.42
Median (range)	0 (0–22,339,125)	0 (0–93,250)	...	0.25
<i>Megasphaera</i>-like type 1				
Positive n (%)	13 (13)	6 (30)	2.77 (0.73–9.45)	0.09
Median (range)	0 (0–38)	0 (0–125)	...	0.1
<i>S. amnii</i>				
Positive n (%)	19 (20)	4 (20)	1.03 (0.22–3.71)	>0.99
Median (range)	0 (0–929,125)	0 (0–33,750)	...	0.97
<i>A. vaginae</i>				
Positive n (%)	36 (37)	6 (30)	0.73 (0.21–2.24)	0.62
Median (range)	0 (0–301,375)	0 (0–16,750)	...	0.43
<i>S. sanguinegens</i>				
Positive n (%)	19 (20)	3 (15)	0.72 (0.12–2.91)	0.76
Median (range)	0 (0–6,190,009)	0 (0–155,125)	...	0.72
<i>Prevotella</i> spp.				
Positive n (%)	68 (70)	16 (80)	1.71 (0.49–7.59)	0.43
Median (range)	2,000 (0–6,943,000)	688 (0–182,211,625)	...	0.84

*original urine sample

<https://doi.org/10.1371/journal.pone.0214425.t002>

Table 3. Adjusted odds ratios (OR_{adj}) with 95% confidence intervals (CI) and p-values in asymptomatic male controls (n = 97) and in men with symptomatic idiopathic urethritis (n = 20) after multivariate logistic regression analysis for eight bacterial vaginosis associated bacteria and *Ureaplasma parvum*.

	OR _{adj}	95% CI	p-value
<i>U. parvum</i>	1.7	0.5 to 5.7	p = 0.38
<i>G. vaginalis</i>	0.4	0.1 to 1.3	p = 0.13
BVAB2	0.5	0.1 to 3.1	p = 0.48
<i>Eggerthella</i> -like bacterium	0.9	0.2 to 4.4	p = 0.88
<i>Megasphaera</i> -like type 1	4.6	1.2 to 18.3	p = 0.03
<i>S. amnii</i>	1.3	0.3 to 5.9	p = 0.72
<i>Prevotella</i> spp	2.1	0.66 to 7.8	p = 0.24
<i>A. vaginae</i>	0.7	0.2 to 3.0	p = 0.60
<i>S. sanguinegens</i>	0.7	0.1 to 3.9	p = 0.72

<https://doi.org/10.1371/journal.pone.0214425.t003>

in men with high grade urethritis ($p = 0.03$) suggesting a possible suppression of this species by the inflammatory response.

Co-occurrence between BV associated bacteria and ureaplasma in all samples

Just from looking at the unanalysed data, it was obvious that BV associated bacteria were present mainly in ureaplasma positive samples, and for both ureaplasma species, the bacterial load of all BV associated bacteria except *Megasphaera*-like type 1 were found to correlate with the ureaplasma load (data not shown). As it was speculated that the production of ammonia from urea by the ureaplasmas with the associated increase in pH could be the explanation for the increased bacterial load of BV associated bacteria, the combined ureaplasma DNA load (*U. urealyticum* plus *U. parvum*) was compared with the DNA load of each of the eight BV associated bacteria in all 141 samples (Fig 2). All BV associated bacteria, except *Megasphaera*-like type 1 ($p = 0.63$), were strongly positively correlated with the total ureaplasma load ($p < 0.0001$ – $p < 0.0005$) using Spearman's correlation coefficient. The strongest correlation was found for *G. vaginalis* (Rho = 0.60), the weakest for BVAB2 (Rho = 0.29).

The correlation between BV associated bacteria and ureaplasmas was weaker when the two ureaplasma species, *U. parvum* and *U. urealyticum* were compared separately.

Discussion

In this cross-sectional case-control study, the role of eight BV associated bacteria in men with idiopathic urethritis (IU) was examined. *G. vaginalis*, BVAB-2, *Eggerthella*-like bacteria, *Megasphaera*-like type 1, *S. amnii*, *A. vaginae*, *S. sanguinegens* and *Prevotella* species were found equally often in men with IU and in controls. However, in a multivariate model adjusting for all tested pathogens, *Megasphaera*-like type 1 was associated with urethritis with an ORadj of 4.6, but the bacterial load of this species was very low and thus, the association is most likely not clinically relevant.

BV-associated species have been detected in penile skin, semen and urine specimens by others [10, 19, 30]. Although there is no strong evidence that male partners to women with BV should be prescribed antibiotics to prevent recurrence, a study by Liu *et al.* showed that the BV-like microbiota in the coronal sulcus skin specimens had a close match to the vaginal microbiota of their female partners [31]. The role of anaerobic bacteria as a cause of urethritis in men is still unclear. In this study only *Megasphaera*-like type 1 was associated with IU with an ORadj of 4.6 and there was a trend towards it being detected more often and in higher loads in IU than in controls although this did not reach statistical significance. *Megasphaera*-like type 1 is a recently discovered vaginal bacterium and has not yet been placed in a taxonomic group. It is susceptible to metronidazole and clindamycin in vitro [32]. Further studies are suggested to confirm the importance of this finding. In the present dataset, *Megasphaera*-like type 1 was present in a very low abundance in five of the 20 IU patients, thus, a cut-off such as has been suggested to increase the specificity of the detection of *U. urealyticum* [6] could not be calculated.

Manhart *et al.* found that *S. amnii* (previously *Leptotrichia amnionii*) and/or *S. sanguinegens* was more common in men with NGU than in controls [10], but this finding was not confirmed in the present study although the assay used in the present study did not cross-react with *S. sanguinegens*. Even if the sum of *S. amnii* and *S. sanguinegens* was used in the analysis, the two *Sneathia* species were neither more common nor present at higher loads in IU patients than in asymptomatic controls ($p = 0.82$ and 0.73 , respectively). It is unclear, however, how the assay developed by Fredricks *et al.* [33] differs in the inclusivity of other *Sneathia* species as

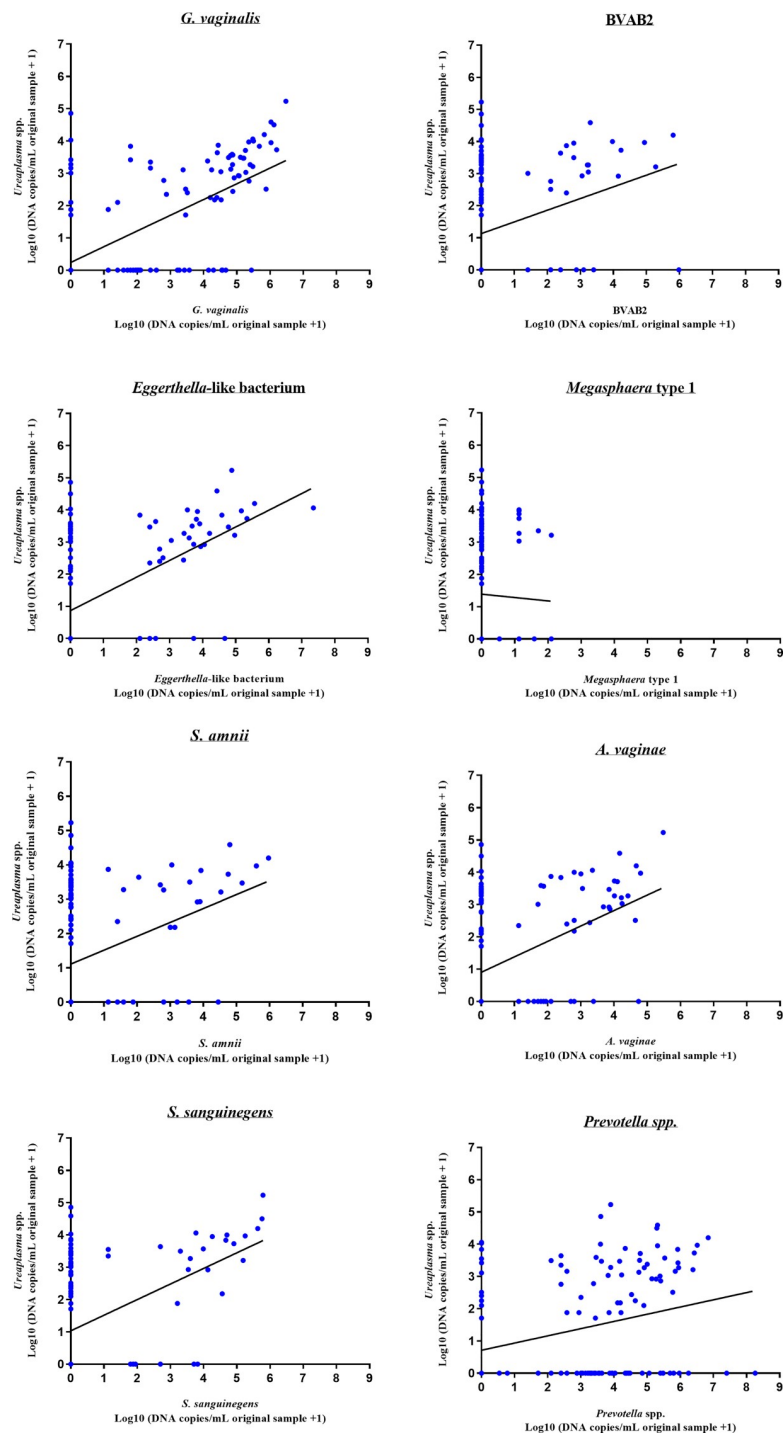


Fig 2. Comparison of the eight BV associated bacteria and total ureaplasma load (*U. urealyticum* and *U. parvum*) in all 141 samples. Regression lines are shown.

<https://doi.org/10.1371/journal.pone.0214425.g002>

compared to the sum of the two species specific assays used in the present study. Similarly, we were not able to replicate our previous finding that *U. urealyticum* is associated with NMGNCNGU [6]. The reasons for these discrepancies are not clear, but our study was limited by the relatively small number of NMGNCNGU cases (n = 24) enrolled. In the present study,

we further eliminated the *U. urealyticum* and virus positive patients to yield a strictly defined group of symptomatic IU, which limited the number of cases to 20. Actually, in order to obtain a power of 0.8 with a p-value of 0.05, an odds-ratio in univariate analysis should be as high as 10 in order to demonstrate a significant association with this number of cases and controls and this is a significant limitation of the study. On the other hand, none of the studied bacterial species, except for *Megasphaera*-like type I and *Prevotella* spp showed OR > 1.5 in univariate analysis whereas several of the studied species showed ORs < 1. Thus, expanding the study may not have changed the conclusions.

To our surprise, all BV associated bacteria, except *Megasphaera*-like type 1, were found to have an increasing bacterial load with increasing total ureaplasma load. This correlation existed even after including a subset of patients with urethritis caused by *C. trachomatis* or *M. genitalium*. The correlation was present for both ureaplasma species when analysed separately, but increased when the bacterial load from *U. parvum* and *U. urealyticum*, was summed. Other studies examining the female vaginal microbiota using sequencing and/or species specific PCRs have not shown this relationship [34]. This could possibly suggest a symbiotic relationship found only in the urethra and possibly just the male urethra. It could be speculated that the presence of ureaplasmas resulted in an elevated pH due to the release of ammonia from urea hydrolysis, the main energy-generating mechanism used in ureaplasmas. Similar to the suggestions that alkalisation of vaginal fluid by menstruation and semen increases the growth of BV associated microbiota [35], the same pH change elicited by ureaplasmas in the urethra where an abundance of urea is available, could play a role. As the correlation between ureaplasma and BV associated bacteria was found unexpectedly a long time after sample collection, pH measurements of the urethral secretions were not possible. Nothing is known about the pH gradient along the urethra in urethritis patients and in healthy men, but with the development of pH micro-sensors it would be possible to study this in relation to the microbiota composition. The role of *U. urealyticum* in male NGU is still not settled, as studies have shown diverging results [5, 6, 36, 37] and the present study did not find any correlation.

The strength of the study is the broad testing for suspected NGU pathogens and the use of well-validated quantitative PCR assays for ureaplasmas and BV associated bacteria. A major limitation is the low number of IU cases examined and possibly the inclusion of asymptomatic controls attending for partner tracing. If BV was present in a large proportion of the female partners due to the strong association between chlamydia and BV, a possible difference may have been masked. In Sweden, contact tracing is mandatory according to the legislation. Usually, contact tracing reaches back one year, and thus, many partners had the contact before the index person acquired her chlamydial infection. One Swedish study reported that 35% of notified partners, where the outcome from testing was known were in fact *C. trachomatis* test negative [38], and all controls in the present study were *C. trachomatis* negative. That the status of the asymptomatic control may have some influence was suggested by a higher load of *G. vaginalis* in the 56 asymptomatic men attending for partner tracing as compared to the 41 men attending for an STI check-up ($p = 0.02$). However, using only the 41 men attending for check-up as controls for the 20 IU cases, did not change the lack of association between BV-associated bacteria and IU. Furthermore, the association between ureaplasma load and the load of BV-associated bacteria was independent of the urethritis status of the study subjects.

The ureaplasmas were not examined to serotype level, as this marker is not stably associated with the genetic relationship [39]

In summary BV associated bacteria were common in both men with and without urethritis and there was a strong correlation between these bacteria and the total ureaplasma load. These correlations are important to control for in studies of idiopathic urethritis. *Megasphaera*-like type 1 was associated with IU, but further studies are needed to confirm this finding.

Supporting information

S1 Datafile. Complete dataset with qPCR bacterial load and metadata.
(XLSX)

Acknowledgments

We are most grateful to the staff at the STD-clinic in Norrköping. Susanne Cramer Johansson is thanked for excellent technical assistance.

Author Contributions

Conceptualization: Maria Frølund, Lars Falk, Peter Ahrens, Jørgen Skov Jensen.

Data curation: Maria Frølund, Lars Falk.

Formal analysis: Maria Frølund, Lars Falk, Jørgen Skov Jensen.

Funding acquisition: Lars Falk, Jørgen Skov Jensen.

Investigation: Maria Frølund, Lars Falk.

Methodology: Maria Frølund, Lars Falk, Peter Ahrens, Jørgen Skov Jensen.

Project administration: Jørgen Skov Jensen.

Resources: Maria Frølund, Lars Falk, Peter Ahrens, Jørgen Skov Jensen.

Supervision: Jørgen Skov Jensen.

Validation: Jørgen Skov Jensen.

Visualization: Maria Frølund, Jørgen Skov Jensen.

Writing – original draft: Maria Frølund, Lars Falk.

Writing – review & editing: Maria Frølund, Lars Falk, Peter Ahrens, Jørgen Skov Jensen.

References

1. Bachmann LH, Manhart LE, Martin DH, Sena AC, Dimitrakoff J, Jensen JS, et al. Advances in the Understanding and Treatment of Male Urethritis. *Clin Infect Dis*. 2015; 61 Suppl 8:S763–S9.
2. The Public Health Agency of Sweden. Gonorrhoea, Annual Report Stockholm: The Public Health Agency of Sweden; 2018 [cited 2018 23-10-2018]. Available from: <https://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistikdatabaser-och-visualisering/sjukdomsstatistik/gonorre/>.
3. Cowan S, Bjerre KD, Hoffmann S. Gonorrhoea 2017. *EPI-NEWS*. 2018; [https://www.ssi.dk/English/News/EPI-NEWS/2018/No%2034-35%20-%202018.aspx\(34-35\)](https://www.ssi.dk/English/News/EPI-NEWS/2018/No%2034-35%20-%202018.aspx(34-35)).
4. Falk L, Fredlund H, Jensen JS. Symptomatic urethritis is more prevalent in men infected with *Mycoplasma genitalium* than with *Chlamydia trachomatis*. *Sex Transm Infect*. 2004; 80(4):289–93. <https://doi.org/10.1136/sti.2003.006817> PMID: 15295128
5. Moi H, Reinton N, Randjelovic I, Reponen EJ, Syvertsen L, Moghaddam A. Urethral inflammatory response to ureaplasma is significantly lower than to *Mycoplasma genitalium* and *Chlamydia trachomatis*. *Int J STD AIDS*. 2017; 28(8):773–80. <https://doi.org/10.1177/0956462416666482> PMID: 27558163
6. Frølund M, Lidbrink P, Wikström A, Cowan S, Ahrens P, Jensen JS. Urethritis-associated pathogens in urine from men with non-gonococcal urethritis: A case-control study. *Acta Derm Venereol*. 2016; 96(5):689–94. <https://doi.org/10.2340/00015555-2314> PMID: 26658669
7. Bradshaw CS, Tabrizi SN, Read TR, Garland SM, Hopkins CA, Moss LM, et al. Etiologies of nongonococcal urethritis: bacteria, viruses, and the association with orogenital exposure. *J Infect Dis*. 2006; 193(3):336–45. <https://doi.org/10.1086/499434> PMID: 16388480
8. Wetmore CM, Manhart LE, Lowens MS, Golden MR, Jensen NL, Astete SG, et al. *Ureaplasma urealyticum* is associated with nongonococcal urethritis among men with fewer lifetime sexual partners: a case-control study. *J Infect Dis*. 2011; 204(8):1274–82. <https://doi.org/10.1093/infdis/jir517> PMID: 21917901

9. Keane FE, Thomas BJ, Gilroy CB, Renton A, Taylor-Robinson D. The association of *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Mycoplasma genitalium* with bacterial vaginosis: observations on heterosexual women and their male partners. *Int J STD AIDS*. 2000; 11(6):356–60. <https://doi.org/10.1258/0956462001916056> PMID: 10872907
10. Manhart LE, Khosropour CM, Liu C, Gillespie CW, Depner K, Fiedler T, et al. Bacterial vaginosis-associated bacteria in men: association of *Leptotrichia/Sneathia* spp. with nongonococcal urethritis. *Sex Transm Dis*. 2013; 40(12):944–9. <https://doi.org/10.1097/OLQ.0000000000000054> PMID: 24220356
11. Thorsen P, Vogel I, Molsted K, Jacobsson B, Arpi M, Moller BR, et al. Risk factors for bacterial vaginosis in pregnancy: a population-based study on Danish women. *Acta Obstet Gynecol Scand*. 2006; 85(8):906–11. <https://doi.org/10.1080/00016340500432655> PMID: 16862466
12. Eriksson K, Adolfsson A, Forsum U, Larsson PG. The prevalence of BV in the population on the Åland Islands during a 15-year period. *APMIS*. 2010; 118(11):903–8. <https://doi.org/10.1111/j.1600-0463.2010.02678.x> PMID: 20955464
13. Beamer MA, Austin MN, Avolia HA, Meyn LA, Bunge KE, Hillier SL. Bacterial species colonizing the vagina of healthy women are not associated with race. *Anaerobe*. 2017; 45:40–3. <https://doi.org/10.1016/j.anaerobe.2017.02.020> PMID: 28238844
14. Peipert JF, Montagna AB, Cooper AS, Sung CJ. Bacterial vaginosis as a risk factor for upper genital tract infection. *Am J Obstet Gynecol*. 1997; 177(5):1184–7. PMID: 9396917
15. Haggerty CL, Hillier SL, Bass DC, Ness RB. Bacterial vaginosis and anaerobic bacteria are associated with endometritis. *Clin Infect Dis*. 2004; 39(7):990–5. <https://doi.org/10.1086/423963> PMID: 15472851
16. Fredricks DN, Fiedler TL, Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. *N Engl J Med*. 2005; 353(18):1899–911. <https://doi.org/10.1056/NEJMoa043802> PMID: 16267321
17. Keane FE, Thomas BJ, Whitaker L, Renton A, Taylor-Robinson D. An association between non-gonococcal urethritis and bacterial vaginosis and the implications for patients and their sexual partners. *Genitourin Med*. 1997; 73(5):373–7. PMID: 9534747
18. Dong Q, Nelson DE, Toh E, Diao L, Gao X, Fortenberry JD, et al. The microbial communities in male first catch urine are highly similar to those in paired urethral swab specimens. *PLoS One*. 2011; 6(5): e19709. <https://doi.org/10.1371/journal.pone.0019709> PMID: 21603636
19. Nelson DE, Dong Q, Van der Pol B, Toh E, Fan B, Katz BP, et al. Bacterial communities of the coronal sulcus and distal urethra of adolescent males. *PLoS One*. 2012; 7(5):e36298. <https://doi.org/10.1371/journal.pone.0036298> PMID: 22606251
20. Frølund M, Wikström A, Lidbrink P, Abu Al-Soud W, Larsen N, Harder CB, et al. The bacterial microbiota in first-void urine from men with and without idiopathic urethritis. *PLoS One*. 2018; 13(7): e0201380. <https://doi.org/10.1371/journal.pone.0201380> PMID: 30052651
21. Shipitsyna E, Roos A, Datcu R, Hallen A, Fredlund H, Jensen JS, et al. Composition of the vaginal microbiota in women of reproductive age—sensitive and specific molecular diagnosis of bacterial vaginosis is possible? *PLoS ONE*. 2013; 8(4):e60670. <https://doi.org/10.1371/journal.pone.0060670> PMID: 23585843
22. Datcu R, Gesink D, Mulvad G, Montgomery-Andersen R, Rink E, Koch A, et al. Vaginal microbiome in women from Greenland assessed by microscopy and quantitative PCR. *BMC Infect Dis*. 2013; 13:480. <https://doi.org/10.1186/1471-2334-13-480> PMID: 24131550
23. Horner P, Donders G, Cusini M, Gomberg M, Jensen JS, Unemo M. Should we be testing for urogenital *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* in men and women?—a position statement from the European STI Guidelines Editorial Board. *J Eur Acad Dermatol Venereol*. 2018; 32(11):1845–51. <https://doi.org/10.1111/jdv.15146> PMID: 29924422
24. Tabrizi SN, Ling AE, Bradshaw CS, Fairley CK, Garland SM. Human adenoviruses types associated with non-gonococcal urethritis. *Sex Health*. 2007; 4(1):41–4. PMID: 17382037
25. Ripa T, Nilsson PA. A *Chlamydia trachomatis* strain with a 377-bp deletion in the cryptic plasmid causing false-negative nucleic acid amplification tests. *Sex Transm Dis*. 2007; 34(5):255–6. <https://doi.org/10.1097/OLQ.0b013e31805ce2b9> PMID: 17483723
26. Jensen JS, Björnelius E, Dohn B, Lidbrink P. Use of TaqMan 5' nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. *J Clin Microbiol*. 2004; 42(2):683–92. <https://doi.org/10.1128/JCM.42.2.683-692.2004> PMID: 14766837
27. Jensen JS, Borre MB, Dohn B. Detection of *Mycoplasma genitalium* by PCR Amplification of the 16S rRNA Gene. *J Clin Microbiol*. 2003; 41(1):261–6. <https://doi.org/10.1128/JCM.41.1.261-266.2003> PMID: 12517858
28. Jensen JS, Bradshaw CS, Tabrizi SN, Fairley CK, Hamasuna R. Azithromycin treatment failure in *Mycoplasma genitalium*-positive patients with nongonococcal urethritis is associated with induced

- macrolide resistance. *Clin Infect Dis*. 2008; 47(12):1546–53. <https://doi.org/10.1086/593188> PMID: 18990060
29. Frølund M, Björnelius E, Lidbrink P, Ahrens P, Jensen JS. Comparison between culture and a multiplex quantitative real-time polymerase chain reaction assay detecting *Ureaplasma urealyticum* and *U. parvum*. *PLoS ONE*. 2014; 9(7):e102743. <https://doi.org/10.1371/journal.pone.0102743> PMID: 25047036
 30. Plummer EL, Vodstrcil LA, Danielewski JA, Murray GL, Fairley CK, Garland SM, et al. Combined oral and topical antimicrobial therapy for male partners of women with bacterial vaginosis: Acceptability, tolerability and impact on the genital microbiota of couples—A pilot study. *PLoS One*. 2018; 13(1): e0190199. <https://doi.org/10.1371/journal.pone.0190199> PMID: 29293559
 31. Liu CM, Hungate BA, Tobian AA, Ravel J, Prodder JL, Serwadda D, et al. Penile Microbiota and Female Partner Bacterial Vaginosis in Rakai, Uganda. *MBio*. 2015; 6(3):e00589. <https://doi.org/10.1128/mBio.00589-15> PMID: 26081632
 32. Petrina M, Cosentino L, Rabe L, Hillier S. Susceptibility of bacterial vaginosis (BV)-associated bacteria to secnidazole, metronidazole, tinidazole and clindamycin. *American Journal of Obstetrics & Gynecology*. 2016; 215(6):S833.
 33. Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, Marrazzo JM. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J Clin Microbiol*. 2007; 45(10):3270–6. <https://doi.org/10.1128/JCM.01272-07> PMID: 17687006
 34. Onderdonk AB, Delaney ML, Fichorova RN. The Human Microbiome during Bacterial Vaginosis. *Clin Microbiol Rev*. 2016; 29(2):223–38. <https://doi.org/10.1128/CMR.00075-15> PMID: 26864580
 35. Norris Turner A, Carr Reese P, Snead MC, Fields K, Ervin M, Kourtis AP, et al. Recent Biomarker-Confirmed Unprotected Vaginal Sex, But Not Self-reported Unprotected Sex, Is Associated With Recurrent Bacterial Vaginosis. *Sex Transm Dis*. 2016; 43(3):172–6. <https://doi.org/10.1097/OLQ.000000000000414> PMID: 26859804
 36. Zhang N, Wang R, Li X, Liu X, Tang Z, Liu Y. Are *Ureaplasma* spp. a cause of nongonococcal urethritis? A systematic review and meta-analysis. *PLoS One*. 2014; 9(12):e113771. <https://doi.org/10.1371/journal.pone.0113771> PMID: 25463970
 37. Shimada Y, Ito S, Mizutani K, Sugawara T, Seike K, Tsuchiya T, et al. Bacterial loads of *Ureaplasma urealyticum* contribute to development of urethritis in men. *Int J STD AIDS*. 2014; 25(4):294–8. <https://doi.org/10.1177/0956462413504556> PMID: 24047884
 38. Carre H, Boman J, Osterlund A, Garden B, Nylander E. Improved contact tracing for *Chlamydia trachomatis* with experienced tracers, tracing for one year back in time and interviewing by phone in remote areas. *Sex Transm Infect*. 2008; 84(3):239–42. <https://doi.org/10.1136/sti.2007.028068> PMID: 18216154
 39. Paralanov V, Lu J, Duffy LB, Crabb DM, Shrivastava S, Methe BA, et al. Comparative genome analysis of 19 *Ureaplasma urealyticum* and *Ureaplasma parvum* strains. *BMC Microbiol*. 2012; 12:88. <https://doi.org/10.1186/1471-2180-12-88> PMID: 22646228