Detection of *Ureaplasma* Species by a Semi-Quantitative PCR Test in Urine Samples: Can It Predict Clinical Significance?

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ABSTRACT: **Background:** *Ureaplasma* species (*Usp*) are the most prevalent genital *Mycoplasma* isolated from the urogenital tract of both men and women. *Usp* may be commensals in the genital tract but may also be contributors to a number of pathological conditions of the genital tract. Because they can also just colonize the genital tract of healthy people, their pathogenic role can be difficult to prove.

Objectives: The aim of the study was to evaluate the efficacy of a quantitative polymerase chain reaction (qPCR) method for the discrimination between infection and colonization by measuring prevalence of *Usp* in asymptomatic versus symptomatic patients.

Methods: Urine samples were tested for *U. parvum* and *U. urealyticum* using a semi-quantitative multiplex PCR technique for sexually transmitted diseases (Anyplex™ STI-7 Detection Kit, Seegene, South Korea). A total of 250 symptomatic and 250 asymptomatic controls were included.

Results: A strong positive result for *U. parvum* was significantly more prevalent in symptomatic compared to asymptomatic patients. This finding was observed especially in women and in the young group (15–35 years of age). No significant differences were observed between the prevalence in symptomatic and asymptomatic patients of *U. parvum* with low strength of positivity and for *U. urealyticum* in all groups by age, gender, and strength of positivity.

Conclusions: The significant difference between the symptomatic and asymptomatic group in the highest positivity group for *U. parvum* using the Anyplex™ STI-7 detection kit in urine may indicate a high probability of infection rather than colonization, especially in women and young patients.

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KEY WORDS: *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Ureaplasma* species (*Usp*), quantitative polymerase chain reaction (qPCR), sexually transmitted infection (STI)

Sexually transmitted infections (STIs) are a broad but relatively well-defined group of infections, usually characterized by an acute presentation that can progress to a chronic clinical condition. Both men and women are affected [1]. Genital *Ureaplasma* species *(Usp)* (i.e., *U. urealyticum* and *U. parvum)* are considered natural inhabitants of the lower urogenital tract of humans as they are often isolated from asymptomatic healthy individuals, with prevalence rates of more than 20% [2,3]. A recently published study by Marovt and colleagues [4] showed no significant association between the detection of *U. parvum* or *U. urealyticum* and symptom status in women. Moreover, some authors emphasized that men with suspected urethritis should undergo evaluation to confirm urethral inflammation and etiologic cause by *U. urealyticum* [5]. *U. urealyticum* is associated with 16–26% of non-gonococcal urethritis (NGU) cases and *U. parvum* has not been associated with NGU [6].

However, *Usp* are involved in a variety of syndromes in women. There are several reports confirming the association of *U. urealyticum* with non-gonococcal urethritis, infertility, postpartum endometriosis, chorioamnionitis, spontaneous abortion, stillbirth, premature birth, and perinatal morbidity and mortality. *Usp* have become increasingly recognized as relevant pathogens during pregnancy and have been associated with chorioamnionitis, spontaneous abortion, and stillbirth. It has been proposed that heavy colonization with these bacteria in the genital tract is associated with premature labor and may lead to adverse perinatal outcomes. *Usp* cause inflammation potentially leading to spontaneous preterm birth and preterm premature rupture of membrane (pPROM) as well as post-delivery infectious complications and neonatal infections [7-11]. Furthermore, Kwak and co-authors [12] found that pregnant women co-infected with *Mycoplasma hominis* and *U. urealyticum* simultaneously had more severe adverse pregnancy outcomes and presented significantly decreased gestational age at birth and birth weight in preterm labor or pPROM. Placental colonization of *U. urealyticum* was also significantly related to chorioamnionitis and neonatal colonization of *U. urealyticum,* and leukocytosis at birth were found to be risk factors for infection-related chronic lung disease of the neonate and even mortality [13,14]. Recently, it has been found that *U. parvum* colonized the amniotic fluid and caused uterine inflammation [15]. Thus, in pregnant women, the pathogenic role of *Usp* cannot be ignored.

Usp also play a potential pathogenic role in genital infections and infertility in men. The pathogenic role of *Usp* in NGU has been reported, especially when high bacterial loads of biovar 2 are present [16-19].

Many authors raised the hypothesis that pathogenicity of *Usp* correlates with high numbers of the bacteria, while low numbers are related to colonization status. A study that quantified *Usp* in urine specimens, in which *U. parvum* and *U. urealyticum* were distinguished from each other, showed that the bacterial loads of *U. urealyticum* were significantly higher in symptomatic men with NGU than in those without urethritis, and as the bacterial loads increased, inflammatory responses developed more intensely [20]. Other studies showed that both *U. parvum* and *U. urealyticum* were present at higher concentrations in non-specific cervicitis patients than in controls, suggesting that *Usp* in the cervix may be associated with this clinical condition [21].

In this controversial scenario, when *Usp* could be a colonizer or a pathogen depending on the species and bacterial load, laboratory tests should offer the most informative results possible to better support the clinician decision on patient management. Since culture could be a problematic and time-consuming method, a quantitative polymerase chain reaction (qPCR) that differentiates between the two *Ureaplasma* species could be a very attractive option to discriminate between infection and commensalism.

Based on the previously mentioned concerns, the main goal of the present study was to evaluate the ability of a commercial qPCR method to differentiate between infection and colonization by measuring prevalence of *Usp* in asymptomatic versus symptomatic patients. In addition, the study attempted to determine whether there is a correlation between the strength of positivity (+, ++, or +++) and the number of DNA copies in the sample, and between strength of positivity and infection status.

PATIENTS AND METHODS

CLINICAL SPECIMENS

A total of 250 urine samples from patients admitted to an STI outpatient clinic because of clinical symptoms of urethritis (at least one of the following: dysuria, urgency, or frequency in the presence of a negative urine culture) between October 2013–June 2014 were randomly included and divided into four groups by gender and age (15–35 and > 35 years of age). Samples found positive for additional pathogens except *Usp* (i.e., *Mycoplasma genitalium*, *Mycoplasma hominis, Chlamidophila trachomatis*, *Neisseria gonorrhoeae*, *and Trichomonas vaginalis*) by Anyplex™ STI-7 Detection Kit (Seegene, South Korea) were excluded from the sample set. In addition, urine specimens were collected from 250 asymptomatic people (gender and age matched), a range of 3 years or less was defined for each age matched pair. The asymptomatic group was defined as people without symptoms of urethritis who had a normal urinalysis test (negative nitrites, leukocytes, and erythrocytes), negative urine culture, and no previous molecular test for STI. The study was approved by the ethics committee of HaEmek Medical Center. Informed consent was obtained from all individual participants included in the study.

PRETREATMENT OF CLINICAL SPECIMENS AND DNA EXTRACTION

Urine samples (approximately 10 ml) were centrifuged at 4000 × g for 25 minutes, supernatant was removed, and the pellet was re-suspended in 1 ml of phosphate-buffered saline. Genomic DNA was extracted from the pretreated specimens using the STARTMag 96 Tissue Extraction Kit (Seegene, South Korea) on the Nimbus platform according to the manufacturer's protocol.

VALIDATION OF SEMI-QUANTITATIVE RESULTS

To validate the semi-quantitative categories of positive results by the Anyplex *STI-7*, a comparison with a second real-time qPCR method was conducted. The goal was to find a positive correlation between strength of positivity of *U. parvum* tested with the Anyplex STI-7 detection kit and bacterial load of *U. parvum* genome equivalents normalized to 100,000 human tested cells tested by AmpliSens® *Ureaplasma* spp.-screen-titre-FRT PCR (AmpliSens, Slovak Republic).

PCR ASSAYS

Genomic DNA extracted from urine samples was tested for *Usp* using a semi-quantitative multiplex PCR for STIs technique (Anyplex STI-7) as compared to a specific qPCR for *Usp* (AmpliSens) according to manufacturer protocols. The Anyplex STI-7 is a multiplex real-time PCR assay that can simultaneously detect and differentiate target nucleic acids of *Chlamydophila trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Ureaplasma parvum*, and *Trichomonas vaginalis*. PCR was performed using a real-time PCR instrument (CFX96 Real-time PCR System, Bio-Rad, Hercules, CA, USA) under the following conditions: (1) primary denaturation at 95°C for 15 minutes, (2) 50 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute and extension at 72°C for 30 seconds, and (3) final cooling down at 55°C for 30 seconds. Results were analyzed using Anyplex II STI-7 Detection (v2.01.002) software (Seegene, Seoul, South Korea), which finally interpreted the results as negative or positive with one of three semi-quantitative measures of strength of positivity.

In the AmpliSens, the concentration of *Usp* DNA can be determined in two variants. In the first variant, the number of genome equivalents of microorganism cells per ml of clinical sample (GE/ml) is determined. These obtained values reflect the absolute concentration of microorganisms in the clinical material. In the second variant, the instrument software automatically calculates the initial number of *Usp* DNA copies as well as the number of human DNA copies in reaction (reflecting the

material sampling quality). The obtained *Usp* genome equivalents are reported as number of copies to 100,000 human cells.

STATISTICAL ANALYSIS

Fisher's exact test was used to calculate the prevalence of *U. parvum* and *U. urealyticum* in symptomatic and asymptomatic patients. Spearman's rank correlation coefficient was determined between the strength of positivity and *Usp* genome equivalents normalized to 100,000 human cells.

The Wilcoxon rank-sum test was used to calculate the prevalence *of U. parvum* in symptomatic and asymptomatic people with different strength of positivity. All statistical analyses were two-tailed and were performed with significance set at $P < 0.05$. Statistical analyses were performed with JMP Version 5.0.1J (SAS Institute Japan, Tokyo, Japan).

RESULTS

DISTRIBUTION OF *U. PARVUM* AND *U. UREALYTICUM* IN THE SYMPTOMATIC AND ASYMPTOMATIC GROUPS

A total of 250 symptomatic patients and 250 asymptomatic controls (gender and age matched) were randomly chosen. The prevalence of *U. parvum* was 30% and 17% ($P < 0.001$) in all symptomatic and asymptomatic groups, and 13% vs. 2% ($P < 0.001$), respectively in the group with the highest strength of positivity (+++ by the Anyplex STI-7, which is equivalent to more than 100,000 copies/ml according to the manufacturer, and more than 500,000 copies/ml as we found by AmpliSens). The prevalence of *U. parvum* in symptomatic and asymptomatic women was also significantly different (50% vs. 28%, $P < 0.0001$), especially in the group with the highest strength of positivity (27% vs. 4%, $P < 0.0001$). In all young individuals (15–35 years of age) prevalence was 36% and 18% ($P < 0.001$) in all symptomatic and asymptomatic samples and 19% and 1% ($P < 0.001$) in the group with highest strength of positivity. No significant differences were found in the prevalence of *U. parvum* in men in all sub-groups as well in older people (> 35 years) and sub-groups with lower strength of positivity (+, ++, data not shown).

There were no significant differences in the prevalence of *U. urealyticum* between symptomatic and asymptomatic patients in all groups and sub-groups by age and gender [Table 1].

CORRELATION IN STRENGTH OF POSITIVITY FOR *U. PARVUM* BY THE TWO ASSAYS

A positive correlation was found between strength of positivity of *U. parvum* tested with the Anyplex STI-7 and bacterial load of *U. parvum* genome equivalents normalized to 100,000 human tested cells ($r = 0.70$, $P < 0.0001$). Figure 1 shows the correlation between values obtained by two qPCR techniques for bacterial loads of *U. parvum* in symptomatic and asymptomatic patients. The ends of the rectangle indicate the upper and lower quartiles, the middle line of the box indicates the median, and the tips of the projecting bars show the locations of the minimum and maximum values within the inner fences. According to these results, a strength of positivity of +++ by the Anyplex STI-7corresponds to more than 750,000 genome equivalents/ml of *U. parvum* by AmpliSens.

PREVALENCE OF *U. PARVUM* IN SYMPTOMATIC AND ASYMPTOMATIC PEOPLE WITH DIFFERENT STRENGTHS OF POSITIVITY

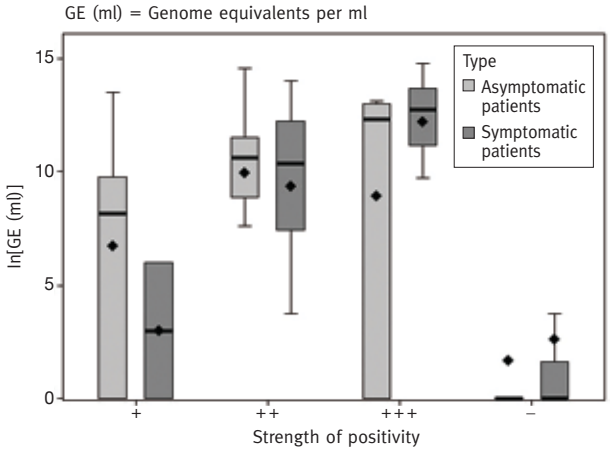
To determine whether there is a cut-off value that differentiates a colonization state from clinical infection (in terms of strength of positivity or number of DNA copies), we looked at the distribution of bacterial loads of *U. parvum* in symptomatic and asymptomatic patients with different strength of positivity. That the

Table 1. Comparative prevalence of *Ureaplasma parvum* and *Ureaplasma urealyticum* by quantitative polymerase chain reaction in symptomatic and asymptomatic patients

	Prevalence of *Ureaplasma* in urine by Anyplex™ STI-7 Detection Kit (Seegene, South Korea)					
	Ureaplasma parvum			*Ureaplasma urealyticum*		
	Symptomatic % (n)	Asymptomatic % (n)	*P*-value	Symptomatic % (n)	Asymptomatic % (n)	*P*-value
Total	30 (75)	17 (44)	$P < 0.001$	10 (25)	10 (25)	N/S
Total +++ [a]	13 (33)	2 (4)	$P < 0.001$			
Men	16 (23)	9 (14)	N/S	8 (12)	10 (13)	N/S
Men +++	3.4 (5)	0 (0)	N/S			
Women	50 (51)	28 (30)	$P = 0.0001$	13 (13)	11 (12)	N/S
Women +++	27 (28)	4 (4)	$P < 0.0001$			
Age 18–35 years	36 (54)	18 (26)	$P < 0.001$	13 (19)	11 (16)	N/S
Age 18–35 years +++	19 (29)	1 (2)	$P < 0.001$			
Age › 35 years	20 (20)	16 (18)	N/S	6 (6)	9 (9)	N/S
Age › 35 years +++	4 (4)	2 (2)	N/S			

[a]with a +++ strength of positivity
N/S = not statistically significant

Figure 1. Correlation of bacterial loads of *Ureaplasma parvum* obtained by two quantitative polymerase chain reaction techniques in symptomatic and asymptomatic patients



The graph shows a positive correlation between the *Ureaplasma parvum* load (expressed in genome equivalents per ml) tested by AmpliSens® *Ureaplasma* spp.-screen-titre-FRT PCR (AmpliSens, Slovak Republic) and strength of positivity (- , +, ++, or +++) by Anyplex™ STI-7 (Detection Kit, Seegene, South Korea)

semi-quantification kit gives a clear indication for quantitative results (there was a positive correlation $P < 0.0001$) [Table 1]. In the strength of +++ all patients with a value of above 490740 GE/ml were from the symptomatic group. A strong positive result (+++) for *U. parvum* using the new qPCR Seeplex® kit for urine samples may indicate a high probability of infection instead of colonization.

DISCUSSION

All genital *Usp* are sexually transmitted, although their role in genital tract disease has long been controversial [22]. In addition, most commercially available PCR techniques only offer qualitative results. As *Usp* are often colonizers rather than pathogens, the reporting of only qualitative positive results could lead to unnecessary antibiotic treatment. In this study, we evaluated the prevalence of *Usp* using a semi-quantitative PCR kit that tests separately *U. urealyticum* and *U. parvum*. In addition, the kit reports positivity in three different categories of strength (+, ++, or +++). A positive correlation between those categories and the number of DNA copies in the sample was observed comparing the kit with a quantitative alternative kit, which reported a number of copies.

In the present study, we observed a high prevalence of *U. parvum* in the symptomatic group compared to the asymptomatic group (30% vs. 17%, $P < 0.001$). A significant difference was observed when prevalence of *U. parvum* in symptomatic and asymptomatic patients with high positivity score (+++) were compared (13% vs. 2%, $P < 0.001$). This trend was also seen in other patient categories. A high prevalence of *U. parvum* in the symptomatic versus asymptomatic women of any age (50% vs. 28%, $P < 0.0001$) was found, especially in those with the highest strength of positivity (+++ -27 vs. 4%, $P < 0.0001$). In young people (15–35 years of age) prevalence for *U. parvum* was 36% and 18% ($P < 0.001$) and 19% and 1% ($P < 0.001$) in symptomatic and asymptomatic for all strength and highest strength of positivity respectively. A significant difference in prevalence rates between symptomatic and asymptomatic was not observed in all other categories.

We are fully aware of the limitations of the present study. For example, we were not able to define *Ureaplasma* subtypes or serovars. However, although some authors postulated than different serovars might present differences in pathogenicity [23], more recent studies contradict this postulate. Xiao et al. [24] suggested that serovar designation is not a reliable subspecies marker for determining the differential pathogenicity of *Ureaplasma*.

As previously mentioned, Marovt and colleagues [4] did not find a significant difference in the detection rate of *Usp* between symptomatic and asymptomatic women, which contradicts our findings. However, a possible explanation may be that in our study we used a semi-quantitative interpretation of the qPCR. Using such approach, we found significant differences between symptomatic and asymptomatic young women with a strong positive result for *U. parvum*.

Since the decision regarding the need of antibiotic treatment is based on laboratory detection of *Usp*, we wondered if a qPCR could be a precise tool to identify infected patients who should be treated.

The treatment decision is especially controversial in patients belonging to age and gender groups with high rates of colonization by these organisms. The findings of our study suggest that there is a positive correlation between *U. parvum* loads in urine and symptomatic urethritis, mainly in young women, which are the patient category with higher risk of complication if not treated.

CONCLUSIONS

Based on our findings and the hypothesis that pathogenicity of *Usp* correlates with high bacterial load, while low bacterial load is generally related to colonization status, we concluded that a strong positive result (++ or +++) for *U. parvum* by the Anyplex STI-7, while negative for all other STI pathogens in a symptomatic young women, suggested infection rather than colonization. Such information is relevant for young women because of the risk developing infertility, and it is especially critical for pregnant women, who are included in this group, because of the risk of negative newborn outcomes. A future prospective study using a more precise quantitative PCR for *Ureaplasma*, with a larger group of patients could be helpful in finding a more accurate cutoff value able to distinguish between infection and colonization status mainly in patients with mild clinical symptoms.

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Capsule

Earliest phase of systemic sclerosis typified by increased levels of inflammatory proteins in the serum

Patients with definite systemic sclerosis (SSc) who lack fibrotic features can be stratified into an intermediate stage of disease severity between preclinical/early SSc (EaSSc) and fibrotic subsets (limited cutaneous SSc [lcSSc] and diffuse cutaneous SSc [dcSSc]). **Cossu** and colleagues aimed to molecularly characterize nonfibrotic SSc and EaSSc on the basis of a broad panel of serum markers of inflammation and tissue damage to increase the knowledge of the pathophysiologic mechanisms underlying SSc progression before the development of fibrosis. Based on the results of the explorative analysis, 16 molecules were selected for testing in the replication set. The results showed that CXCL10, CXCL11, tumor necrosis factor receptor type II (TNFRII), and chitinase 3–like protein 1 levels were significantly increased in patients with EaSSc and those with nonfibrotic SSc, compared to healthy controls. The disease in patients with high concentrations of CXCL10 and TNFRII was also characterized by a faster rate of progression from EaSSc and from nonfibrotic SSc to worse disease stages. The authors concluded that SSc patients with preclinical/early SSc and those with established, yet nonfibrotic, disease exhibit clear molecular alterations that are associated with faster rates of disease evolution. These data open novel avenues for disease interception in SSc.

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