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Original Article

Ureaplasma spp. in male infertility and its relationship with semen quality and seminal plasma components

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Abstract *Objective:* We investigated the prevalence of *Ureaplasma* spp. in semen samples of infertile men in Shanghai, China and evaluated the correlation between the sperm parameters (seminal volume, sperm concentration, progressive motility and non-progressive) and the secretary function in these infectious populations.
Methods: Semens were collected from 540 infertile men and 260 fertile control group in shanghai, China and subjected to standard bacterial and *Ureaplasma* spp. culture. Positive *Ureaplasma* spp. isolates were further tested by PCR to detect the biovars and serotypes of *Ureaplasma* spp. Sperm seminological variabilities were analyzed by Computer-Assisted Semen Analysis according to the fifth edition of World Health Organization (WHO) laboratory manual for the examination and processing of human semen. Seminal markers were measured by the automatic analyzer.
Results: The prevalence of *Ureaplasma* spp. in semen specimens was 39.6% (214/540) and 19.2% (50/260) in infertile and control group, respectively. Significant difference was observed between the two groups ($P < 0.001$). Among all clinical isolates from infertile men (n = 214), 59.3% (n = 127) was *Ureaplasma parvum* (UPA), 26.2% (n = 56) was *Ureaplasma urealyticum* (UUR), and 14.5% (n = 31) was mixed species. While those numbers in control group (n = 50) were 64.0% (n = 32), 20.0% (n = 10), 16.0% (n = 8), respectively. There was no significant difference between any two groups ($P > 0.05$). The progressive motility and the NAG activity of infertile men infected with UPA and mixed species were significantly lower than those of UUR infected subgroup ($P < 0.05$).

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Conclusions: The infection of *Ureaplasma* spp. plays an important pathogenic role in male infertility. UPA has higher pathogenicity on the progressive motility and the secretary function of epididymis than UUR.

Introduction

Ureaplasma spp. has been identified as an etiology of male infertility since 1967.[1] Friberg and Gnarpe[2] demonstrated a higher frequency of *Ureaplasma* spp. isolated from the semen of men with unexplained infertility (76%) compared with fertile men (19%). Since then the relationship between *Ureaplasma* spp. infection and male infertility has been studied widely. The frequency of *Ureaplasma* spp. isolated from the semen of infertile male patients in several studies varied from 5% to 58%, while the numbers from fertile men was 3%—31%.[3,4] Fowlkes[5] described the restrictive quantitative and qualitative semen parameters (volume, number of sperm, motility and morphology) in patients with *Ureaplasma* spp. infection. Spermatogenesis, sperm function and sperm transportation should been affected by such infection.[6]

The mechanisms of male infertility caused by *Ureaplasma* spp. infection are still not clear. Reports were controversial about the effects of *Ureaplasma* spp. on sperm seminological variables. Some studies did not find any correlation between the infection and semen alterations[7,8]; while others reported that the presence of *Ureaplasma* spp. in semen was related to the decrease in sperm concentration[9—11] and motility[12] and altered morphology.[13—15] There was also a dual effect of *Ureaplasma* spp. infection on the sperm activity — inhibition of sperm motility at low pHs and increase of sperm velocity at higher pHs.[16] *Ureaplasma* spp. infection caused sperm membrane changes could induce the production of antisperm antibodies, which were also associated with higher risk of infertility.[17]

To date, *Ureaplasma* spp. consists of 14 serovars which could be divided into 2 species, *Ureaplasma parvum* (UPA) and *Ureaplasma urealyticum* (UUR), based on differential growth responses to manganese, 16S rRNA gene sequences, the 16S—23S rRNA intergenic region, urease gene, and differences in the multiple-banded antigen (MBA) genes. UPA comprises four serovars (UPA1, UPA3, UPA6, and UPA14), while UUR includes the remaining ten serovars.[18,19] Most of the previous reported studies have discussed the role of *Ureaplasma* spp. in male infertility without discriminating the two species. The two species may have differential pathogenicity. However, the data are limited and controversial.[20—22]

We aimed to investigate the prevalence of the two *Ureaplasma* spp. in semen collected from infertile men and fertile men from a large population in Shanghai, China. The semen quality in different infectious populations was also studied.

Materials and methods

Subjects

A total of 540 infertile men attending the fertility clinic from January 2010 to December 2011 were included in this study (aged 21—45 years). Infertility was defined as a failure to conceive after at least 12 months of unprotected intercourse. The selection criteria for enrollment were (1) infertility without female factor subfertility because of fallopian tube pathology, menstrual cycle abnormalities or endometriosis, (2) male without reproductive system abnormalities (varicocele, hydrocele, undescended testis, or inguinal hernia) and without hormonal abnormalities. The patients did not take any antibiotic therapy in the last two weeks. The control group included 260 fertile men who were attending the clinics during the same study period (aged 22—42 years). They had normal semen parameter and/or whose wives have had non-assisted pregnancies in the past. Ethics committee approval for study protocol and written informed patient consent were taken for this study.

Clinical specimens

Prior to semen analysis, the men were asked to abstain by masturbation for 2—7 days of sexual abstinence, with urine voided before masturbation. Men were given detailed instructions regarding semen collection. Water intake was increased the day before collection to ensure a "cleaning" of the urethra. The hands were first washed carefully with liquid soap and dried with a paper towel, and the skin and the free glans penis were cleaned with chlorhexidine solution. Sperm samples were collected into sterile plastic containers provided by fertility clinic that had previously been shown not to have any cytotoxic effects on human spermatozoa according to the methods outlined by the fifth edition of World Health Organization (WHO) laboratory manual for the examination and processing of human semen.

Materials and reagents

Broth culture medium kits were purchased from Antobio Bio-Company (Zhengzhou, China). The agar culture medium was provided by Zhongaisheng Hebei Bioscience Technology INC (Xingtai, China). Reference strains were provided as gift from Autobio Diagnostics co., Ltd, Zhengzhou, China. Strains ATCC 27813, ATCC 27618 and *Mycoplasma hominis* (Mh) ATCC 15488 were taken as quality control. Semen

quality control and SQA-V Full Automatic Sperm Quality Analyzer was provided by Israel Medical electronic technology co., LTD.

Ureaplasma spp. isolation procedure

The standard *Ureaplasma* spp. culture procedures were performed as described.[23] Briefly, semen were placed in an incubator immediately after collection, and liquefied at 37 °C for up to 30 min before inoculation. The liquefied semen was inoculated in the broth medium and on the selective agar medium and incubated at 37 °C in an atmosphere of 5% CO_2 parallelly. The concentration of *Ureaplasma* spp. isolated from each semen sample more than 10^3 CFU/ml or 10^4 CCU/ml was defined as infection. The bacteria and *M. hominis* culture were also performed on these specimens. The results of bacterial cultivation and *Chlamydia trachomatis* screen of semen sample were negative. PCR assay was used to identify the *Ureaplasma* spp. clinical strains.

Sperm seminological variables

Semen analysis was carried out according to the guidelines of the fifth edition of World Health Organization (WHO) laboratory manual for the examination and processing of human semen. The following characteristics were determined: seminal volume, sperm concentration, progressive motility (PR), non-progressive (NP), total motility (PR + NP). Computer-Assisted Semen Analysis (SQA-V) was used for the semen analysis.

Seminal plasma biochemical test

The remaining semen after the above assays was centrifuged at 1000 g for 10 min. Seminal plasma without sperm was collected and stored at 80 °C for analysis. Elastase, neutral alpha-1, 4-glucosidase (NAG), zinc, and fructose were measured by the automatic ChemWell BRED analyzer.

Ureaplasma spp. species- and UPA serovar-specific PCR

The primers used for identification of UPA and UUR and UPA serovars were showed in Table 1.[24,25] Detection of *Ureaplasma* spp. *biovars* was done using 16S-rRNA gene primers UMS 57-UMA 222 for the detection of UPA (327 bp), as well as UMS 170—UMA 263 for the detection of UUR (476 bp), respectively. Serovars of UPA could be identified with new primer pairs as follows: UMS-83—UMA 269 amplified only serovar 1; UMS-125—UMA 269 amplified only serovar 3 or 14; and UMS-54—UMA 269 amplified only serovar 6. There is only 3 bps of the difference between serovar 3 and 14, so both serovars are indistinguishable by the methods used.

The PCR assay was performed in 25 μL of reaction mixture containing 1 μL from each extracted DNA, 2.5 μL buffer, 1 μL 1 U/μLTaq, 1 μLdNTP (2.5 mM), 2 μL of each primer (forward and reverse) (A + S 10 pmol/μL), 12.5 μL of master mix, and 3 μL of nuclease-free water. PCR was performed using the ProFlex™ PCR System. The PCR cycling conditions used to amplify the *Ureaplasma* spp. gene was as the following: an initial cycle at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 60 s, with a final cycle at 72 °C for 10 min. In each reaction, positive control (27813, serovar 1 as UPA, 27618 serovar 14 as UUR) and negative controls were processed in parallel with the tested samples to detect false-negative results or contamination.

Statistical analysis

Data analysis was performed using SPSS, version 21. Chi-square test was used for comparison of qualitative variables. Continuous data are presented as mean ± SD. Means were calculated in the three subgroups (UPA infected, UUR infected and the mixed biovars infected of infertile men) for semen characteristics: sample volume, sperm concentration, PR, PR + NP. The data were not normally distributed and nonparametric techniques (Wilcoxon rank-sum test) were used to test the significance of the differences among the three subgroups. $P < 0.05$ was considered statistically significant.

Results

Isolation and prevalence of *Ureaplasma* spp

Every *Ureaplasma* spp. strain isolated from clinical sample was confirmed by PCR test. Two hundred and fourteen *Ureaplasma* spp. strains were obtained from 540 infertile

Table 1 Primers of UPA and UUR identification and subtyping.

Target	Primer	Sequence (5′—3′)	Length (bp)
UPA	UMS-57	(T/C) AAATCTTAGTGTTCATATTTTTTAC	327
	UMA-222	GTAAGTGCAGCATTA AATTCAATG	
UUR	UMS-170	GTATTTGCAATCTTTATATGTTTTCG	476
	UMA-263	TTTGTTGTTGCGTTTTCTG	
UPA 1	UMS-83	TACTGTAGAA ATTATGTAAGATTGC	398
	UMA-269	CCA AATGACCTTTTGTAACTAGAT	
UPA 3/14	UMS-125	GTATTTGCAATTTTATATGTTTTCG	369
	UMA-269	CTAAATGACCTTTTTCAAGTGTAC	
UPA 6	UMS-54	CTTAGTGTTCATATTTTTTACTAG	442
	UMA-269	CCAAATGACCTTTTGTAACTAGAT	

Table 2 Distribution of *Ureaplasma* spp. species isolated from 540 infertility and 260 fertile men.

Ureaplasma spp.	From infertile men	From fertile men	P value
UPA	127 (59.3%)	32 (64.0%)	0.428
UUR	56 (26.2%)	10 (20.0%)	0.468
Mixed	31 (14.5%)	8 (16.0%)	0.825
Total isolates	214 (39.6%)	50 (19.2%)	0.001

patients (39.6%) while 50 from 260 fertile men (19.2%) (Table 2). There was a significant difference in the overall prevalence of *Ureaplasma* spp. in infertile versus fertile men ($P < 0.001$). However, there was no significant difference in the isolated rate between the two *Ureaplasma* spp. species.

UPA isolates were further subtyped into different serovars (Table 3). Among the UPA strains detected in the 127 infertile patients, the prevalence of serovars1, 3/14 and 6 were found to be 12 (9.4%), 60 (47.2%) and 49 (38.6%), respectively. In addition, there were 4 (3%) cases contained two or more subtypes. Among the 32 strains from the fertile control, the prevalence of serovars1, 3/14 and 6 were found to be 3 (9.3%), 13 (40.6%), and 15 (46.9%), respectively. There's only 1 case (3%) contained two subtypes. Although serovars 3/14 and 6 were more common than serovar 1 and the mixed serovars, the difference between the two groups was not significant.

Of the 127 infertile man, 30 (14.1%) were aged 21—25 years, 86 (40.2%) were aged 26—30 years, 62 (29.0%) were aged 31—35 years, and 36 (16.8%) were over 36 years old (Table 4). Approximately 40% of all of the *Ureaplasma* spp. were isolated from patients aged 26—30. No correlation between the age and the *Ureaplasma* spp. was found.

Table 3 Subtyping of UPA results of the infertile and the fertile groups by PCR.

Group	Serovar 1	Serovars 3/14	Serovar 6	Mixed	Total number
Infertile	12 (9.4%)	60 (47.2%)	49 (38.6%)	4 (3.1%)	127
Fertile	3 (9.3%)	13 (40.6%)	15 (46.9%)	1 (3.1%)	32
P value	0.990	0.555	0.424	0.994	

Table 4 The distribution of *Ureaplasma* spp. in different age groups of 214 infertile men.

Age group (range, years)	UPA	UUR	The mixed	Total positive rate (%)
21—25	17 (13.4%)	10 (17.8%)	3 (9.7%)	30 (14.1%)
26—30	48 (37.8%)	25 (44.6%)	13 (41.9%)	86 (40.2%)
31—35	41 (32.3%)	9 (16.1%)	12 (38.7%)	62 (29.0%)
>35	21 (16.5%)	12 (21.4%)	3 (9.7%)	36 (16.8%)
P value	0.428	0.468	0.825	

Correlation between *Ureaplasma* spp. infection and the sperm seminological variables as well as the secretary function of accessory sex gland

Because of severe oligozoospermia or azoospermia, 72 patients were excluded from these seminological variables test. Semen quality and secretary function of accessory sex glands of the infertile and fertile men were showed in Table 5. The activity of NAG and the level of fructose were calculated according to the volumes of the semen. The progressive motility of the semen from infertile men infected with UPA or the mixed species were significantly lower than that infected with UUR ($P < 0.05$). The NAG activity of UPA and the mixed-species infected group was significantly lower than that of the UUR infected group ($P < 0.05$). There were no significant differences among the three infected groups regarding other semen characteristics, such as volume, sperm concentration, PR + NR, elastase, fructose and Zn levels ($P > 0.05$).

Discussion

Our study demonstrates that infertile men have significantly higher prevalence of *Ureaplasma* spp. (39.6%) compared with fertile men (19.2%); UPA is more common in infertile men than UUR. This finding is in agreement with the previously reported results.[22,26] Some studies showed that UUR may be more potentially pathogenic in playing an etiologic role in both genital infections and male infertility.[14,27—29] The controversy could be due to the different ethnic and social populations, varying sample sizes and examination methods. In this study, we found no significant difference about the prevalence of UPA, UUR and the mixed species as well as each UPA serovar between the two groups, suggesting that there is no relationship between the infertility and the distribution of species or serovar genotypes.

To investigate the different impacts of *Ureaplasma* spp. on the semen quality, we compared the sperm seminological variables and the seminal plasma biochemical characters of the infertile men infected with different *Ureaplasma* spp. species. Among the UPA-, UUR- and the mixed-species-infected groups, the sperm seminological variables had no significant differences in the mean values of seminal volume, sperm concentration and PR + NR. There was a decline of PR in UPA infected and the mixed infected group compared with UUR infected. Seminal plasma elastase has been proven a reliable marker of silent male genital tract inflammation. The concentration of fructose was the marker of seminal vesicle function.[30] Zn and NAG was considered as functional markers of prostate and epididymis.[31,32] There were no significant differences in the levels of seminal plasma elastase and the amount of fructose and Zn between the three infected groups. The NAG activity of UPA and the mixed species infected group was significantly lower than that of the UUR infected group, which was in accordance with the lower PR of UPA infected infertility. Epididymal secretions play a crucial role for the acquisition of PR of spermatozoa.[33] PR is one of the important contributing factors for reaching the fertilization goal in the natural cycles. Our study suggests that the two

Table 5 Seminological variables of the infertile men.

	UUR	UPA		Mixed	
	(n = 38)	(n = 77)	*P1*	(n = 27)	*P2*
Semen characteristics					
Volume (ml)	3.5 ± 1.1	3.5 ± 1.5	0.423	3.6 ± 0.8	0.811
Sperm concentration (10^6/ml)	55.1 ± 33.3	63.7 ± 35.4	0.176	66.6 ± 33.1	0.082
PR + NR (%)	51.4 ± 21.3	45.1 ± 16.8	0.068	46.7 ± 19.8	0.252
PR (%)	39.4 ± 19.5 △	33.7 ± 14.9[a]	0.000	35.9 ± 16.9	0.003
Seminal plasma characteristics					
Elastase (ng/ml)	1114.9 ± 1420.4	1002.1 ± 1254.6	0.741	1143.8 ± 1382.7	0.837
Neutral a Glucosidase (mU)	97.0 ± 37.4 △	76.3 ± 35.1[a]	0.003	76.1 ± 33.9	0.024
Fructose (μmol)	49.1 ± 36.2	38.5 ± 25.8	0.089	46.7 ± 26.1	0.926
Zn (μmol)	8.9 ± 4.5	10.1 ± 4.8	0.204	10.8 ± 5.8	0.206

Note: Data are means ± Standard Error; a and △, there was significant difference, UPA compared to UUR: a $P1 < 0.05$; UUR compared to the mixed biovars: △ $P2 < 0.05$.

Ureaplasma spp. species have no obvious different impact on semen quality, except that UPA infection is correlated with the decrease of PR and the epididymal secretions.

There are some limitations in our study. We didn't determine the bacterial loads of the infected specimens, either by quantitative culture or real-time PCR. The bacterial loads have been shown to be related to the outcome of the diseases.[34]

Conclusion

The study demonstrates that infertile men have higher prevalence of *Ureaplasma* spp. compared with fertile men, *Ureaplasma* spp. infection is correlated with the infertility. UPA is more prevalent than UUR in semens from both infertile and fertile men. Serovars 3/14 and 6 were most commonly detected UPA serovars in these isolates. No correlation between the infertility and the distribution of *Ureaplasma* spp., or serovars/genotypes. UPA has higher pathogenicity affecting the progressive motility and secretary function of epididymis of the semen compared with that of UUR.

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