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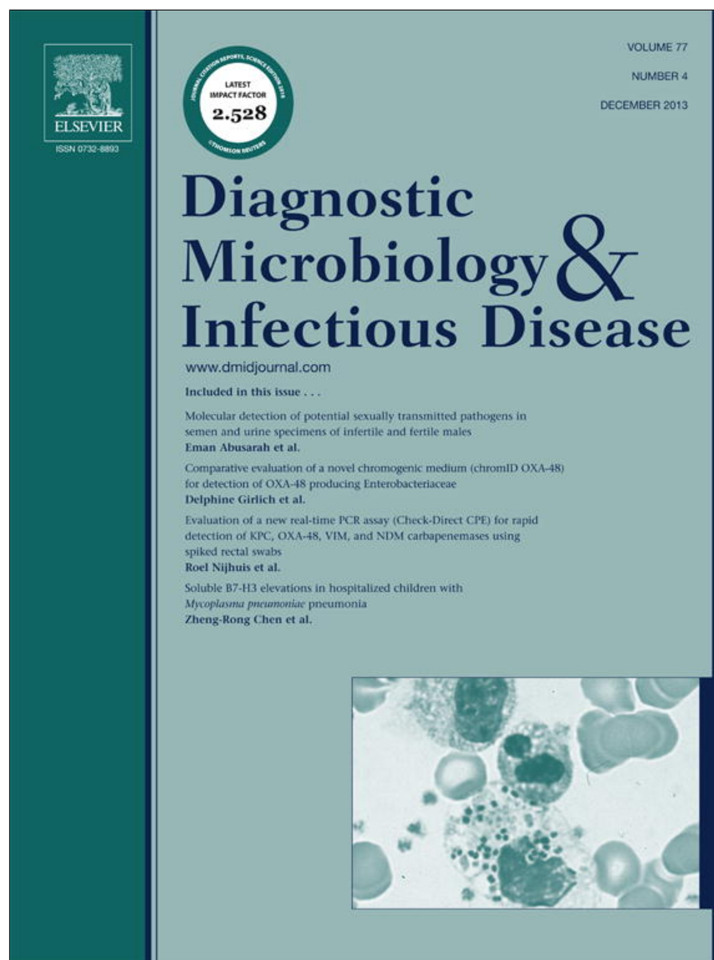

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Bacteriology

Molecular detection of potential sexually transmitted pathogens in semen and urine specimens of infertile and fertile males

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ABSTRACT

A total of 93 infertile and 70 fertile men attending various urology and gynecology clinics in Jordan were investigated in this prospective study. First void urine and the corresponding semen specimens were collected from 96% of the patients. Presence of *Neisseria gonorrhoeae* (NG), *Chlamydia trachomatis* (CT), *Ureaplasma urealyticum* (UU), and *Mycoplasma genitalium* (MG) DNA in specimen was detected using polymerase chain reaction. The distribution of NG, CT, UU, and MG in semen and FVU specimens among infertile versus fertile men was 6.5% versus 0%, 4.3% versus 1.4%, 10.8% versus 5.7%, and 3.2% versus 1.4%, respectively. Two of infertile and 1 of fertile men harbored mixed pathogens. The highest number of positive potential pathogens was found among young men aged 20–29 years old. The present study found a very high concordance between the detection of CT, UU, and MG DNA in semen and the corresponding FVU specimens, while NG DNA found only in semen and not in the corresponding FVU specimens. This study also revealed that *Ureaplasma parvum* species is more prevalent than *Ureaplasma urealyticum* in specimens of infertile men (90%). The study demonstrates that infertile men have higher prevalence of NG, CT, UU, and MG compared with fertile men and NG as significantly associated with infertile men.

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1. Introduction

There is still controversy on the role of sexually transmitted infections (STIs) as a significant cause of male infertility (Çalışkan et al., 2010; Ochsendorf, 2008; Pellati et al., 2008; Waugh, 2003) despite the fact that certain genital bacterial infections affecting not only sperm cell function but also the whole spermatogenesis (Peerayeh et al., 2008). *Neisseria gonorrhoeae* (NG), *Chlamydia trachomatis* (CT), *Ureaplasma urealyticum* (UU), and *Mycoplasma genitalium* (MG) are considered among the most prevalent sexually transmitted pathogens and have a worldwide (Gdoura et al., 2008a; Krause, 2008). In particular, NG is associated with several serious urogenital complications in men (Krause, 2008; Ng and Martin, 2005). While genital tract infections, caused by CT, are the most frequent cause of nongonococcal urethritis (NGU) and epididymitis in men, approximately 50–70% of chlamydial infections in men and women are asymptomatic and remain undiagnosed and untreated (Bebear and de Barbeyrac, 2009). Several studies have suggested that CT has pathological effects on female and male reproductive tract; however, no direct link has been demonstrated to infertility (Carey and Beagley, 2010; Gdoura et al., 2008a).

UU and *Ureaplasma parvum* are commensal organisms of the lower genitourinary tract of sexually active men and women, and ureaplasmal infection is strongly associated with urethritis, prostatitis, epididymitis, endometritis, chorioamnionitis, spontaneous abortion, and prematurity/low birth weight, but the exact role of these agents in the male infertility remains a controversial subject (Zeighami et al., 2009).

There is increasing evidence that MG could be associated with NGU in men (Al-Sweih et al., 2012; Shehabi et al., 2009), but there are not enough studies supporting the contention that this bacterium can cause epididymitis and prostatitis and male infertility (Taylor, 2005). Polymerase chain reaction (PCR) and serological studies of women have associated MG with pelvic inflammatory disease, cervicitis, endometritis, and infertility (Taylor, 2005).

Diagnosis of STIs is currently best made by using Nucleic Acid Amplification Tests because such tests show a good specificity and high sensitivity and do not require invasive procedures for specimen collection and culture (McKechnie et al., 2009).

This study aims to compare the prevalence of NG, CT, UU, and MG in semen and urine specimens of infertile men versus fertile men using molecular assay.

2. Patients and methods

2.1. Patients

A total of 93 infertile men attending in 5 urology and gynecology clinics in Amman and Zarka regions of Jordan (approximately represent 45% of the Jordanian population), over the period from

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May 2011 to October 2011, were included in this study. Infertile men were defined in this study as those who had a history of infertility and abnormal semen parameter and had no clinical signs of genitourinary tract infections. The control group included 70 fertile men who were attending the clinics during the same study period for urology checkup and were clinically asymptomatic for any infection. Fertile men were defined as men who had normal semen parameter and/or whose wives have had non-assisted pregnancies in the past. All subjects were assessed by history of infertility and semen analysis and were of sexually active age. Semen analysis was performed using the criteria outlined by Gdoura et al. (2008b). Men who showed any symptoms of genitourinary infections or had history of antibiotic use in the previous 2 weeks were excluded from the study. An informed consent was obtained from all patients and controls enrolled in the study. The study has been approved by the high graduate committees of the Faculty of Medicine, the ethics committee of Jordan Hospital University, and Faculty of Graduate Studies/University of Jordan, Amman, Jordan.

2.2. Collection of urine and semen specimens

First void urine (FVU) and the corresponding semen specimens were collected from most of the patients and controls. Out of the 93 infertile patients, 12 failed to give the FVU specimens, and of the 70 controls group, 9 failed to submit FVU specimens. Semen specimens were obtained by masturbation and collected into sterile plastic containers. All FVU and semen specimens were stored and transported at 2–8 °C within 24 h to the microbiology research labs, Faculty of Medicine, University of Jordan, for DNA extraction.

2.3. Detection of NG, CT, UU, and MG DNA by PCR

For each male patient, 500 μL of semen specimens and 3–4 mL of corresponding FVU samples were used for DNA detection of NG, CT, UU, and MG using PCR according to the following investigational protocols: The bacterial DNA was extracted from specimens using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol for isolation of bacterial DNA from biological fluids. The extracted DNA was first tested for human β-globin gene to check for potential PCR inhibitors in the samples using primer BgloF (5′- CAACTTCATCCACGTTCACC-3′) and Primer BgloR (5′- GAAGAGCCAAGGACAGGTAC-3′), which were capable of amplifying a 268-bp fragment in the exon 1 of the human β-globin.(Krajinovic et al., 1999). All β-globin–positive samples were examined for the presence of UU, MG, and CT as described by Lee et al., 2007, whereas NG was detected according the method of Mahony et al. (1997). Table 1 shows the specific primers used in detection these agents. Detection of CT, UU, and MG was done by multiplex PCR. The PCR assay was performed in 25 μL of reaction mixture containing 5 μL from each extracted DNA, 1.5 μL $MgCl_2$, 0.5 μL of each primer—forward and reverse—(20 pmol/μL), 12.5 μL of master mix (Kapa Biosystems, Woburn, MN, USA), and 3 μL of nuclease-free water. Detection of UU biovars was done using primers UMS 57 (5′-TAAATCT TAGTGTTCATAT TT TTTAC-3′) and UMA 222 (5′-GTAAGTGCAGCATTAAATTCAATG-3′) for the detection of *U. parvum* (326 bp), and UMS 170 (5′-GTATTTGCAATCTTTA-TATGTTTTCG-3′) and UMA 263 (5′-TTTGTTGTTGCGTTTTCTG-3′) for the detection of UU (476 bp), respectively, whose amplification target is the multiple-banded antigen gene of UU (De Francesco et al., 2009). The PCR reaction for both *U. parvum* and UU was performed in 2 separate tubes as described by De Francesco et al., 2009. Positive and negative controls were included in all PCR runs. The positive controls used in this study were the extracted DNA of CT, UU, MG, and NG provided by Dr Ekatherina Charvalos, Central Labs IASO Hospital (Kifissias 37–39, Athens Greece 151 23), whereas the negative control consisted of all the components of the PCR reaction mixture except for the purified DNA, to exclude any contamination of the PCR components or the master mix itself. After the PCR was performed, 7 μL of the amplified material was analyzed by electrophoresis in 1.5% agarose gel (Promega, Madison, WI, USA) in 1X Tris-Borate-EDTA buffer containing ethidium bromide, and was visualized by UV illumination (UVP, Upland, CA, USA). An amplicon yield of 390-bp DNA band detected by uniplex PCR is considered positive for NG. There are 3 patterns of DNA bands produced by multiplex PCR representing the following: 200 bp for CT, 346 bp for MG, and 423 bp for UU. The amplicon sizes of 326 bp or 476 bp detected by the uniplex PCR system are considered positive for *U. parvum* and UU, respectively.

Table 1
Primers sequence used for the detection of NG, CT, UU, and MG.

Pathogenic bacteria	Primers	Oligonucleotide sequence5'-3'	Target gene	Product size (bp)
CT[a]	Forward	5'-CTAGGCGTTTGTACTCCGTCA	Orf8[a]	200
	Reverse	5'-TCCTCAGGAGTTTATGCACT		
MG[a]	Forward	5'-AGTTGATGAAACCTTAACCCCTTG	Mgpa	346
	Reverse	5'-CATTACCAGTTAAACCAAAGCCT		
UU[a]	Forward	5'-GAAACGACGTCCATAAGCAACT	UreA-B	423
	Reverse	5'-GCAATCTGCTCGTGAAGTATTAC		
NG[b]	Forward	5' -GCT ACG CAT ACC CGC GTT GC	cppB gene of 4.2-kb cryptic plasmid	390
	Reverse	5'-CGA AGA CCT TCG AGC AGA CA		

[a] Lee et al. (2007).
[b] Mahony et al. (1997).

2.4. Data analysis

Data acquisition and analysis were performed with the Statistical Package of Social Science Program (SPSS, Chicago, IL, USA), version 17. Continuous data are presented as mean ± SD, and qualitative data are presented as frequencies. Chi-square test and Fisher's exact test were used for comparison of qualitative variables. A *P*-value of <0.05 was considered as statistically significant. To assess the agreement between the detection of NG, CT, UU, and MG in semen and corresponding FVU specimens, we used k (nominal scale variables) as proposed by Landis and Koch (1979). Guidelines for the interpretation of k were as follows: $k < 0.20$, poor agreement; $k = 0.21–0.40$, fair agreement; $k = 0.41–0.60$, moderate agreement; $k = 0.61–0.80$, good agreement; $k= 0.81–1.00$, very good agreement.

3. Results

A total of 163 men infertile and fertile men participated in the study. The mean age of the 93 infertile men was 33 years (range 20–58; SD ±8.07), while the mean age of the 70 fertile men was 32 years (range 20–48, SD ±6.74) as shown in Table 2. The results show that 20/93 (21.5%) of infertile versus 5/70 (7.1%) of fertile men were positive for 1 or more organisms in semen or FVU specimens, while the total of positive patients infected with potential urogenital pathogens was 25/163 (15.3%). The highest number of positive pathogens was found among young men aged between 20 and 29 years old (Table 2). However, no significant difference was observed between age groups according to the presence or absence of urogenital pathogens ($P = 0.689$), while there was a statistically significant difference in the overall prevalence of these pathogens in infertile versus fertile men ($P = 0.009$) (Table 2).

The prevalence of genital NG, CT, UU, and MG in semen or FVU specimens among infertile versus fertile men was 6.5% (6/93) versus 0% (0/70), 4.3% (4/93) versus 1.4% (1/70), 10.8% (10/93) versus 5.7% (4/70), and 3.2% (3/93) versus 1.4% (1/70), respectively. Only NG represents a statistically significant difference in bacterial prevalence between infertile and fertile men (Table 3). Two (2.2%) of infertile

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Table 2
The overall prevalence of the uropathogens in infertile versus fertile men.

Groups[a]	PCR results		Total	*P*-value
	No. of positive (%)	No. of negative (%)		
Infertile men[b]	20 (21.5%)	73 (78.5%)	93	0.009
Fertile men[c]	5 (7.1%)	65 (92.9%)	70	
Total	25 (15.3%)	138 (84.7%)	163	

[a] The highest number of positive potential pathogens was found among young men aged between 20–29 years old.
[b] Age (years), mean ± SD; 33 ± 8.07.
[c] Age (years), mean ± SD; 32 ± 6.74.

Table 4
Distribution of NG, CT, UU, and MG DNA in semen and FVU specimens of the 163 infertile and fertile men.

Detected organism[a]	Total no. of positive	Semen specimens, no. of positive	FVU specimens, no. of positive	Semen-FVU concordance (%)
NG	6	6	0	Null
CT	5	5	5	100
UU	14	13	10	85
MG	4	4	4	100

[a] NG was positive only in semen, whereas CT and MG were detected in 5 and 4 men in both semen and corresponding FVU specimens, and UU was found in 13 of the semen specimens and in 10 of the FVU specimens.

men harbored 2 pathogens (UU plus MG, NG plus CT), and 1 (1.4%) of fertile men harbored both UU and MG. Table 3 shows the number and type of mixed infections in the infertile and fertile and where infection with UU was found to be *U. parvum* in 9/10 (90%) of the infertile men and 2/4(50%) of the fertile men. Table 4 shows the distribution of positive NG, CT, UU, and MG DNA in each semen and FVU specimen of the 163 examined patients. NG was positive only in semen, whereas CT, MG, and UU were detected in both semen and urine. A total of 14 out of the 163 investigated men were positive for UU in semen or FVU specimens. This organism was detected in 13 of the semen specimens and in 10 of the FVU specimens. Nine men have both their semen and the corresponding FVU specimens positive, while four men have positive semen specimens only, and 1 has positive FVU specimen only.

4. Discussion

Previous studies from Jordan have reported that the prevalence of sexually transmitted agents such as NG, CT, and MG are very low among general Jordanian population (Al-Ramahi et al., 2008; Awwad et al., 2003; Mahafzah et al., 2008; Shehabi et al., 2009). This prospective study has revealed that there is a statistically significant difference in the overall prevalence of STD agents in infertile versus fertile men (21.5% versus 7.1%). A recent study reported by Al-Sweih et al. (2012) from Kuwait indicated that there was no statistically significant difference in the overall prevalence of CT, MG, UU, and *Mycoplasma hominis* infections in infertile versus fertile men. The prevalence of NG in this study was 6.5% among only infertile men, and this represents a statistically significant difference ($P = 0.032$). This result was higher than that previously reported in recent studies conducted in other countries such as in Canada (Domes et al., 2011) and Tunis (Gdoura et al., 2008b). The prevalence of CT (4.3%) in our examined infertile men is nearly similar to that reported in other recent studies (Al-Sweih et al., 2012; Awwad et al., 2003; Bezold et al., 2007; Hamdad-daoudi et al., 2004; Hosseinzadeh et al., 2004), but was also not in agreement with other studies (Domes, et al., 2011; Gdoura, et al., 2008a; Golshani et al., 2007). The results of this study also revealed that the prevalence of MG (3.2%) in infertile men is nearly similar with that reported by Al-Sweih et al. (2012) and Gdoura et al. (2008a) but was higher than that reported by Kjaergaard et al. (1997). This study found no statistically significant difference in the prevalence of MG and UU infections between infertile and fertile men (3.2% versus 1.4%) and (10.8% versus 5.7%), respectively. The frequency of UU (10.8%) in our infertile men is nearly similar to other reported studies (Levy et al., 1999; Zeighami et al., 2009), while at the same time are not in agreement with other studies (Al-Sweih et al., 2012; Gdoura et al., 2008a; Knox et al., 2003; Peerayeh et al., 2008; Zhang et al., 2011). Few of our infertile (1.1%) and fertile (1.4%) men had mixed MG and UU infections, a finding comparable to that reported by Al-Sweih et al. (2012)). In general, the prevalence of potential STD agents in male infertility patients as reported in the previous mentioned studies worldwide varies widely from 0 to 43.3%.

Table 3
Prevalence of NG, CT, UU, and MG infections in infertile versus fertile men.

Species	Infertile men (n = 93)	Fertile men[a] (n = 70)	*P*-value
	No. of positive (%)	No. of positive (%)	
NG	6 (6.5%)	0 (0)	0.032
CT	4 (4.3%)	1 (1.4%)	0.284
UU	10(10.8%)[b]	4 (5.7%)[a]	0.198
MG	3 (3.2%)	1 (1.4%)	0.424

[a] 2/4 (50%) were *U. parvum*.
[b] 9/10 (90%) were *U. parvum*.

This study demonstrates a high concordance between the detection of CT, UU, and MG DNA in semen and FVU specimens, while no concordance was established between the detection of NG DNA only in semen and not in FVU specimens. This result may indicate that NG is harbored in the epididymis, prostate, or seminal vesicles. In addition, the concordance between the detection of CT and MG in semen and corresponding FVU specimens was 100%. A very good agreement between the 2 tested specimens was found ($k = 1$), and the concordance between the detection of UU in semen and the corresponding FVU specimens was 85% ($k = 0.89$). The study of Gdoura et al. (2008b) has also demonstrated a high concordance between the detection of CC, ureaplasmas, and mycoplasmas DNA in semen and the corresponding FVU specimens.

Most of the previous studies have reported the prevalence of ureaplasmas in infertile males without discriminating between UU and *U. parvum*, while our study indicates that *U. parvum* species was the most prevalent isolate detected among infertile men (90%). This finding is in agreement with the result reported by Knox et al. (2003), but it is in contrast to other studies, which found UU species to be more common among infertile men (Gdoura et al., 2008a; Zeighami et al., 2009).

The highest number of positive potential pathogens in this study was found among young men aged 20–29 years. A similar result has been reported by Golshani et al. (2007), whereas Zeighami et al. (2009) found that the age of the patients who were positive for UU varied from 21 to 41 years. All variation mentioned on the prevalence of potential sexually transmitted pathogens could be due to the fact that most studies were conducted on different ethnic and social populations, with varying sample sizes and examination methods.

In conclusion, this study demonstrates that there is a overall tendency towards increased prevalence of the potential sexually transmitted pathogens: NG, CT, UU, and MG among infertile men compared with fertile men. Further studies are needed to confirm the potential of these urogenital pathogens in male infertility.

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