Human papillomavirus and *Chlamydia trachomatis* in semen samples of asymptomatic fertile and infertile men: prevalence and relation between semen parameters and IL-18 levels

Fertil ve infertil asemptomatik erkeklerin semen örneklerinde human papillomavirüsü ve *Chlamydia trachomatis:* prevalans ve semen parametreleri ile IL-18 arasındaki ilişki

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Abstract

Objective: To determine the prevalence of human papillomavirus (HPV) and *Chlamydia trachomatis (C. trachomatis)* infections, and the relationship of these infections to semen parameters and proinflammatory cytokine (IL-18) levels in both sexually active and asymptomatic men.

Materials and methods: One hundred and seventy-five (144 infertile and 31 fertile as control) men were included in this study. The presence of HPV and *C. trachomatis* were investigated by polymerase chain reaction (PCR) assay in semen samples. IL-18 levels were measured using ELISA method in seminal plasma.

Results: The prevalence rates were 1.1% and 8.6% for HPV and *C. trachomatis*, respectively. All HPV positive samples subtyped as high-risk HPV. There was no significant difference for both HPV and *C. trachomatis* rates between fertile and infertile groups ($p>0.05$). Although the seminal parameters between infertile and fertile groups were different ($p<0.001$), the differences of mean IL-18 levels were statistically insignificant ($p>0.05$).

Conclusion: The prevelance rates of HPV and *C. trachomatis* in asymptomatic sexually active men in our region were comparable with the other studies. These findings might provide a basic data for preventive measures and policy development for sexually transmitted infections in Turkish men.

Key words: *Chlamydia trachomatis*; cytokines; human papillomavirus; seminal plasma.

Özet

Amaç: Seksüel olarak aktif ve asemptomatik erkeklerde human papillomavirüs (HPV) ve *Chlamydia trachomatis* (*C. trachomatis*) enfeksiyonlarının prevalansının ve bu infeksiyonlar ile semen parametreleri ve seminal proinflamatuvar sitokin (IL-18) düzeyleri arasındaki ilişkinin belirlenmesi.

Gereç ve yöntem: Çalışmaya 144 infertil ve kontrol grubu olarak 31 fertil olmak üzere, toplam 175 erkek alındı. Semen örneklerinde HPV ve *C. trachomatis* varlığı polimeraz zincir reaksiyon (PCR) yöntemiyle araştırıldı. IL-18 düzeyleri ise seminal plazmada ELISA yöntemi ile belirlendi.

Bulgular: HPV ve *C. trachomatis* için prevalans oranları sırasıyla %1.1 ve %8.6 olarak bulundu. Tüm HPV pozitif örnekler yüksek risk grubu olarak tiplendirildi. Fertil ve infertil gruplar arasında, HPV ve *C. trachomatis* varlığı açısından anlamlı fark bulunmadı ($p>0.05$). İnfertil ve kontrol grupların seminal parametreleri arasında fark istatistiksel olarak anlamlı iken ($p<0.001$), iki grup arasındaki ortalama IL-18 düzeylerinde anlamlı fark bulunmadı ($p>0.05$).

Sonuç: Bölgemizdeki asemptomatik seksüel aktif erkeklerde HPV and *C. trachomatis* prevalans oranları diğer çalışmalarla karşılaştırılabilir düzeydeydi. Bu sonuçlar Türk erkeklerinde cinsel yolla bulaşan hastalıklara yönelik önlemler almak ve koruyucu programlar geliştirmek için temel verileri sağlayacaktır.

Anahtar sözcükler: *Chlamydia trachomatis*; human papillomavirüs; seminal plazma; sitokinler.

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Sexually transmitted infections (STI) are a major public health problem throughout the world.[1] STI can affect fertility by destroying sperm function or by obstructing seminal tract.[2] Human papillomavirus (HPV) is a small DNA virus that is responsible for fastest spreading STI in humans. The vast majority of sexually active men and women will become infected with HPV in their lives. Currently, there are neither good methods for preventing, nor comprehensive and effective treatments for the clinical consequences of HPV infection. Fortunately, the majority of those infected with HPV will not develop clinical disease or symptoms because the host immune system resolves most infections.[3] *Chlamydia trachomatis* (*C. trachomatis)* is the main bacterial agent in the etiology of STI in sexually active men and women. *C. trachomatis* infection is also associated with more persistent HPV infection, which may contribute to the increased risk of clinical complications of HPV in individuals co-infected with *C. trachomatis*.[4]

The prevalence of HPV and *C. trachomatis* infections is variable in communities. In European countries, the prevalence of STI with *C. trachomatis* present an increasing trend, though detection is difficult.[5] In Turkey, there is lack of population-based studies demonstrating prevalence of asymptomatic STI in asymptomatic male.

Cytokines, which principally regulate inflammatory and immune responses, are released by various cells in the male urogenital tract.[6] Human semen contains several cytokines and their soluble receptors.[7] Seminal tract infections may lead to an increased release of proinflammatory cytokines such as interleukin-18 (IL-18) in seminal plasma (SP).[8]

In this study, we first aimed to demonstrate the prevalence of HPV and *C. trachomatis* in sexually active and asymptomatic Turkish men. We also investigated the relationship between these infections and semen parameters, and finally assessed the seminal proinflammatory cytokine (IL-18) response.

Materials and methods

Study population

One hundred and seventy-five (144 infertile and 31 fertile) men who were referred to outpatient urology clinic in 2006 were included in this point-prevalence study. The study was approved by the institutional ethics committee of Adnan Menderes University School of Medicine. Informed consent was taken from all of the participants. After taking medical history and detailed genital examination, semen parameters of each patient were determined with WHO methods using a Makler counting chamber.[9] Two semen parameters, sperm count and motility, were evaluated. The semen samples were obtained by masturbation into a sterile container after abstinence for 3-4 days and examined within 60 min after liquefaction at 37°C. All men had no history of genital warts and without symptoms of genitourinary infections.

Cytokine analysis

For IL-18 analysis, the semen sample was centrifuged at 4,000 rpm for 10 min to pellet sperm. SP was separated and stored at -80ºC until analysis. Before use, the samples were thawed overnight at +4ºC and diluted with trisma buffer with a ratio of 1:1. Diluted samples of 100 µl were used for cytokine analysis. IL-18 measurements in SP were determined by using commercially available solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) kit (Cat: KHC0181, Biosource CA, USA) according to the instructions of the manufacturer. Calibrators prepared at 0 to 1000 pg/mL concentrations. The minimal detectable dose is 12.5 pg/mL in this kit. All samples were assessed in duplicate. Absorbance of calibrators and samples was read using micro plate reader at 450 nm. The concentration of IL-18 was expressed as pg/mL.

DNA extraction

Total DNA was obtained by a commercial NucleoSpin Tissue mini column (Macherey-Nagel, Duren, Germany) from semen samples. Total 100 µL aliquots of the samples were analyzed. Semen samples were initially digested at 56°C for 3 hours in the T1 buffer supplied with the kit, sperm cells were removed by centrifugation at 1,600 rpm for 10 min, and the supernatant was extracted by following the manufacturer's protocol. A final volume of 100 µl was obtained.

HPV DNA detection and typing

HPV DNA of semen samples was studied by polymerase chain reaction (PCR). To detect a broad range of HPV genotypes simultaneously, the consensus primers MY09 and MY11 were used.[10] These primers amplify a fragment of 450 base pairs. Amplifications were carried out in a Mastercycler (Eppendorf, Germany) denaturation at 94°C for 45 sec, primer annealing at 55°C for 45 sec, DNA exten-

sion at 72°C for 1 min, and last DNA extension at 72°C for 7 min. A total of 35 cycles were used for amplification. A positive result was indicated by a DNA band of approximately 450 base pairs. HPV positive samples were typed by the HPV line assay kit (GenID GmbH, Straβberg, Germany). Firstly, HPV DNA was amplified and then genotyped with reverse dot blot hybridization by following the manufacturer's protocol. Briefly, 5 µL of each sample was amplified with biotinylated primers, which amplify the L1 open reading frame. Thereafter, the biotin-label amplified DNA was hybridized with sequence-specific oligonucleotide probes (SSOP) for HPV high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53) and the low-risk types (6 and 11). The results of typing were indicated as HPV high-risk group, low-risk group, type 16, type 18, type 45, 30th groups, 50th groups, type 6 and 11.

C. trachomatis DNA detection

C. trachomatis DNA was studied using cryptic plasmid primers by PCR. Amplifications were carried out in a Mastercycler (Eppendorf, Germany) denaturation at 94 °C for 60 sec, primer annealing at 55°C for 60 sec, DNA extension at 72°C for 2 min, and last DNA extension at 72°C for 7 min. A total of 35 cycles were used for amplification. All amplification products were analyzed in 2% polyacrylamide gel and stained with ethidium bromide. A positive result was indicated by a DNA band of 241 base pairs.[11] Clinical samples were checked for DNA quality and the absence of inhibitors of amplification by analysis for the human ß-globin gene.[12]

Statistical analysis

According to Shapiro-Wilk test, the distribution of the age, IL-18, sperm counts and motility were not normal in groups. Therefore the descriptive statistics were demonstrated as median (25-75 percentile) and the comparisons between groups were made by Mann Whitney U test. The difference between HPV positive and negative groups and C. *trachomatis* positive and negative groups were compared by using the Fisher exact test. P value of less than 0.05 was considered as statistically significant.

Results

A total of 175 men aged between 19 and 47 years participated in the study. The median age of the infertile group (n=144) was 32.0 (range 18-50.0) years. It was 32.0 (range 23-46.0) years in fertile group (n=31). There was no statistically significant difference between the mean ages of the groups (p=0.664).

There was also no significant difference in IL-18 levels between infertile and fertile groups (p=0.108). Both sperm count and sperm motility parameters were found to be higher in fertile group compared to infertile group (p<0.0005). Semen parameters, mean ages of the patients, and the prevalence of sexually transmitted infections were given in Table 1.

The prevalence rates of HPV and *C. trachomatis* were found to be 1.1% (n=2) and 8.6% (n=15), respectively. All HPV positive samples subtyped as high-risk HPV. There was no significant difference for HPV rate between fertile and infertile groups (3.2% vs. 0.7%, p=0.324). The difference of *C. trachomatis* positivity between these groups was also insignificant (9.7% vs. 8.3%, p=0.732). IL-18 was detected in substantial quantities in both groups. Although the semen parameters between infertile and fertile groups were different (p<0.001), the difference of median IL-18 levels was statistically insignificant (28.71 pg/mL vs. 34.71 pg/mL, p=0.108).

Discussion

STIs are caused by myriad of bacteria, viruses, parasites, and fungi. STIs still create an important public health problem almost every part of the world. According to the WHO data, approximately 333 million people are infected with a new causal agent of STI every year. The distribution of these cases is reported to be 12 million syphilis, 62 million gonorrhoea, 89 million *Chlamydia* infection, and 170 million trichomoniasis. Except for these cases, every year 30 million HPV infection and 20 million genital herpes infection are reported.[13] There is still controversy on STI as an etiologic factor in male infertility. The presence of HPV and *C. trachomatis* in semen emphasizes the potential risk of this route of transmission, and underlines the need to determine its prevalence in asymptomatic male partners of infertile couples. There is no study on the prevalence of HPV and *C. trachomatis* in asymptomatic sexually active Turkish men in the literature. The introduction of commercial PCR in the routine clinical microbiology laboratory is an important advance to diagnose asymptomatic HPV and *C. trachomatis* infections in male.[14,15]

HPV, one of the most common STI, is a member of small DNA viruses inducing proliferation of epithelial

Table 1. Semen parameters, the presence of human papillomavirus (HPV) and *C. trachomatis*, and IL-18 levels in study groups [median (25-75 percentile) or n (%)]

	Fertile group (n=31)	Infertile group (n=144)	p
Median age (years)	32 (27-36)	32 (29-35)	0.664
Sperm count (x10^6/mL)	102.0 (45-150)	14.0 (0.42-45)	<0.0005
Sperm motility (A+B %)	65.0 (55-70)	50.0 (30-60)	<0.0005
IL-18 (pg/mL)	34.71 (23.43-49.90)	28.71 (15.86-48.62)	0.108
HPV	1 (3.2)	1 (0.7)	0.324
C. trachomatis	3 (9.7)	12 (8.3)	0.732

cells. It is prevalent in all sexually active populations and frequently presented clinically as anogenital warts in both males and females. High-risk, oncogenic, HPV types (including HPV 16 and HPV 18) are associated with 99.7% of all cervical cancers; low-risk HPV types (HPV 6 and HPV 11) are responsible for almost all cases of genital warts.[3] Although it is primarily transmitted via direct epithelial contact, high-risk HPV types were detected in semen.[16,17] Similarly, we found high-risk HPV types in both HPV positive semen samples.

Currently, there are neither effective means of preventing HPV transmission nor cures for clinical manifestations. Unfortunately, sperm washing does not eliminate the risk of transmission.[15,16] Condom use protects against some clinical sequel of HPV infection and clearance of clinical symptoms, even if it does not prevent primary infection. Preventative vaccines that lower the incidence of HPV infection and its associated diseases may offer a promising alternative to current therapies. At present, prophylactic HPV vaccines that confer protection against both high- and low-risk types are costly, especially for developing countries.[3] The role of the vaccine in males is still controversial.

As recently reviewed, HPV infection rates in men ranged from 1.0% to 82%. This wide range may be due to the variation in the clinical material analyzed such as penile surface, glans, scrotum, urethra, semen, and urine. Some investigators reported that the most sensitive sample site are penile shaft, glans penis or coronal sulcus, while others studied urethral brushing or semen sample.[18,19] Giovannelli et al.[19] compared penil brushing, urethral brushing, and semen samples. They found the highest HPV rate in penil brushing, followed by urethral brushing and semen, and suggested that penil brushing combined with urethral brushing is the best sampling method for testing men for HPV. As an alternative, combination of penil brushing and semen could be applied to improve the rate of detection of HPV DNA. In a study, HPV-DNA was detected in semen of only 2% of patients without HPV associated lesions.[20] The results indicated low HPV prevalence in semen from men without detectable lesions. This is in accordance with the result (1.1%) of our study. In addition, previously reported higher prevalence rates may be due to the fact that uncircumcised men have an increased risk of HPV infection.[21] The presence of HPV was not associated with deterioration of semen parameters. Some studies indicated negative association between HPV and semen parameters,[17,22] while another study did not confirm this finding.[16] Thus, additional research is needed to determine whether HPV infection contributes to male infertility.

C. trachomatis often causes asymptomatic genital tract infections in both men and women, and the high number of unrecognized infected individuals provides a reservoir for spreading the infection to men and women via sexual contact.[23] Men are less likely to be infected than women, and most men with urethral infection are free of symptoms. On the other hand, there are few data on the duration of *C. trachomatis* infection in men; it may be several months or years.[24] Semen is one of the preferred specimens for diagnosing asymptomatic *C. trachomatis* infections in men. It has been suggested that testing for the presence of genital *C. trachomatis* in males is incomplete if semen samples are not included.[25] Transmission of *C. trachomatis* due to insemination with infected semen has also been reported.[26]

PCR analysis is a more sensitive detection method and yields higher rates of prevalence. Although, there was a recent increase in the prevalence of asymptomatic *C. trachomatis* infections, our rate is not consistent with that in earlier publications.[27,28] The 8.6% preva-

lence rate of asymptomatic *C. trachomatis* infection among the population is not also in accordance with the reported prevalences among male in France and Netherlands (4% and 6.3%, respectively).[15,29] But it should be noted that prevalence estimates vary depending on the technique used to assess *C. trachomatis*. In another study, Hosseinzadeh et al.[28] indicated 4.9% prevalence rate of asymptomatic seminal *C. trachomatis* infection in UK infertile male population. They concluded that semen quality is unaffected by *C. trachomatis*. This is in agreement with our findings. Some studies have found a correlation between genital *C. trachomatis* infection and sperm quality,[30,31] while others have reported contradictory findings.[14,32] This may be due to different design or diagnostic methods of the studies. Additionally, the prevalence rates may also be different due to the different sample sizes of the studies. The smaller sample size in our study may be the reason of not being in accordance with the reported prevalence rates.

Although hormonal factors are essential for successful spermatogenesis, cytokines are involved in the local control mechanisms of testicular function.[33] Human semen contains several cytokines and their soluble receptors.[7] They may be produced by the testis, epididymis or released by immunocompetent cells even in the absence of genital infection. Their production occurs in response to foreign antigens, pathogens and chronic inflammation. Some cytokines were shown to effect sperm motility, viability and ova penetration capacity. Previous reports indicated the presence and fluctuations of the some cytokines in certain andrological diseases and in infertile patients.[6-8] The proinflammatory cytokine IL-18 has been implicated as important in the regulation of both innate immunity and acquired immune responses.[34] Lu et al.[35] have shown that cell lines infected with *C. trachomatis* do in fact produce the active form of IL-18 after cleaving its proform with caspase 1. There is only one study in the literature showing the role of IL-18 in seminal plasma in response to genital tract infections.[8] The authors of this study concluded that IL-18 might be used in the diagnosis of seminal tract infections. However, in the present study, there was no statistically significant difference in the levels of IL-18 between *C. trachomatis* infected and uninfected males. On the other hand, cytokines are not released solely in response to infection. Although leukocytes are more important, spermatozoa have to have capacity to generate reactive oxygen species which can amplify the production of cytokines.[36]

In conclusion, STI pathogens in semen were not associated with poor semen parameters or increased IL-18 levels in our study. The results support the findings that presence of *C. trachomatis* or HPV in semen does not have an effect on sperm function or trigger cytokine response (at least IL-18). Since the presence of these pathogens in semen may threaten the health of partners, appropriate measures should be taken to diagnose and treat asymptomatic males. Our findings might provide a basic data for preventive measures and policy development for STI in Turkish men.

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Conflict of interest

No conflict of interest was declared by the authors.

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