INFLUENCE OF BACTERIAL INFECTIONS ON REPRODUCTIVE HEALTH

Ph.D thesis

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Szeged, 2017

Publications directly related to the thesis

I. Juhász E., Ostorházi E., Pónyai K., Silló P., **Párducz L.**, Marschalkó M., Rozgonyi F.: Ureaplasmas: from commensal flora to infectious diseases.
Rev. Med. Microbiol. 2011, **22**: 73-83.**(IF:0.370)**

II. Farkas B, Ostorházi E, Pónyai K, Tóth B, Adlan E, **Párducz L**, Marschalkó M, Kárpáti S, Rozgonyi F. Az *Ureaplasma urealyticum* és a *Mycoplasma hominis* antibiotikum-érzékenysége és gyakorisága szexuálisan aktiv egyének genitális mintáiban [Frequency and antibiotic resistance of *Ureaplasma urealyticum* and *Mycoplasma hominis* in genital samples of sexually active individuals].
Orv Hetil. 2011 Oct 16;152(42):1698-702.

III. Pónyai K, Mihalik N, Ostorházi E, Farkas B, **Párducz L**, Marschalkó M, Kárpáti S, Rozgonyi F. Incidence and antibiotic susceptibility of genital mycoplasmas in sexually active individuals in Hungary.
Eur J Clin Microbiol Infect Dis. 2013 Nov;32(11):1423-6. **(IF:2.544)**

IV. Nemes-Nikodém E, Vörös E, Pónyai K, **Párducz L,** Kárpáti S, Rozgonyi F, Ostorházi E. The importance of IgM positivity in laboratory diagnosis of gestational and congenital syphilis.
Eur J Microbiol Immunol (Bp). 2012 Jun;2(2):157-60.

V. **Párducz L**, Eszik I, Wagner G, Burián K, Endrész V, Virok DP. Impact of antiseptics on *Chlamydia trachomatis* growth.
Lett Appl Microbiol. 2016 Oct;63(4):260-7. (**IF:1.579)**

Publications with results related to the thesis

I. **Párducz L**., Ostorházi E., Pónyai K., Mihalik N., Párduczné Szöllősi A., Rozgonyi F.: A reprodukciót befolyásoló fiatalkori bakteriális STD fertőzések.
Nőgyógy. Szül. Továbbk. Szemle 2013, **15:** 97-101.

II. **Párducz L**.: Az alsó genitális tractus fertőzései. In: A Nőgyógyászat kézikönyve (Szerk.: Papp Z.) Medicina Könyvkiadó Zrt, Budapest, 2017. 836-856.

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List of abbreviations

2D-PAGE	two-dimensional-polyacrylamide gel electrophoresis
BPD	bronchopulmonary dysplasia
CDC	Center for Disease Control and Prevention
COFRADIC	combined fractional diagonal chromatography
COMC	*Chlamydia* outer membrane complex
EGFR	epidermal growth factor receptor
EIA	enzyme immunosorbent assay
ELISA	enzyme linked immunosorbent assay
FP	fluorescence polarization
FTA-ABS	fluorescent treponemal antibody absorption assay
LGV	lymphogranuloma venereum
LPS	lipopolysaccharide
MIBC	minimal inhibitory biofim concentration
MIC	minimal inhibitory concentration
MOI	multiplicity of infection
MQ	Milli-Q
MSM	men sex with men
NGU	non-gonococcal urethritis
NGNCU	non-gonococcal-non-chlamydial urethritis
NTT	nontreponemal tests
qPCR	quantitative PCR
RPR	rapid plasma reagin
RT	room temperature
SPG	sucrose-phosphate-glutamate
STDs	sexually transmitted diseases
STIs	sexually transmitted infections
TPHA	*T. pallidum* hemagglutination assay
TPPA	*T. pallidum* particle agglutination
TTS	Type-III secretion system
VDRL	venereal disease research laboratory test

1. Summary

Various bacterial infections can influence the reproductive health in both sexes by either as a direct infection of the reproductive organs or as a hematogenous dissemination of a generalised infection. In the later case the foetus is always involved in the infection to a certain extent. Bacterial sexually transmitted diseases (STD) are caused by the species of *Chlamydia trachomatis (C. trachomatis), Neisseria gonorrhoeae (N. gonorrhoeae), Treponema pallidum (T. pallidum), Haemophilus ducreyi, Gardnerella vaginalis, Streptococcus agalactiae, Mycoplasma genitalium (M. genitalium), M. hominis, Ureaplasma urealyticum (U. urealyticum)* and *U. parvum* [1].

A number of studies focused on the STDs in Hungary [2]-[5], however the infections caused by the intracellular parasite *Mycoplasma* family are not well described. Due to the recent technical development in culturing and antibiotic sensitivity testing of mycoplasmas, we have been able to culture mycoplasmas *in vitro* and perform antibiotic susceptibility testing of isolates routinely. To study the incidence and antibiotic susceptibility of Hungarian *Ureaplasma* strains during a four-year period 4154 samples were obtained to culture for the presence of *Ureaplasma*. Out of these 2114 samples were collected from female patients. 2047 samples were cervix swabs and 11% of these were *Ureaplasma* positive by culture. Out of 59 urethra samples 7% was positive and all of the 12 urine samples were negative. It can be stated that cervix swabs are the most suitable samples for *Ureaplasma* cultivation in female patients. 2040 samples were collected from male patients. The vast majority of these samples were obtained from the urethra. Only 15 samples were glans swab, ejaculatum or urine, none of these showed positive culture result. 3.01% of the urethra samples showed *Ureaplasma* positivity. These data show that non-gonococcal urethritis (NGU) is the main symptom in *Ureaplasma* positive male patients. *Mycoplasma hominis/genitalium* positivity was below 1% in both sexes, therefore, *Mycoplasma* was left out from the further studies. *Ureaplasma* positive females and males covered a broad age spectrum, from less than 16- to over 60-year old. However, the age distribution of *Ureaplasma* positive females and males showed a rather different picture.

The ratio of symptom-free persons among *Ureaplasma urealyticum/ parvum* positive individuals was above 40% in both sexes. The dominant clinical symptoms in females were vaginal discharge (23.5%), genital pruritus (9.2%) and colpitis (11.7%). The dominant clinical symptoms in males were urethritis (35.5%), balanitis (14%) and

urethral discharge (10.8%). These data suggest that NGU is the main symptom in *Ureaplasma* positive male patients. Antibiotic sensitivity testing of 373 *Ureaplasma* strains showed that no fully sensitive *Ureaplasma* strain was found. Extremely high incidence of resistance was exhibited by the *Ureaplasma* strains against erythromycin (81.23%) and clindamycin (75.06%). Every fourth strain was resistant to ofloxacin. Doxycycline proved to be the most effective agent with 97.32% susceptibility, followed by tetracycline (95.9%) and azithromycin (85.79%). Consequently, for a successful therapy, identifying the infective agent with culturing or molecular genetic methods are not enough, the determination of antibiotic susceptibility is also necessary.

In another study of 3-year period 33.753 serum samples were examined for syphilis. *Treponema pallidum* infections were confirmed in 241 pregnant women. Four children born to inadequately or untreated syphilitic women were confirmed to have connatal syphilis. The rapid plasma reagin (RPR) titer was measured to determine the stage of the infection and to examine the success of the antiluetic therapy. The diagnosis of maternal syphilis was confirmed with enzyme linked immunosorbent assay (ELISA), *T. pallidum* particle agglutination (TPPA) and IgG and IgM immunoblots. It was concluded that maternal IgM immunoblot results identify mothers at risk of delivering babies with connatal syphilis better than the maternal RPR titer. The standard serological tests are less useful in newborns because of the IgG transfer across the placenta. IgM test which depends on the infant's response has more specificity in diagnosing connatal syphilis.

In a third series of experimerts we measured the antichlamydial effects of iodine aqueous solution, povidone-iodine, chlorhexidine and borax. These antiseptics are being used for the treatment of bacterial vaginosis, but their effect on *Chlamydia trachomatis* infections has not yet been investigated. Bacterial vaginosis is a frequent dysbiosis, where the normal lactobacillus-dominated flora is replaced by an anaerob/aerob polymicrobial flora. Bacterial vaginosis increases the risk of acquiring sexually transmitted infections (STIs) including the most frequent *C. trachomatis* infections. Intravaginal antiseptics are part of the bacterial vaginosis treatment, and ideally they should also inhibit the bacterial vaginosis-related STIs. Therefore, we tested the antichlamydial activity of four antiseptics: iodine aqueous solution, povidone-iodine, chlorhexidine and borax. First, we measured the impact of antiseptics on the viability of the HeLa cervical epithelial cells, and calculated the maximum nontoxic concentrations. Next, we infected the HeLa cells with *C. trachomatis* preincubated for 1 h with the particular antiseptic. The chlamydial

growth was measured by direct quantitative PCR (qPCR) of the infected cells. The minimal inhibitory concentrations (MIC) of chlorhexidine and povidone-iodine were 3.91 and 97 μg/ml respectively; however, MIC of chlorhexidine was close to its maximum nontoxic concentration. The iodine aqueous solution and the borax showed no antichlamydial activity. Our *in vitro* studies showed that chlorhexidine and particularly povidone-iodine are potentially able to limit the bacterial vaginosis-related *C. trachomatis* infection.

2. Introduction

2.1 Incidence and antibiotic susceptibility of genital *Ureaplasmas* in sexually active individuals in Hungary

Several species in the class of *Mollicutes* have been shown to cause colonization and disease in the human body; regarding the genital tract the *Mycoplasma* species: *M. hominis*, *M. genitalium*, as well as the *Ureaplasma* species: *U. urealyticum, U. parvum*. Neither the two *Mycoplasma* species nor the two *Ureaplasma* species can be distinguished on the basis of their phenotypical appearences. Genetic analysis is necessary to distinguish between them but we did not have the techniques to perform genetic analysis. Therefore, the name *U. urealyticum* is used for both *Ureaplasma* species and *M. hominis* is used for both *Mycoplasma* species. Genital mycoplasmas are found relatively frequent on the genital mucosa of sexually active people without signs of a manifest disease. After puberty both *U. urealyticum* and *M. hominis* can be detected in a significant part of the sexually active population, but *U. urealyticum* is more common. The frequency of colonization increases with sexual experience and the number of partners [4][6].

Genital mycoplasmas can cause acute and chronic inflammations in the urogenital tract, and are proven etiological factors of bacterial vaginosis, salpingitis and pelvic inflammatory disease, postpartum sepsis, urethral syndrome and habitual abortion, chorioamnionitis and consequential premature birth, neonatal infections, such as pneumonia and sepsis [4][7][8]. In male patients they also cause acute or chronic urethritis – primarily the role of *U. urealyticum* and *M. genitalium* is proved –, acute and chronic prostatitis, epididymitis, epididymoorchitis as well as oligoasthenospermia [9]. They can cause renal calculi, pyelonephritis, Reiter-syndrome and sexually acquired reactive arthritis in both sexes [4][9]. *Ureaplasmas,* originally called “T-mycoplasmas”,

were first described by Shepard in 1954. Since then these organisms have been studied thoroughly, but it seems like that our knowledge about *Ureaplasmas* is still insufficient, especially in the field of their antibiotic resistance, ability of biofilm formation and their role in chronic and mixed infections. Studying human *Ureaplasmas* is important, because of their high prevalence and pathogenicity.

Ureaplasmas are eubacteria in the class *Mollicutes*. For a long time, *Ureaplasma urealyticum* was the only known species in human *Ureaplasma* infections. Based on 16S rRNA sequences, this species was subdivided into two separate species (instead of the previous biovar 1, biovar 2 classification): *U. parvum* containing serovars 1, 3, 6, and 14, and *U. urealyticum* containing serovars 2, 4, 5, 7, 8, 9, 10, 11, 12 and 13 [10].

Because the lack of cell wall, *Ureaplasmas* are extremely susceptible to drying and other adverse environmental conditions. Therefore, careful attention have to be given to specimen collection. The inoculation of transport media is recommended at bedside whenever possible. Proper transport conditions are also needed to get viable cells in the diagnostic laboratory to culture *Ureaplasmas*. *Ureaplasma* spp. can be detected by several laboratory methods. Culture is the reference standard for detection. Specialized media: Shepard's 10 B broth, A7 and A8 agar can be used for culturing. Culture media are completed with antibacterial agents (Penicillin G, broad-spectrum semisynthetic penicillins) in order to minimize bacterial overgroth, and with pH indicator, such as phenol red, in order to detect *Ureaplasma* growth. Broths that have changed color should be subcultured within a short time into a fresh broth and onto agar plates, because the culture can loose viability rapidly (within a few hours). A7 agar is used traditionally for *Ureaplasma* isolation. Microscopic examination of the colonies is neccessary. Agar plates have to be incubated in the presence of 5-10% CO_2 or in an anaerobic environment of 95% N_2 plus 5% CO_2 for the best growth. Colonies typically grow in 2 to 5 days. The morphology of the colonies are granular, brown, 15 to 60 μm in diameter. According to the colonial morphology and urease production, genus level identification is possible. Cultures can not be considered negative until 7 days of no growth. A8 agar does not contain manganese salts that inhibit some *Ureaplasma* serotypes, therefore it has become the preferred growth medium in many laboratories. Commercially available diagnostic kits such as Mycoplasma Duo (Bio-Rad) and Mycoplasma IST 2 (bioMérieux) are simplified alternatives to conventional cultures. These rapid diagnostic kits contain selective and differential liquid media. Growth of organisms are detectable after 24 to 48 h of incubation by biochemical activity related pH change. Specifically, they test the urea

hydrolysis of *Ureaplasma* spp. or the arginine hydrolysis of *Mycoplasma hominis* via a phenol red pH indicator that detects ammonia liberation. These rapid culture techniques have relatively similar sensitivities, specificities, and positive predictive values as traditional culture methods [11]. Culture provides only an initial positive or negative result. Inhibitory substances present in a clinical specimen also affect negatively the result of culturing. Subculture and molecular technique is necessary to determine species type and the serovar [12]. *Ureaplasma* cultures can be overgrown by other microorganisms such as *Proteus* spp. and yeasts, spoiling the specificity/ sensitivity of the culture method.

Nucleic acid amplification tests have also been developed for *Ureaplasma* spp. The first PCR method for detection of human ureaplasmas in clinical sample was published in 1992 by Lee et al. [13]. Since then PCR methods have been increasingly used in the diagnosis of *Ureaplasma* infections. PCR methods have advantages over traditional culture methods: organism identification is possible even if the numbers of bacteria are low, viability is not necessary, rapid identification within 24 h is possible [14]. Subtyping of isolates can be performed faster with PCR methods (24–48 h after sample collection) than with culture methods. According to multiple studies, the detection of ureaplasmas in female genitourinary specimens, including cervical swab, amniotic fluid, and vaginal specimens by PCR is comparable or superior to that by culture. Reported sensitivities for PCR detection ranged from 90–100%, whereas culture yielded detection sensitivities of 40–91%. The PCR method has been used as a diagnostic tool for rapid detection of ureaplasmal infection in neonates with lower respiratory infections. The possible role of ureaplasmas in bronchopulmonary dysplasia (BPD) has also been examined by PCR [7]. Because of the high sensitivity of PCR, false positive results can occur, principally due to inter-sample cross contamination. Gene targets for PCR assays used to detect ureaplasmas and to define species and subtypes included the subunits of urease gene [15], 16S rRNA genes and the MB antigen gene. Heterogeneity of the MB antigen gene can be the basis of serotype identification [15]. Sophisticated nucleic acid amplification tests are necessary to discriminate between the two *Ureaplasma* species, leading to difficulties in species determination. Recently, multiplex real-time PCR assays have been developed for the quantitative detection of the two species separately [16]. PCR can also be used to discriminate among all of the serovars. Real-time TaqMan PCR assays have been developed that allow rapid, specific, sensitive, and quantitative detection with a 100 times greater sensitivity than conventional PCR [16]. Convenient differentiation of *U. parvum* and *U. urealyticum* is also possible with real-time TaqMan

PCR assays. Discrimination between harmless commensal colonization and clinically significant *Ureaplasma* infection can de done by the application of quantitative PCR techniques and PCR serotyping [12].

Pulse field gel electrophoresis (PFGE) is a valuable tool for characterization of genetic relatedness of different *Ureaplasma* isolates, but it can not be considered as a gold standard for genotyping of ureaplasmas [10][17]. Although PFGE is suitable for differentiating *U. parvum* from *U. urealyticum*, the four serovars of *U. parvum* and the serovars of *U. urealyticum* except 7, 11 and 4, 12 [10].

Multiplex PCR assay for simultaneous detection of *Trichomonas vaginalis, Mycoplasma hominis*, and *U. urealyticum* has also been developed [39]. Multiplex PCR-based microarray for detecting *N. gonorrhoeae, C. trachomatis* and *U. urealyticum* simultaneously in the presence of the internal control is also available [18]. A fluorescence polarization (FP) assay has been developed for simultaneous detection of *U. urealyticum, U. parvum, M. hominis* and *M. genitalium*. The FP assay (based on the asymmetric PCR coupled with hybridization-FP assay) could detect co-infection more effectively. FP assay might serve as an alternative for the detection of multiple genitourinary infections [19]. Serological tests (enzyme immunoassay, microimmunofluorescenc) for *Ureaplasma* spp. have also been developed and are mainly used in research [8].

Methods for antimicrobial susceptibility testing by microbroth, agar dilution and agar gradient diffusion have been utilized [8][11]. Broth microdilution is the most practical, widely used method [11]. The inoculum size, the pH of media and the incubation time alter the MIC values of antibiotics dramatically, therefore standardized antimicrobial susceptibility testing methods are needed [20]. Methodological guidelines and quality control parameters have been developed by the Clinical and Laboratory Standards Institute (CLSI) Subcommittee [21]. SIR Mycoplasma (Bio-Rad) is a liquid medium antibiogram. It contains the folllowing antibiotics at two different concentrations: doxycycline, tetracycline, azithromycin, josamycin, erythromycin, ofloxacin and the following ones at a single concentration: clindamycin, pristinamycin. The growth of ureaplasmas is objectively measured by the metabolic activity (hydrolysis of urea). The breakpoints for the antimicrobials are as follows in mg/L: tetracycline $S \leq 4$, $R \geq 8$; doxycycline $S \leq 4$, $R \geq 8$; azithromycin $S \leq 2$, $R \geq 4$; erythromycin $S \leq 1$, $R \geq 4$; josamycin $S \leq 2$, $R \geq 8$; ofloxacin $S \leq 1$, $R \geq 4$; clindamycin $S < 2$, $R > 2$; pristinamycin $S < 2$, $R > 2$.

Mycoplasma IST-2 (bioMerieux) kit contains strips that give information on the presence or absence of *M. hominis* and *U. urealyticum*, give an estimate of the density of each organism (>10^4 CFU) and provide additional information on antibiotic susceptibility to erythromycin, clarithromycin, azithromycin, doxycycline, tetracycline, ciprofloxacin, ofloxacin, josamycin and pristinamycin. The development of red colour indicates the growth of bacteria and the resistance to a given antimicrobial agent. The breakpoints for the antimicrobials tested (according to the guidelines of CLSI) are as follows in mg/L: tetracycline S $\leq$ 4, R $\geq$ 8; doxycycline S $\leq$ 4, R $\geq$ 8; clarithromycin S $\leq$ 1, R $\geq$ 4; azithromycin S $\leq$ 0.12, R $\geq$ 4; erythromycin S $\leq$ 1, R $\geq$ 4; josamycin S $\leq$ 2, R $\geq$ 8; ciprofloxacin S $\leq$ 1, R $\geq$ 2; ofloxacin S $\leq$ 1, R $\geq$ 4 [21].

SIR Mycoplasma kit has the advantage that the CFU of the inoculum used for antibiotic susceptibility testing can be choosed according to the density of bacteria (below or up to 10^4 CFU) in the specimen. Another advantage of this kit is that the antibiotic susceptibility of *M. hominis* and *U. urealyticum* can be tested separately, even if both bacteria are present in the clinical sample. With Mycoplasma IST-2 kit, just the common effective antibiotics can be determined in double *Mycoplasma* and *Ureaplasma* infections.

The biofilm forming property of *Ureaplasma* isolates should be examined routinely, at least in patients with recurrent symptoms. By the commercially available antibiotic susceptibiliy testing kits, only the sessile *Ureaplasma* cells are tested and the planktonic cells are out of observation. Therefore, it can occur that an *Ureaplasma* isolate is determined as susceptible to an antibiotic, however the biofilm forming, planktonic form of the bacteria is resistant. This can result in therapeutic failure.

Ureaplasma spp. are originally susceptible to bacteriostatic agents such as protein synthesis-inhibiting macrolides and tetracyclines as well as bactericidal agents including fluoroquinolones [22]. These drugs are the major antibiotics in treatment of *Ureaplasma* infections. Azithromycin (1g single dose *per os*) and doxycycline (2x100 mg daily for 7 days *per os*) are the first drug of choice. Resistance to all three antibiotic classes has been described in clinical *Ureaplasma* isolates [22][23]. Macrolides are often used as first-line therapies for *U. urealyticum* infections. This could be changed in the near future, since recent epidemiological investigations have revealed substantial resistance [24]. New macrolides are the most promising antibiotics for neonatal ureaplasmal and mycoplasmal infections. Traditionally erythromycin has been the most commonly used antibiotic in infants with *Ureaplasma spp.* infection. The eradication of ureaplasmas with

erythromycin from the respiratory tract of neonates was shown to be effective at variable rates. *In vitro* studies with azithromycin showed good inhibitory activity against *Ureaplasmas*. Azithromycin has combined antimicrobial and anti-inflammatory properties, in addition, the special pharmacokinetic properties allow higher intracellular and tissue concentration, better tolerance, fewer adverse effects and fewer drug interactions when compared with erythromycin. Therefore, azithromycin is an attractive agent for use in premature infants with *Ureaplasmal* infections, but it has not been studied thoroughly in that population up to now. Timing of treatment may be crucial, the early treatment and eradication of *Ureaplasmas* may be necessary to interrupt the inflammatory cascade generated by them and therefore the development of BPD can be prevented. At present, data obtained from clinical trials are insufficient to determine whether antibiotic treatment of *Ureaplasma* in neonates has any influence on the development of BPD and its comorbidities [25][7]. Eradicating *Ureaplasma* spp. from the genital tract of pregnants and the lungs of fetus or newborns may be difficult. The effect of macrolide antibiotics can be altered by mutations in 23S rRNA, co-infection with *M. hominis* or protective biofilm formation [8]. In case of biofilm forming *Ureaplasma* infection, clarithromycin is the drug of choice, since it can penetrate the biofilm and then bacterial membrane to reach ribosome in order to inhibit biofilm synthesis [26]. Due to potential toxicities, fluoroquinolons are usually not recommended for use in neonates or pregnants. Instead of macrolides, doxycycline is the most commonly used antibiotic in the treatment of NGU. In case of tertracycline resistance, fluoroquinolons and doxycycline seem to be the most suitable treatment alternatives. Josamycin and pristinamycin are also therapeutic options, even as first choice drugs when empirical therapy is required [27].

Since ureaplasmas lack cell wall peptidoglycan, they are not affected by beta-lactams or glycopeptides. They are not susceptible to sulfonamides or trimethoprim since they do not synthesize folic acid. Rifampicin are also inactive against them. They are intrinsically resistant to lincosamides except at high concentrations. Linezolid is not active against *Ureaplasma* spp. The extent of aquired resistance varies geographically. The antimicrobial therapy policies and the prior antimicrobial exposure in different populations determine the antibiotic resistance rates [23]. Fluoroquinolones are active against *Ureaplasmas*, but resistance has been reported [28]. It is likely that the widespread use of fluoroquinolones for treatment of respiratory and urogenital infections promoted the appearance and the spread of quinolone resistance in *Ureaplasma* spp. Mutations in the *gyrA* and *parC* genes of the DNA gyrase/topoisomerase IV complex occurred in the

presence of antimicrobial selective pressure [29]. Amino acid substitution in the type II topoisomerase proteins was proposed to be the reason of fluoroquinolone resistance. The most commonly noted substitutions were the followings: Ser83Leu in ParC protein, Asp112Glu in GyrA protein along with Ala125Thr and Ala136Thr in ParC protein (triple substitution). All are within the quinolone resistance determining regions. It was proved that the latter triple mutation is not related to quinolone resistance. It is a species-specific polymorphism, which is found in all *Ureaplasma* serovars. This fact highlights the importance of species determination of *Ureaplasma* isolates. The Gln100Arg amino acid transitions in GyrA could potentially be responsible for the ciprofloxacin MIC of 128 mg/L [22]. The Ser83Asn and Asp87Tyr or Val in ParC (corresponding to changes at amino acid positions 80 and 84 in *Escherichia coli* ParC) were commonly observed in fluoroquinolone-resistant strains of *Mycoplasma* and *Ureaplasma* spp. [30]. Naturally occurring fluoroquinolone resistance in *Ureaplasma* spp. from the United States was first reported in 2005. Until 2009, a total of 33 fluoroquinolone-resistant *Ureaplasma* isolates have been described [31]. 75% of the fluoroquinolone-resistant strains identified to date were *U. urealyticum*, even though this species is isolated only in ~20% of all *Ureaplasma* clinical isolates. The ParC Ser83Leu (or Ser80Leu in *E. coli*) mutation was found in 58% of the fluoroquinolone-resistant *Ureaplasma* strains. This mutation is a strong candidate for fluoroquinolone resistance. Homologous mutations has been identified in many other fluoroquinolone-resistant bacteria, such as *Streptococcus pneumoniae* and *Staphylococcus aureus* [22][20]. In case of Ser83Leu, a point mutation leads to an amino acid substitution. The substitution is two amino acids downstream from the proposed active site of the ParC protein [22][30][31]. The proposed Gram-positive origin of ureaplasmas may be responsible for the higher incidence of mutations within the topoisomerase IV proteins [22]. The drug resistance rates in *U. urealyticum* strains isolated from Turkish pregnant women were 92.6% to ciprofloxacin and 85.2% to ofloxacin. Resistance to josamycin was not observed, although some strains had intermediate resistance [23]. The mechanism of resistance for many fluoroquinolone resistant strains is still unknown. Site-directed mutagenesis methods can be useful to confirm the reported mutations whether they are fully account for the fluoroquinolone resistant phenotype in ureaplasmas.

U. urealyticum was considered as an intrinsically sensitive species to macrolides. Recently, resistance to macrolides has been widespread in *Ureaplasma* spp. The phenotype of aquired macrolid resistance mainly to roxithromycin and azithromycin was

detected [32]. The molecular characteristics of resistance to macrolides are well understood in bacteria, but are still undefined in *U. urealyticum*. The only reported mechanisms accounted for acquired resistance to macrolides in *U. urealyticum* up to now are the transition mutations in 23S rRNA [24]. The C2243N (T or C) transition in the 23S rRNA sequence might be associated with the acquired resistance to roxithromycin and azithromycin. This mutation has an influence on the tertiary structure of 23S rRNA and the binding of roxithromycin and azithromycin to 23S rRNA [32]. High-level erythromycin resistance is extremely uncommon in ureaplasmas. A mutation of the erythromycin-binding site around nucleotide position 2067 (2058 in *E. coli*) near the peptidyl transferase loop in domain V of one of the 23S rRNA operons was found consistently in erythromycin resistant *Ureaplasma* strains. Additional mutations of the associated L4 or L22 protein was found occasionally. Selected macrolide-resistant mutants of *U. parvum* was associated with a variety of mutations in 23S rRNA and in L4 and L22 ribosomal proteins [33]. A deletion of two adjacent amino acids in the L4 protein was detected for the highly resistant strain (MIC 64 μg/ml). Other resistance mechanisms, such as DNA methylation, expression of antibiotic modifying proteins, drug efflux via ion channels may be also responsible for the increased tolerance to erythromycin for ureaplasmas [20]. To detect the presence of a putative efflux phenotype, the susceptibilities of erythromycin resistant *Ureaplasma* mutants were determined in the presence and absence of the efflux pump inhibitor reserpine. No significant difference in MIC values was detected between the two groups, the putative efflux mechanism was not proved [33]. *U. urealyticum* harbors the *erm*B, *msr*A, *msr*B, and *msr*D genes. These genes confer resistance to macrolides and (or) lincosamides. The *erm*B gene, closely associated with the int-Tn gene, may be located in transposon in *U. urealyticum*. The *erm*B gene (a subtype of the *erm* gene) encodes a protein that can post-transcriptionally methylate the binding sites of macrolides, the 23S rRNA. This results in a resistant phenotype, while affecting macrolide binding to the 23S rRNA. The *msr* genes are common active efflux genes. They confer low level resistance to 14-, 15-membered macrolides (M phenotype) or 14-, 15-membered macrolides and streptogramin B (MS phenotype) in many bacteria. *Msr* genes, mainly the *msr*B and *msr*D genes, are also commonly observed molecular mechanisms of resistance to macrolides in *U. urealyticum*. The *erm*B and *msr* genes can be located in plasmids, transposons, or in the pathogenicity islands of enteric pathogens. The resistance genes might have been transmitted by these mobil genetic elements from enteric bacteria to other bacterium species, probably to *U. urealyticum,* too. The three *msr*

gene subtypes were not associated with the int-Tn gene, so they were not suggested to be located on transposon in *U. urealyticum* [24]. It is possible that *U. urealyticum* isolates have inherently increased tolerance to erythromycin, comparing with *U. parvum* isolates [20].

Tetracyclines are generally effective against *Ureaplasma* spp., but resistance is becoming more common. Acquisition of the *tetM*-transferable genetic element, that mediates tetracycline resistance was first described in *Ureaplasma* by *Roberts* and *Kenny* in 1986 [34]. Currently, this is the only mechanism of tetracycline resistance described in ureaplasmas. Tetracycline resistance in ureaplasmas has been reported to occur in approximately 1-3% of clinical isolates [35]. *Ureaplasma* strains resistant to tetracycline with a functional TetM protein were detected to be additionally resistant to doxycycline [20]. Screening for the *tetM* gene could detect tetracycline-resistant strains, but a false-positive strain was already found. This fact is a note of caution for the use of PCR screening for antibiotic resistance [22]. The sessile culture form of *Ureaplasma* which forms biofilm shows reduced antibiotic susceptibility, comparing with the planktonic culture form [26]. The major differences between the two types of cell growths were observed in the case of erythromycin and telithromycin. These antibiotics are more active against planktonic cultures. The most active antibiotic against biofilm forming *Ureaplasma* is clarithromycin. All examined *Ureaplasma* strains were fully susceptible to clarithromycin, independently of the type of culture [26].

PCR testing of the amniotic fluid in second trimester can help to identify women at risk for preterm labor and delivery because of *Ureaplasma* infection [36]. Real-time PCR assays could also be used to quantitatively screen vaginal swabs for *U. parvum* and *U. urealyticum* as risk factors in pregnant women [37]. Early antimicrobial treatment of infants based on the result of susceptibility testing could play a role in interrupting the *Ureaplasma* caused inflammatory cascade, hereby improving respiratory and other outcomes. Additional studies using azithromycin as an adjunctive therapy in preterm labor are now warranted [8] [38] .

2.2 Serological identification of gestational and congenital syphilis in the Hungarian population

The venereal syphilis causing Treponemas are included into the phylum Spirochaetes. The phylum contains five groups with markedly different genotypes and phenotypes: *Treponema*, *Leptospira*, *Borrelia*, *Spirochaeta* and *Brachyspira*. The *Treponema* genus can be further divided into the venereal disease causing *T. pallidum*

subsp. *pallidum* and the other non-venereal subspecies and species *T. pallidum* subsp. *endemicum*, *T. pallidum* subsp. *pertenue* and *T. carateum*. It is important to note that the oral flora, especially the periodontal pockets contains various other *Treponema* species, such as *T. denticola, T. vincentii, T. parvum* that morphologically hard to distinguish from *T. pallidum* subsp. *pallidum.* This similarity makes the direct identification of *T. pallidum* subsp. *pallidum* in mucosal lesions uncertain. Also, the seroresponse to oral treponemas could lead to false positivity of the serological detection of *T. pallidum* subsp. *pallidum.*

The socioeconomical impact of venereal syphilis is significant. According to the Centers for Disease Control and Prevention (CDC) the syphilis prevalence was approximately 400 cases/ 100000 population in the 1940s, before the antibiotics era [39]. The syphilis prevalence then steadily decreased and reached 2.1 cases per 100.000 population in 2000-2001. After 2001, the number of syphilis cases started to increase to 7.5 cases per 100.000 population by 2015. The prevalence of syphilis was correlated with sex and sexual behavior (highest among men, especially among men sex with men (MSM)), age (highest prevalence age groups were 20–24 and 24–29 years). Syphilis prevalence was also different between races and regions. Importantly, parallel to the increase of the adult acquired syphilis cases, the congenital syphilis cases also increased during the 2000s, reaching 12.4 cases per 100.000 live births at 2015. The Hungarian epidemiology data show similar changes [40]. In 2012 the number of syphilis cases increased by 10% compared to 2011, with more than three times more males than females. The highest syphilis prevalence was measured at the age group of 25-29 for males, and of 20-24 for females. The regional incidence was the highest in Budapest, and Bács-Kiskun county at the countryside.

Syphilis is principally a sexually transmitted disease, the exudative lesions of primary and secondary syphilis containing a large number of Treponemas are the most important in transmission. Besides the sexual route, syphilis can be transmitted vertically, the bacterium can cross the placenta leading to congenital infection. Neonates can also become infected by direct contact to *T. pallidum* containing lesions and/ or the mothers' blood during mainly the delivery. The clinical manisfestations of syphilis can be grouped into three different stages. After 10-90 days of incubation, the primary syphilis appears as a painless indurated ulcer (chancre) at the site of the inoculation. The secondary syphilis starts 3-8 weeks after the appearance of the primer chancre and predominantly appears as mucocutaneous lesions. The lesions disappear in 4-12 weeks and the patients enters the latent syphilitic stage when the pathogen is not detectable from mucosal and

cutaneous samples, but the specific anti-treponemal tests are present and the non-specific infection/ inflammation related seroresponses may (*syphilis latens recens*) or may not be (*syphilis latens tarda*) present. Years from the secondary syphilis the tertiary phase of the disease starts with gumma formation at various organs including skin, bone, internal organs and the central nervous system. The cardiovascular syphilis is also a predominant sign of the tertiary phase, mainly involving the aorta manifesting as aorta aneurysm and insufficiency. The vertical transmission to the child leads to congenital syphilis, with no or minor signs at birth in the majority of the cases. Secondary syphilis can be observed in some of the newborns, where the lesions are not localized to the mucocutaneous surfaces only, but involves internal organs. After two years of age late congenital syphilis starts that is equivalent to adult tertiary syphilis, and has several clinical manifestations including interstitial keratitis, deafness and blunted upper incisor teeth (Hutchinson's triad), neurosyphilis and bone deformities.

The detection of syphilis infection can be performed either by the direct detection of the bacterium in infected tissues or the detection of the antibody response against the pathogen. A specialty in syphilis diagnosis is that non-specific serological reactions detecting antibodies specific for self-antigens released during the course of the active infection is included in the diagnostic protocol. The direct detection is useable in primary and secondary syphilis, where the bacterium concentration is high enough in the infected lesions. *T. pallidum* is very thin (0.2 μm diameter) spirillum, therefore the normal light microscopy is not usable, staining, especially Gram staining is not effective, but dark-field microscopy is useable for detection. Besides the microscopy, PCR methods are also under development for direct detection. Serological assays can be grouped to nontreponemal tests (NTT) and treponemal tests. NTTs measure the IgG and IgM autoantibodies generated against the lipids released from infected/ inflamed human cells. NTTs use a mixture of cardiolipin, cholesterol and lecithin as antigens in two major types of precipitation (flocculation) reactions, the venereal disease research laboratory test (VDRL), and the rapid plasma regain test (RPR). While these tests are relatively cheap, both sensitivity and specificity problems are well known. NTT sensitivity is about 75% [41], due to various reasons, such as inactive or treated infection, or due to the prozone effect where the high antibody level is prohibiting the precipitation reaction leading to false negative results. False positivity could also be detected due to various reasons, such as pregnancy, fever, autoimmune diseases and active tuberculosis. It is important to note, that the rapid decrease of NTT titer during treatment can be used to monitor the treatment

efficiency. The specific treponemal tests screen the *T. pallidum* specific antibody responses and opposite to the NTTs are able to screen the IgM and IgG levels separately. Treponemal tests include agglutination reactions such as *T. pallidum* hemagglutination assay (TPHA) and *T. pallidum* particle agglutination assay (TPPA) where the treponemal antigens are attached to the surface of particles. The fluorescent treponemal antibody absorption assay (FTA-ABS) uses fixed bacteria to screen the patients serum after it was depleted by a non-pathogenic treponemal strain. The binding is detected by a secondary immunofluorescent antibody. ELISA reactions using various treponemal antigens has been developed and used with high specificity and sensitivity. A more detailed screen of antitreponemal seroresponse is also possible by Western-blot and line immunoassay technologies, where multiple *T. pallidum* antigens are attached to a solid membrane surface. Simlar to ELISA, these assays also can screen the IgM and IgG responses separately with high specificity and sensitivity. Various diagnostic algorithms apply the NTTs and treponemal tests in combination to achieve the highest sensitivity and specificity considering the cost of the screening, the laboratory background and the prevalence of syphilis in the population. The traditional algorithm uses the NTTs first as a cost-effective screening step, and a treponemal test as a confirmatory step. Since this algorithm starts with an NTT, all the above mentioned sensitivity and specificity problems are encoded in the procedure. The reverse algorithm uses treponemal tests first as an identification step, and secondly an NTT for measuring the activity of the disease. If there is no match between the two tests, a second treponemal test is advised. This protocol could achieve close to 100% sensitivity and specificity, easy to automate but more expensive than the regular approach. There is a third approach, where the NTT step is omitted, and a treponemal test and a different other treponemal test is applied as a confirmation.

The identification of congenital syphilis predominantly relies on the screen of the mother's seropositivity by NTT and treponemal tests in the first and third trimesters. Although the risk of fetal infection is much higher during early maternal syphilis (the first year of infection) than during later stages, *T. pallidum* can be transmitted from the bloodstream of the infected woman to her developing fetus at any time during pregnancy [42]. The diagnostic algorithms are identical to the above described ones. Current Hungarian guidelines suggest that all pregnant women should be tested for *T. pallidum* infection in the first trimester [43]. Besides the NTTs sensitivity and specificity problems we mentioned before, the altered immune state of pregnancy could lead to false positivity

for NTT and treponemal tests, therefore the additional validation steps would be necessary. Serological testing of the newborns is also complicated by the transplacental transfer of maternal IgG. Fourfold or higher NTT titer of the newborn versus the mother is highly indicative of the newborn's infection, on the other hand, less than fourfold difference has a low predicitive value. For adults, treponemal tests ELISA or TPPA can be used for screening, in all stages with or without signs and symptoms of syphilis [44]. However, standard serological tests for syphilis detect both immunoglobulin IgG and IgM, including transplacentally acquired maternal IgG, therefore they cannot be used to provide a laboratory diagnosis of congenital syphilis when a single serum sample is tested. On the other hand, IgM ELISA and IgM immunoblot proved to be highly sensitive and specific to detect infant syphilis [45].

In our study, we screened the *T. pallidum* IgM status in 241 mothers with syphilis to determine if positive tests indicate a risk of connatal syphilis better than RPR test, and we also evaluated the use of IgM immunoblots for the identification of infants with congenital syphilis.

2.3 Measurement of the antimicrobial activities of various antiseptics against *C. trachomatis*

C. trachomatis is an obligate intracellular bacterium with cell tropism to epithelial cells of the conjunctiva and the urogenital tract. The bacterium is particularly capable of establishing persistent infections and chronic inflammation. The local inflammation could lead to oviduct fibrosis potentially leading to infertility. Sexually transmitted infections (STI) caused by the urogenital tract pathogens *C. trachomatis* serovars D-K and L1-L3 are the most frequent STIs in the world [46]. While antiseptics are not part of the antichlamydial chemotherapy, they have various intravaginal applications including the prevention of postoperative infections before cesarean section [47][48], trans-vaginal ultrasound-guided ovum pick-up [49], surgical treatment of HPV generated cervical lesions [50] and other invasive procedures [51], and prevention of early-onset neonatal group B *Streptococcus* infection [52]. Besides these applications, antiseptics are being used to prevent/ treat prechlamydial states such as bacterial vaginosis. Bacterial vaginosis is a state where the normal *Lactobacillus* flora of the vagina is disappearing and is replaced by a polybacterial flora. Several studies showed that bacterial vaginosis is a significant risk factor of acquiring STIs including chlamydial STIs with a relative risk of 2.0-3.4 [53][54][55]. The connection between bacterial vaginosis and chlamydia infection is not clear, but one of the possibilities is the increased indole production of the bacterial

vaginosis related bacteria [56]. Urogenital *C. trachomatis* serovars likely propagate in a tryptophan deprived environment due to the interferon-gamma induced indole 2,3-dioxygenase, but the bacterium can survive by producing tryptophan from the exogenous indole. There are several treatment options of bacterial vaginosis including the antibiotics metronidazole, clindamycin, the antiseptics benzydamine, chlorhexidine, hydrogen peroxide, povidone-iodine, borax, acidification, prebiotics and recolonizing the cervicovaginal region with lactobacilli [57]. Despite the various treatments the long term cure rate of bacterial vaginosis can be as low as 20-50% [58] [59], therefore search for more effective therapies including effective antiseptic therapies is necessary. Importantly, an ideal antiseptic should treat the bacterial vaginosis and inhibit the underlying STIs, including the frequent chlamydial STI. Chlamydiae have a complex developmental cycle. The bacterium has two major forms, the extracellular, infectious and metabolically relatively dormant elementary body, and the intracellular, non-infectious, metabolically active reticulate body. The infection of the host cells starts with the attachment of the elementary body to the host cell receptor(s). This is a key step, when the disinfectants can interfere with the chlamydial developmental process. Obviously, the attachment to the host cells requires that the potential chlamydial ligands remained intact. Alteration of the tertiary or quaternary structure of these chlamydial proteins by disinfectants can significantly inhibit the complete developmental cycle.

Various potential chlamydial ligands could be identified as potential disinfectant targets. *C. trachomatis* genome encodes nine polymorphic membrane protein genes. One of them, pmpD is conserved among all *C. trachomatis* serovars and anti-pmp antibodies are capable to neutralize the infection [60]. *C. trachomatis* pmpD null mutants also were not able to attach to human endocervical cells [61]. Another *Chlamydia*, *Chlamydia pneumoniae* (*C. pneumoniae*) has proteins pmp6, pmp20 and pmp21 shown to be involved in the attachment of elementary bodies to human cells. Also, the *C. pneumoniae* pmp21 protein's receptor, the human epidermal growth factor receptor (EGFR) has been identified [62]. Chlamydiae have no capsule, they have a truncated lipopolysaccharide (LPS), the outer surface of the bacterium consists of mainly proteins. Besides the above-mentioned chlamydial proteins, several other proteins can be found in the outer membrane of *C. trachomatis*, the so-called outer membrane complex (COMC) [63] [64]. Birkelund et al. used two-dimensional-polycacrylamide gel electrophoresis (2D-PAGE) and combined fractional diagonal chromatography (COFRADIC) method to separate *C. trachomatis* L2 outer membrane proteins and N-terminal proteolytic fragments resulted

by proteolytic cleavage sites of COMC proteins [63]. The most abundant proteins/ protein cleveage peptides identified were the major outer membrane protein (MOMP), Omp2, CTL0626 (Ct372, OprB), PorB, PmpF, PmpG, CTL0541 (Ct289), CTL0887 (Ct623), PmpH and YscC. Identification of CTL0887 (Ct623) as a relatively abundant protein was particularly interesting, since it has not been described before as a COMC protein. Among the identified COMC proteins several were porins including the MOMP, PorB, Omp85 and the hypothetical protein CTL0626. The potential importance of the identified CTL0626 is supported by the fact that homologues could be found in other serovars/ species such as *C. trachomatis* serovars (A, D), *C. muridarum* Nigg, *C. pneumoniae*, *C. abortus*, *C. caviae* GPIC and *C. felis* Fe/C-56. From the 9 pmp proteins described in *C. trachomatis* L2, pmpB, C, D, E, F, G and H were found in the COMC. Besides porins and other outer membrane proteins, members of the chlamydial Type-III secretion system were also identified, such as the *Yersinia* YscC homologue, and the low calcium response protein D (LcrD).

C. trachomatis outer membrane proteins are likely also involved in the characteristic tyrosine phosphorylation cascade accompanying the attachment/ entry of the elementary bodies. Birkelund et al described first by phosphotyrosine specific Western-blot, that *C. trachomatis* serovar L2 induced the tyrosine phosphorylation of various proteins at the 66, 64, 68, 97 and 140 kDa range [65]. The tyrosine phosphorylated proteins appeared 15 minutes after the attachment of the elementary bodies, indicating that the trigger of the tyrosine phosphorylation was the attachment/ entry of the bacterium. This hypothesis was further supported by the fact that the tyrosin phosphorylation could not be detected when the attachment of the elementary bodies was inhibited by pretreatment with the known attachment inhibitor heparin. Virok et al. showed that there were subtle differences in the pattern of tyrosine phosphorylated proteins between the various *C. trachomatis* serovars, and interestingly the pattern differences correlated with the tissue/host tropism of the tested Chlamydiae [66]. The mucoso-tropic, trachoma causing *C. trachomatis* serovars induced tyrosine phosphorylation of three major proteins in the 75 to 70 kDa range, while the *C. trachomatis* lymphogranuloma venereum (LGV) lympho-tropic biovars induced the tyrosine phosphorylation of the two larger proteins. *C. muridarum* MoPn, a murine chlamydia strain induced the tyrosine phosphorylation of higher molecular weight proteins at the 75 to 85 kDa range. Surprisingly, *C. pneumoniae* and its relative, *C. caviae* did not induce tyrosine phosphorylation. The infectious process were performed and compared in human and murine epithelial cells. The fact that the

observed tyrosine phosphorylation patterns were identical in murine and human cells indicated that the phosphorylated proteins are either strictly conservative host proteins with the same molecular weight in these two species, or more likely chlamydial proteins that retained the same molecular weight during the infection of both host cells. Clifton et al. showed that at least one of the tyrosine-phosphorylated protein had a chlamydial origin CT456 (Tarp) [67].

Genome sequencing data also showed that *C. trachomatis* also possess a Type-III secretion system (TTS), and as we mentioned above components of the TTS could be found in the COMC. TTS forms a needle-like structure and is involved in the translocation (injection) of bacterial proteins into the host cells during the attachment/ entry phase and also at later stages of the developmental cycle. Subtil et al. screened for potential *C. pneumoniae* proteins that could be a substrate of the TTS, and had homologues in *C. trachomatis* and *C. caviae* [68]. They identified 24 potential TTS substrate, including proteins that could be involved in the early critical interaction between the elementary body and the host cell.

All of the above mentioned and functionally important COMC proteins are easily accessible and could be target of disinfectants, but we have to note that the cytoplasmic proteins of *Chlamydia* also could be inhibited by the applied antiseptics. The tested antiseptics were iodine, povidon-iodine, chlorhexidine and borax. Probably the most widely used ones in the clinical practice are povidone-iodine (Betadine) and chlorhexidine. Povidone-iodine is a so-called iodophor where the iodine forms a stable chemical complex with polyvinyl-pyrrolidone carrier. The advantage of povidone-iodine compared to the iodine solution is the better stability, longer release and tissue presence and less irritability. Povidone-iodine is bactericidal, virucidal but it is less effective against spores and fungi. The antimicrobial compound is the elemental iodine [69]. Iodine can oxidize membrane components but also penetrates into the bacteria and can attack intracellular proteins and nucleic acids. Iodine can oxidize sulfhydryl bonds between cysteine and methionine, react with the phenol groups of tyrosine and amines in arginine, histidine and lysine. It reacts with nucleic acid bases and oxidizes secondary bonds in fatty acids chains. Chlorhexidine is a bactericidal compound. Chlorhexidine can also be uptaken efficiently by bacteria, therefore it can target intracellular metabolism, but it is believed, that the primary target is the plasma membrane and outer membrane of Gram negatives. At low concentration it disrupts membrane integrity and cause the efflux of cytoplasmic content, while at higher concentration it coagulate intracellular bacterial

macromolecules [69]. Borax (sodium borate) is a salt of boric acid. The exact antimicrobial mechanism of borax is not well characterized. Its fungistatic activity is likely due to the increase in cell wall permeability and disintegtration of the plasma membrane [70]. Boric acid has been used for the prevention of recurrent vulvovaginal candidiasis [71]. It also showed bacteriostatic activity against *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Escherichia coli* and various other strains of *Enterobacteriaceae* and several *Pseudomonas* species [72].

3. Aims

I. to determine the incidence of *Ureaplasma urealyticum/ parvum* infections in sexually active individuals according to ages.

II. to examine the age distribution of *Ureaplasma*-positive patients and the frequency of manifestation of symptoms.

III. to determine the antibiotic resistance spectrum of *U. urealyticum/ parvum* isolated from these patients.

IV. to evaluate the *Treponema pallidum* IgM immunoblots for the identification of newborns with congenital syphilis compared with rapid plasma reagin (RPR), *T. pallidum* particle agglutination (TPPA), and enzyme immunosorbent assay (EIA) tests.

V. to determine the antichlamydial effects of iodine aqueous solution, povidone-iodine, chlorhexidine and borax.

4. Materials and methods

4.1 *Ureaplasma/ Mycoplasma* detection and antibiotic sensitivity

To study the incidence and antibiotic susceptibility of Hungarian *Ureaplasma* strains during the first period of the study 4154 samples, 2114 from female and 2040 from male patients ranging between 15 and over 60 years of age, were obtained to culture for the presence of *Ureaplasma.* For *Ureaplasma/ Mycoplasma* cultivation, sampling was peformed with cervix/urethra flexible cotton swabs (Biolab, France) from patients with non-gonococcal-non-chlamydial (NGNC) urethritis, or other genitourethral complaints, and from symptom-free promiscuous patients. Majority of the samples were taken from the cervix in the case of female patients and from the urethra in the case of male patients. Some other samples of the urogenital tract were also examined. For culturing samples Mycoplasma Duo kit (Bio-Rad, France) was used, with an incubation period of 48 hours, in 37^{o}C environment with elevated CO_2 tension [73]. The determination of antibiotic sensitivity of 373 *Ureaplasma* strains collected during a six-year period was done in U9 medium with SIR Mycoplasma kit (Bio- Rad®) under the same circumstances [74].

4.2 Antibody detection and measurement in syphilis

After prescreening 33.753 serum samples for the presence of syphilis, we used rapid plasma reagin (RPR) (Omega Diagnostics, UK), *T. pallidum* particle agglutination (TPPA) test (Fujirebio Inc, Japan), and Enzime immunosorbent assay (Syphilis II-EIA) test (BioRad, France) to investigate 241 maternal serum samples previously proven to be syphilitic and 242 serum samples of the neonates. IgM Immunoblots (MAST, UK) were prepared from all the 483 serum samples, but the IgM-IgG complexes, and the maternal IgG were evaluated with MastSorb (MAST, UK) from the infants' serum. IgG Immunoblots (MAST, UK) were prepared from the 241 maternal serum samples. One of the infants had symptoms resembling neurosyphilis, this diagnose was confirmed with Veneral Disease Research Laboratory (VDRL) test (Omega Diagnostics, UK) and TPPA in the cerebrospinal fluid.

4.3 Measurement of the antichlamydial effects of antiseptics

To test the antichlamydial activity of frequently used antiseptics, we infected HeLa cervical epithelial cells with *C. trachomatis* in the presence of iodine aqueous solution, povidone-iodine, chlorhexidine and borax. For chlamydial growth measurement we used the recently published direct qPCR based approach [75].

4.4 *C. trachomatis* propagation and HeLa cell culture

C. trachomatis serovar D strain (UW-3/CX, ATCC) was used, the strain was propagated and partially purified as described previously [76]. HeLa 229 cells (ATCC) were transferred into 96-well plates (Sarstedt, Nümbrecht, Germany) at a density of 6 x 10^4 cells/well in 100 µl of minimal essential medium (MEM) with Earle salts supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1x MEM vitamins, 1x non-essential amino acids, 0,005% Na-pyruvate, 25 µg/ml gentamicin, 1 µg/ml fungisone. HeLa cells were incubated overnight at 37 °C, 5 % CO_2 to obtain a 90 % confluent cell layer.

4.5 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was performed to characterize the maximum non-toxic concentration of the antiseptics. HeLa cells were transferred into the wells of the 96-well plate (Sarstedt) at a density of 6 x 10^4 cells/well in 100 µl of culture medium (see above). The plates were incubated for 1 h at room temperature (RT) and then overnight at 37 °C, 5 % CO_2 to obtain a 90 % confluent cell layer. Next day the medium was supplemented with the serial 2-fold dilutions of the antiseptics in three parallel wells for each concentration. After 48 h incubation, 10 µl of the MTT (SIGMA, St. Louis, MO, USA) labelling reagent (final concentration 0.5 mg/ml was added to each well. The plate was incubated for 4 h at 37 °C, 5 % CO_2. After the incubation, 100 µl of the solubilisation solution (10 % SDS in 1 N HCl) was added into each well. The plate was allowed to stand overnight in the incubator at 37 °C, 5 % CO_2. Next day the optical density of the wells were measured by a microtiter plate reader (Labsystems Multiskan Ex 355, Thermo Fisher Scientific, Waltham, MA, USA). The absorbance of the formazan product was measured at 540 nm. The average viability (OD 540) of three wells with untreated HeLa cells was considered the 100% viability. Viabilities of the treated cells were compared to the untreated controls as follows: Cell Viability (%) = (OD 540 of treated cells/ OD 540 of untreated cells) x 100.

4.6 Antiseptics used for *C. trachomatis* growth inhibition, *C. trachomatis* infection and DNA extraction

Iodine aqueous solution, povidone-iodine (Betadine, Egis, Budapest, Hungary), chlorhexidine-digluconate (Chlorhexamed, GlaxoSmithKline, Brentford, UK) and borax were diluted in sucrose-phosphate-glutamic acid buffer (SPG). Concentration ranges of 100-0.78 µg/ml for iodine aqueous solution and borax, 390-3 µg/ml for povidone-iodine

and 4-0.003 μg/ml for chlorhexidine with 2-fold dilutions were tested. Before infection, *C. trachomatis* elementary bodies were incubated in the antiseptics for 1 h, 37 ^{o}C, 5 % CO_2. HeLa cells were washed twice with 200 μl/well of phosphate buffered saline (PBS), pH 7.4 and were infected at multiplicity of infection (MOI) 8 in 100 μl 0.5 % (w/v) glucose medium for 60 min, RT. The cells were washed twice with PBS and culture medium containing 1 μg/ml cycloheximide was added. The plates were incubated at 37 °C, 5 % CO_2 for 48 h. The cells were washed with PBS twice, resuspended in 100 μl Milli-Q (MQ) water (Millipore, Billerica, MA, USA), and subjected to two freeze-thaw cycles and mixing as it was described before [75]. The mixed lysates were used as a template in the qPCR. All reagents were purchased from SIGMA, St. Louis, MO, USA, unless otherwise indicated.

4.7 Direct qPCR to study the effects of the antiseptics on *C. trachomatis* growth

Direct qPCR was performed as described before [75] in a Bio-Rad CFX96 real-time system, using the SsoFast EvaGreen qPCR Supermix (Bio-Rad, Hercules, CA, USA) master mix and the *C. trachomatis* primer pairs *pykF*: 5'-GTTGCCAACGCCAT-TTACGATGGA-3', 5'-TGCATGTACAGGATGGGCTCCTAA-3'. The PCR mixture consisted of 5 μl SsoFast EvaGreen supermix, 1-1 μl forward and reverse primers (10 pmol each), 1 μl template, and 2 μl MQ water to 10 μl final volume. After the 10 min at 95 °C polymerase activation step, 40 PCR cycles of 20 s at 95 °C and 1 min at 64 °C were performed, with measurement of the fluorescence intensity at the end of the annealing-extension step. The qPCR ended with a melting curve analysis. Student's t-test has been used to compare the statistical differences of Ct values between two experimental conditions as it was described before [75].

4.8 Immunofluorescent monitoring of *C. trachomatis* growth

C. trachomatis growth was evaluated by immunofluorescent staining as it was described before [77]. Briefly, semiconfluent layers of HeLa cells (6 x 10^4 cells/well) in chamber slides (Lab-Tek chamber slide system, Thermo Fisher Scientific, Waltham, MA, USA) were infected with untreated *C. trachomatis* MOI 8 (preincubated in SPG buffer at 37 ^{o}C for 1 h) or *C. trachomatis* preincubated with iodine aqueous solution (100 μg/ml), povidone-iodine (390 μg/ml), chlorhexidine (4 μg/ml) and borax (100 μg/ml) in SPG buffer at 37 ^{o}C for 1h. Before infection the wells were washed with 200 μl/well of PBS. After removal of the PBS solution, the treated and untreated chlamydiae were added and

the cells were incubated at 37 °C under 5 % CO_2 for 1 h. After infection, the inocula were replaced with a culture medium containing 1 µg/ml cycloheximide and were incubated at 37 °C, 5 % CO_2 for 48 h. After removing the culture medium from the slides, the cells were washed twice with PBS (200 µl/well). After detaching the chamber structure from the slides, the cells were fixed with precooled 100 % acetone at -20 °C for 10 min. Anti-chlamydia LPS antibody (AbD Serotec, Oxford, United Kingdom) labeled with Alexa-647, was used at 1:200 dilution for detection of chlamydial inclusions. Following an incubation of 1 h at 37 °C, the cells were washed three times with PBS for 7 min and at last with distilled water. Fluorescence signals were analyzed with an Axon GenePix Personal 4100A DNA chip scanner and GenePix Pro (version 6.1) software (Molecular Devices, Sunnyvale, CA, USA) using the Cy5 channel and a 5 µm resolution. Inclusion counts were determined by the ChlamyCount software as it was described before [77].

5. Results

5.1 Incidence and antibiotic susceptibility of genital *Ureaplasmas* in sexually active individuals in Hungary

5.1.1 Incidence of genital *Ureaplasmas*

During the first period of the study 4154 samples were obtained to culture for the presence of *Ureaplasma* (**Table 1**).

Specimen taken from/by	Number of females (N=)	Ureaplasma positive (N (%))	Number of males	Ureaplasma positive(N (%))
Urethra swab	59	4 (7.02%)	2025	61 (3.01%)
Cervix swab	2047	225 (11.00%)	-	-
Urine	8	0	6	0
Glans swab	-	-	2	0
Ejaculate	-	-	7	0
Subtotal	2114	229 (10.83)	2040	61 (2.99)
Total samples/positive together			4154/290 (6.98%)	

Table 1. Distribution of samples obtained from sexually active individuals to culture for *U. urealyticum/parvum* and the incidence of *Ureaplasma* strains [78].

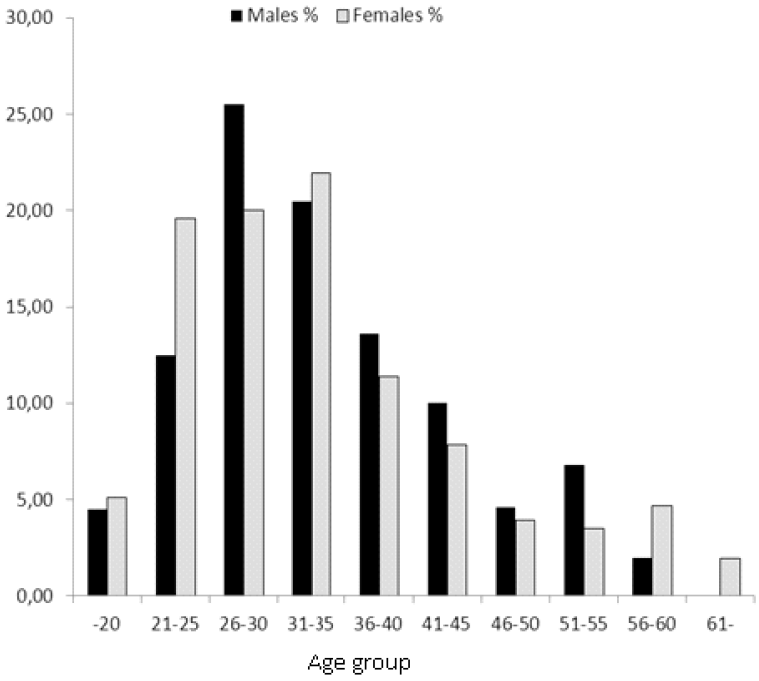
Former microbiological examinations ruled out the possibility of *N. gonorrhoeae* and *C. trachomatis* infections. 2114 samples were collected from female patients. 2047 samples were cervix swab and 11% of these samples were *Ureaplasma* positive by culture. 59 urethra samples and eight urines from females were examined. 7.02% of the urethra specimens were *Ureaplasma* positive, while all the urine samples were negative. Based on these data it can be estimated that cervix swab is the most suitable sample for *Ureaplasma* culturing in female patient.

2040 samples were collected from male patients. The vast majority of these samples were obtained from urethra. Only 15 samples were glans swab, ejaculatum or urine. None of these last ones showed positive culture result. 3.01% of the urethra samples turned out to be *Ureaplasma* positive by culture. According to these data, NGU was the main symptome in male patients. In case of balanithis, also urethra samples should be collected, because glans swabs were not suitable samples for *Ureaplasma* culture. Altogether 229

and 61 *Ureaplasma* strains were isolated in this study period from females and males, respectively. The total prevalence of *Ureaplasma* strains in the whole population examined was 6.98 %. Subsequent study in the next two years slightly modified these tendencies showing an increase in the prevalence of *Ureaplasma* positive patients [74] .

5.1.2 Age distribution and frequency of symptoms in *Ureaplasma* positive patients

Ureaplasma positive females and males covered a broad spectrum of ages from less than 16 to over 60-year old. *Ureaplasma* positive females from 20 years of age increased rapidly reaching a plateau at 26-year old which existed to 36-year old then gradually decreased to the late 60 **(Figure 1)**.



	-20	21-25	26-30	31-35	36-40	41-45	46-50	51-55	56-60	61-
Males %	4,5	12,5	25,5	20,5	13,6	10	4,6	6,8	2	0
Females %	5,1	19,61	20	21,96	11,37	7,84	3,92	3,53	4,71	1,96

Figure 1. Age distribution of *Ureaplasma* positive patients (% of all the patients).

The distribution of *Ureaplasma* positive males showed a different picture. It increased linearly from 5% at 20-year old men reaching a 25% peak at 30-year old then linearly decreased to below 5% at 50-year old following by a small rise between 51-55 year-olds then it decreased again to 0 over 60-year old of age **(Figure 1)**.

The ratio of symptom-free persons among *Ureaplasma* positive individuals was above 40% in both sexes. The dominant clinical symptoms in females were vaginal discharge (23.53%), genital pruritus (9.19%) and colpitis (11.76%). The dominant clinical

symptoms in males were urethritis (35.48%), balanitis (13.97%) and urethral discharge (7.52%).

5.1.3 Antibiotic resistance of *Ureaplasma* strains

Antibiotic	No. of strains tested	Sensitive		Moderately sensitive		Resistant	
		No.	%	No.	%	No.	%
Erythromycin	*373*	47	12.56	23	6.1	303	81.23
Azithromycin	*373*	320	85.79	17	4.55	36	9.65
Clindamycin	*373*	85	22.78	8	2.1	280	75.06
Ofloxacin	*373*	264	71.58	15	4.2	94	25.2
Tetracycline	*373*	358	95.9	0	0	15	4.1
Doxycycline	*373*	363	97.32	1	0.27	9	2.41

Table 2. Distribution of antibiotic sensitivity of 373 *Ureaplasma urealyticum/parvum* strains cultured from genital samples [74]**.**

Table 2 shows that no fully sensitive *Ureaplasma* strain was found. Extremely high incidence of resistance was detected in the *Ureaplasma* strains against erythromycin and clindamycin. However, cross resistance against the two macrolides, the older erythromycin and the newer azithromycin existed only in less than 10% of the strains. Every fourth strain was resistant to ofloxacin as representative of fluoroquinolons. Doxycycline proved to be the most effective agent with 97.32% susceptibility, followed by tetracycline (95.9%) and azithromycin (85.79%).

5.2 Serological identification of gestational and congenital syphilis in the Hungarian population

5.2.1 Clinical staging of pregnant syphilis positive women

Positive syphilis serology was noted in 241 pregnant women out of the 33.753 serum samples during a three year period. **Table 3.** shows the status of 241 pregnant syphilitic women at the time of their laboratory examinations.

Before pregnancy	During pregnancy			At delivery	
succesfully treated	Syphilis I	Syphilis latens recens	Syphilis latens tarda	Syphilis latens recens	Syphilis latens tarda
217	2	9	2	4	7

Table 3. Number and staging of maternal syphilis diagnosis

5.2.2 Serostatus of pregnant syphilis positive women and newborns

230 mothers had adequate prenatal care. 217 of the cared mothers have already been adequatly treated against syphilis before pregnancy, they were advised to repeat therapy, according to the Hungarian guidelines [79]. These women had RPR titer of 0-1:16, results of TPPA, EIA and IgG immunoblot tests were also positive, but IgM Immunoblot results were negative. Children of these mothers had RPR titers of 0-1:8, equal or less than their mothers. All TPPA and EIA reactions were positive in these children, only IgM immunoblots were negative. These infants were uninfected, without any symptoms of connatal syphilis at birth. They received careful follow-up examinations and serological testing at 0, 3, 6, 9 and 12 months. RPR titer was negative of two mothers who acquired syphilis I stage during their pregnancy. The diagnosis was confirmed with positive TPPA, EIA and IgM immunoblot, and with negative IgG immunoblot results. They were immediately treated with intramuscular benzathine-penicillin. None of the two children born to these mothers presented clinical or laboratory evidence of infection, no positive serological test, included IgM immunoblot was found in these two infants. Nine pregnant women received the diagnosis of syphilis latens recens during their pregnancy. RPR titers in this group of mothers were 1:16 and 1:32 in five and four cases, respectively. Except the IgM immunoblot, all the treponemal tests were positive to these mothers. After adequate treatment the RPR titers decreased four-fold. Their infants were born with the same serological results as the mothers, but they were uninfected. Syphilis latens tarda was the diagnosis of two pregnant mothers. RPR results were negative, the TPPA, EIA

and IgG immunoblot tests were positive, but the IgM immunoblot was negative. After adequate treatment during pregnancy, the infants of these mothers had the same serological results as their mothers, but none of them was infected. Finally, eleven women, having received inadequate prenatal care, classified as latent syphilis patients, were first found to have reactive test results for syphilis at delivery. Seven mothers had the diagnosis of syphilis latens tarda, with negative RPR and IgM immunoblot results, but with positive TPPA, EIA and IgG immunoblot tests. At delivery the RPR test results of their infants were negative, however, TPPA and EIA were positive. None of these children had a positive IgM immunoblot result.

	maternal RPR titer	maternal IgM	RPR titer of the newborn child	IgM of the newborn child	diagnosis of the newborn child
1. mother	1:64	negative	1:64	negative	uninfected
2. mother	1:2	positive	twin A: 1:8	positive	connatal syphilis
			twin B: 1:2	positive	connatal syphilis
3. mother	1:256	positive	1:256	positive	connatal syphilis
4. mother	1:64	positive	1:64	positive	connatal syphilis

Table 4. Serological results of mother-child pairs, from cases, when maternal syphilis latens recens diagnosis was observed only at delivery

The other four mothers, as shows, had the syphilis latens recens diagnosis at delivery, with an RPR titer at least 1:2 to a maximum of 1:256. TPPA, EIA and IgG immunoblot tests were all positive (**Table 4)**. One mother with an RPR titer of 1:64 has already had IgM negative immunoblot result. Her baby had the same serological results and had no clinical symptoms of syphilis, after adequate treatment the follow-up examinations and serological testing at 0, 3, 6, 9 and 12 months confirmed the absence of connatal syphilis infection. The last three women were found to have positive IgM results together with the other reactive syphilis tests at delivery. Remarkably, the first of them, an intravenous drug

user, has had an RPR titer only of 1:2, but one of her twin sons has died immediately after birth. The RPR titer of this „A" infant was 1:8. RPR titer of „B" infant was 1:2, his treponemal tests, including IgM immunoblot, were all positive. Clinical symptome of seizures suggested the existence of neurosyphilis in this child. Lumbar puncture was performed to obtain cerebrospinal fluid for VDRL and TPPA tests. VDRL was negative, but TPPA was positive of a 1:80 titer. The second mother-child pair had the same RPR titer of 1:256, all treponemal tests, including IgM immunoblot were positive in both of them, but the infant had no symptoms of connatal syphilis. The last mother had an RPR titer of 1:64, TPPA, EIA and IgG, IgM immunoblots were positive. The premature daughter had RPR titer of 1:64, positive results of TPPA, EIA and IgM immunoblot. Prematurity was the only non-specific clinical manifestation of connatal syphilis in this case.

5.3 Measurement of the antimicrobial activities of various antiseptics against *C. trachomatis*

5.3.1 Cell viability of HeLa cells incubated with antiseptics

First we tested the long term impact of the antiseptics on the viability of the HeLa human cervical epithelial cells. HeLa cell viability was determined by the MTT assay after 48 h incubation with the particular antiseptic dissolved in cell culture medium **(Figure 2 A-D)**. Maximum cytotoxicity was observed at concentrations of =>1562 μg/ml for povidone-iodine, while the viability reached its maximum at concentration of =<390 μg/ml. We considered this value the maximum non-toxic concentration. Maximum cytotoxicity was observed at concentrations of =>8 μg/ml for chlorhexidine, its maximum non-toxic concentration was 2 μg/ml. Maximum cytotoxicity was observed at concentrations of 800 μg/ml for iodine aqueous solution, its maximum non-toxic concentration was 200 μg/ml. Maximum cytotoxicity was observed at concentrations of 800 μg/ml for borax, its maximum non-toxic concentration was 25 μg/ml, however the viability reached 80% at 400 μg/ml.

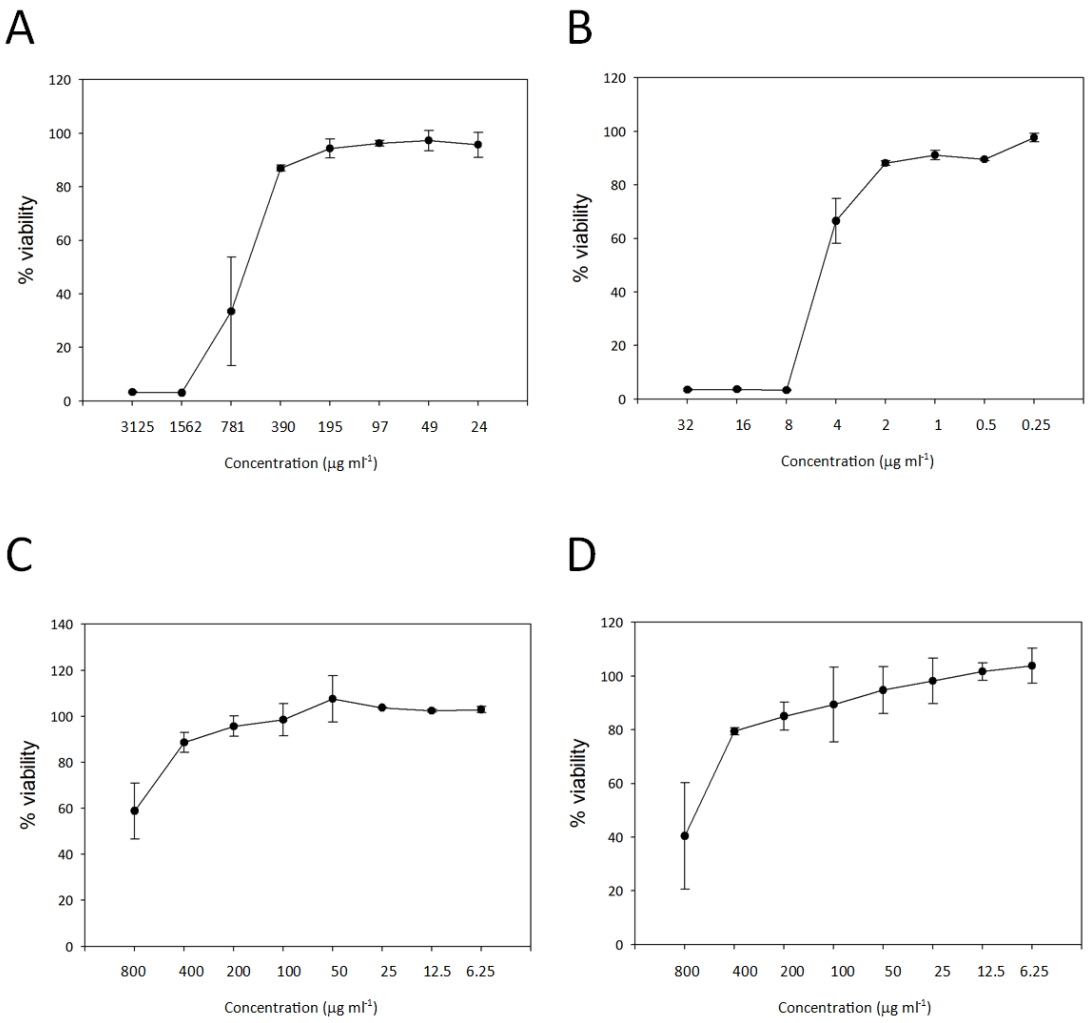


Figure 2. MTT cell viability assay of HeLa cells incubated for 48 h with (A) povidone-iodine, (B) chlorhexidine, (C) iodine aqueous solution and (D) borax dissolved in cell culture medium. Viabilities of the treated cells were compared to the untreated controls. MTT assay was performed as described in the Materials and Methods. Three parallel measurements were performed for each antiseptic concentration. Data are means +/- standard deviations.

5.3.2 Direct qPCR measurement of the impact of antiseptics on *C. trachomatis* growth

The direct qPCR method was used to determine the antichlamydial activity of antiseptics**.** HeLa cells were infected with *C. trachomatis* at MOI 8 after preincubation (37 ^{o}C, 1 h) with serial 1:2 dilutions of povidone-iodine, chlorhexidine, iodine aqueous solution and borax starting with the maximum non-toxic concentrations. The MICs of the antiseptics were calculated as it was described before [44]. Briefly, the chlamydial DNA concentrations (threshold cycle (Ct) values) measured in the three parallel wells of a given

antiseptic concentration were compared with the Ct values measured in the three parallel wells of the highest antiseptic concentration (we considered it as the inoculum) using Student's t-test **(Figure 3 A-D)**. The lowest antiseptic concentration, where the Ct values did not change significantly compared with the inoculum was considered the MIC value. The MIC value of povidone-iodine was 97 µg/ml, the MIC value of chlorhexidine was approximately 4 µg/ml. The iodine aqueous solution and the borax did not show antichlamydial activity in the tested concentration range.

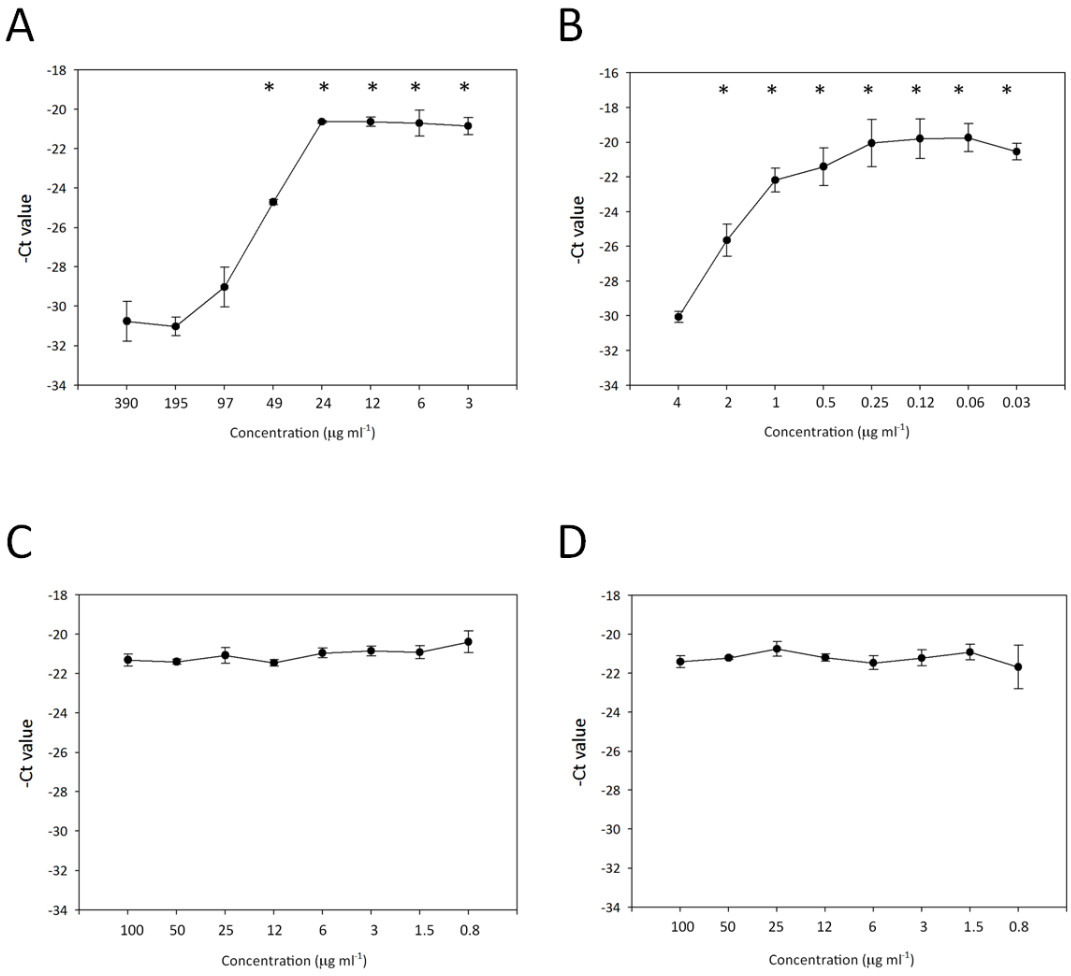


Figure 3. Measurement of antichlamydial activity of the antiseptics by direct qPCR. HeLa cells were infected with *C. trachomatis* (MOI 8) preincubated with various concentrations of (A) povidone-iodine, (B) chlorhexidine, (C) iodine aqueous solution and (D) borax for 1 h 37 ^{o}C. Each infection at a particular antiseptic concentration was performed in 3 parallel wells. At 48 h post infection, the cells were lysed and the chlamydial DNA concentration was measured by direct qPCR. Data are the average −Ct values +/- standard deviations. *: the Ct values are significantly different ($p < 0.05$) from

the values measured in the presence of the highest concentration of antiseptics using Student's t-test.

The measured MIC of the chlorhexidine was lower than that of the povidone-iodine; however its MIC was close to the 4 µg/ml concentration that decreased the viability of the epithelial cells after 48 hours incubation. This data indicates that the chlorhexidine could be an effective antichlamydial agent, but should be applied as a short term rinsing, rather than as a long-term vaginal gel. On the other hand, the povidone-iodine had a MIC of 97 µg/ml, while its maximum non-toxic concentration was 390 µg/ml, suggesting that this antiseptic can be applied long-term intravaginally. Also, it should be noted that the longer presence of povidone-iodine and its longer interaction with the chlamydial elementary bodies might result in an even lower MIC measured after the one hour coincubation.

5.3.3. Estimation of the qPCR inhibitory activity of the antiseptics.

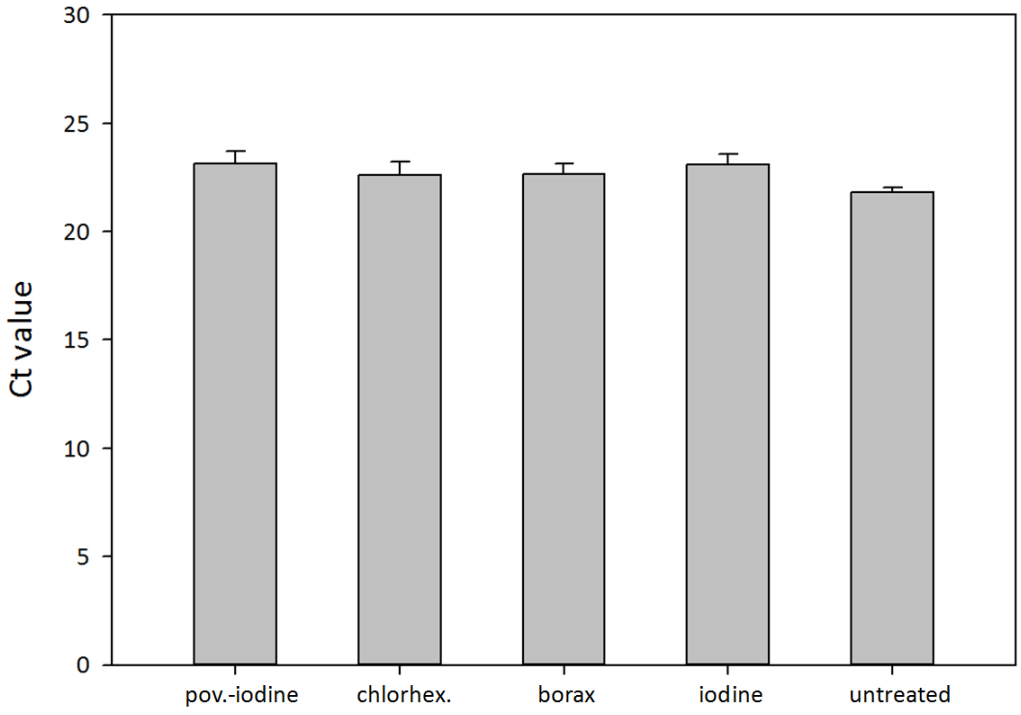


Figure 4. Estimation of the qPCR inhibition by the antiseptics. Cell lysates of HeLa cells infected with untreated *C. trachomatis* (MOI 8) mixed with cell lysates from uninfected HeLa cells treated with SPG solution containing 390 µg/ml povidone-iodine, 4 µg/ml chlorhexidine, 100 µg/ml borax and 100 µg/ml iodine aqueous solution respectively.

Untreated *C. trachomatis* (MOI 8) infected cells are also processed (n=3). Data are the average Ct values +/- standard deviations.

Since the growth related chlamydial DNA synthesis was measured by a qPCR method, we tested whether the applied antiseptics had a direct inhibitory impact on the qPCR. This effect could appear as a false- positive antichlamydial activity. We mixed cell lysates of HeLa cells infected with untreated *C. trachomatis* with cell lysates from uninfected HeLa cells treated with the maximum concentration of antiseptic applied for the *Chlamydia* inhibition. If there was no PCR inhibition then the Ct level of the 1:1 mixture (basically a two-fold dilution of the chlamydial DNA) of the infected and uninfected but antiseptic containing cell lysates would have been ~1 cycle higher (50% less chlamydial DNA concentration) than the *C. trachomatis* infected cells lysate's alone. The Ct levels of the povidone-iodine, chlorhexidine, iodine aqueous solution and borax mixtures were 1.33, 0.79, 0.85 and 1.28 cycles higher than the untreated *C. trachomatis* infected cell lysate's **(Figure 4)**, therefore the observed antichlamydial effect of the antiseptics were not due to the inhibition of the qPCR.

5.3.4 ChlamyCount immunofluorescent measurement of the impact of antiseptics on *C. trachomatis* growth.

To validate the qPCR results with an independent chamber slide infection method [77], we performed *C. trachomatis* infections (MOI 8) in the presence of the antiseptics with the highest concentrations used for qPCR **(Figure 5)**.

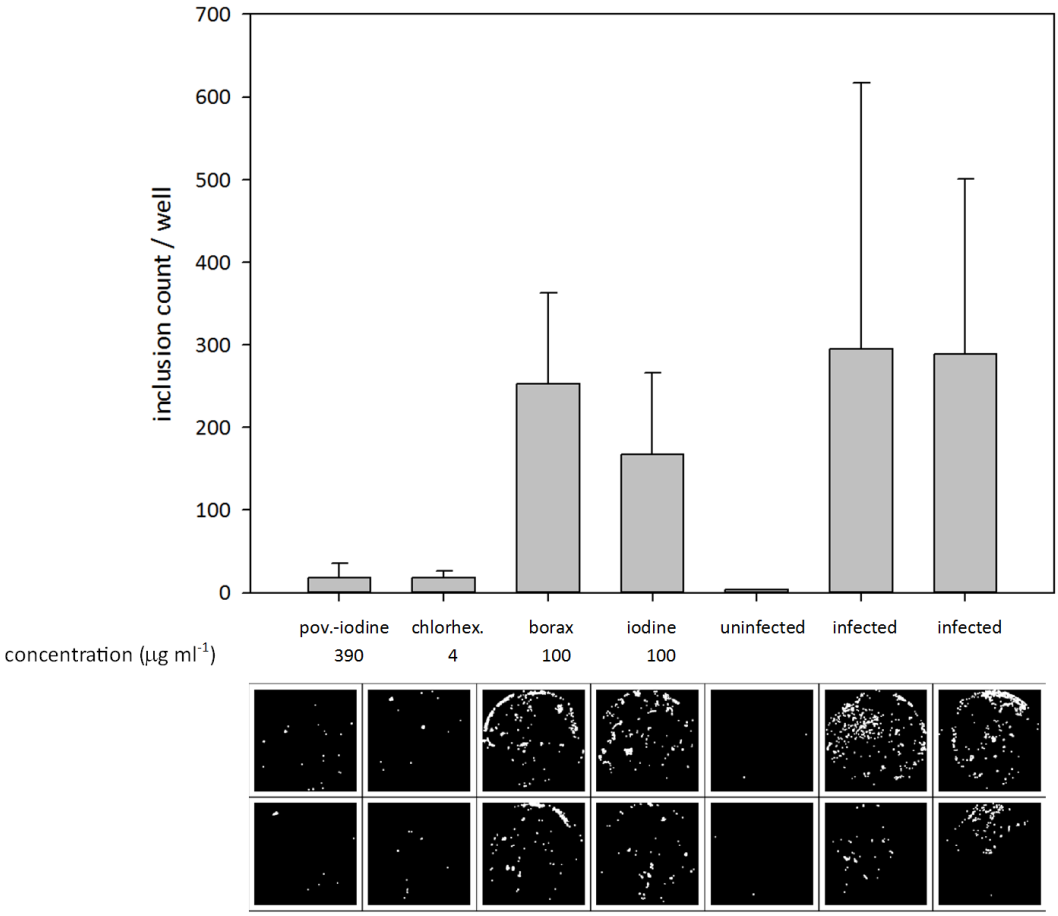


Figure 5. Measurement of antichlamydial activity of the antiseptics by immunofluorescent detection of the chlamydial inclusions. HeLa cells were infected with *C. trachomatis* (MOI 8) in the presence of the tested antiseptics. Four untreated *C. trachomatis* infected wells and uninfected wells were also included as controls. Each infection with a particular antiseptic was performed using parallel wells. The chlamydial inclusions were enumerated by the ChlamyCount software. The ChlamyCount processed well images and the counted inclusion numbers are shown. Data are means +/- standard deviations.

Infected and control cells were fixed 48 h post infection, and the chlamydial inclusions were labeled with an Alexa-647-labelled anti-chlamydia LPS antibody. The slide was scanned with a DNA-chip scanner, and chlamydial inclusions were enumerated by the ChlamyCount software **(Figure 5.).** ChlamyCount inclusion number data showed that the povidone-iodine and chlorhexidine treatment decreased the chlamydial inclusion number approximately 94 % and 94 %, while the iodine aqueous solution and borax decreased 13 % and 43 % respectively the number of chlamydial inclusions.

6. Discussion

6.1 Incidence and antibiotic susceptibility of *U. urealyticum/parvum*

Ureaplasmas are the most common bacteria of the human urogenital tract [9]. Their prevalence in the lower urogenital tract of healthy adults is high, up to 80%. The detection rate is higher for women. Screening of asymptomatic men showed that 11% of them were colonized with human ureaplasmas [12]. Up to 50% of men with Ureaplasma show an urogenital bacterial invasion. Ureaplasma was detected in children (5%), in sexually inactive women during reproductive age (40%), in sexually active women (60-80%) and also in postmenopausal women (25%). In pregnancy the detection rate can be as high as 82% and 24% in the puerperium. These fluctuations in prevalence may reflect oestrogen dependence. Among women with a genital infection, those who have multiple sexual partners, low socioeconomic status, and smokers have a higher rate of colonisation. There is also dependence on the ethnicity [80][23][27]. *U. parvum* is the more commonly isolated species from clinical specimens [16]. Both species may occur simultaneously. *U. parvum* is the predominant species in newborn serum and liquor samples detected by PCR [38]. A high incidence (52%) of *U. urealyticum* was observed also in non-sexually active young females with vaginal discharge [81]. The incidence of genital ureaplasma infections are twice as high in infertile couples as in fertile couples. The colonisation rate of women who had an abortion was showed to be 20% higher in comparision with uninfected women. Women with habitual abortion had 50% higher colonisation rate than uninfected pregnant women [38]. Ureaplasmas can be detected from the endotracheal specimens of newborn infants in up to 40% within 30 min to 24 h after birth. In preterm infants Ureaplasma spp. can be present for long periods in the lower respiratory tract. In full-term infants the Ureaplasma colonization is transient and declines beyond 3 months of age [25].

Based on our results we can state that *U. urealyticum/parvum* strains have high prevalence in both sexes. We were able to detect *U. urealyticum/ parvum*, in all sexually active age groups; the highest number of isolates were from age group of 21-40 years regarding both men and women. Among Ureaplasma positives the symptom free carriers was above 40% in both sexes.

Comparing our resistance results with those published in international journals, we found similar tendencies regarding certain antibiotics like macrolides, clindamycin and tetracycline, as well as the outstandingly high erythromycin and clindamycin resistance and cross-resistance [24], while we found deviations in cases regarding

fluoroquinolones. Regarding erythromycin and clindamycin resistance, the examination method might showed only constitutive cross-resistance. There can be more than one mechanisms in the background of macrolide resistance: eg. active efflux (*msrD, msrB* genes) or 23S rRNS mutation or methylation (*erm* gene). The *msrD* and *msrB* genes – also responsible for active efflux – protect the bacteria from lincosamides; while the changes in the 23S rRNS structure – methylation by *erm* gene or mutation – do not affect lincosamides. According to literature, primarily azithromycin and josamycin are the most effective, but there have already been resistant strains isolated [24]. Azithromycin was found to be considerably effective against Hungarian Ureaplasma strains in our examinations. Fluoroquinolone resistance can develope in various ways, including mutations of gyrase/ topoisomerase and proteins protecting topoisomerase enzymes (*gyrA, parC, parE*). In our study fluoroquinolons were represented by ofloxacin with 25.2% resistance. Concerning our data, the Hungarian situation is significantly more favourable than those in other countries [82][29][83]. Tetracycline was effective in 95.9% of Hungarian Ureaplasma strains. So far only the *tet*M gene was mentioned in the background of resistance, which produces a protective protein bound to the bacterial ribosome [34] [84]. Doxycycline seems to be the drug of the first choice in case of Ureaplasma infections with 2.41% resistance. In case of pregnancy, infancy and allergy or in any other cases when it is contraindicated, the medication to be chosen is azithromycin for Ureaplasma infections [85]. Thus, the present guideline to treat Ureaplasma infection have to be changed by replacing erythromycin with azithromycin. If a simultaneous infection of *C. trachomatis* and *U. urealyticum/parvum* is present, azithromycin is the drug of choice, too, because both bacterium species significantly kept their sensitivity to it [86]. In case of biofilm producing *U. urealyticum/ parvum* strains, the first drug of choice is clarithromycin, because of it is possessed of a capability to cross biofilm and inhibiting its formation.

In case of suspected *U. urealyticum/ parvum* infection we highly recommend to test the antibiotic resistance after the first unsuccessful antibiotic therapy, the most up-to-date procedure is, however, to establish antibiotic sensitivity prior to commencing therapy. Furthermore, it is advisable to perform tests regarding biofilm formation and determine minimal inhibiting biofim concentrations (MIBC) of antibiotics in case of all *U. urealyticum/ parvum* strains [26].

6.2 Serological identification of gestational and congenital syphilis in the Hungarian population

Globally, nearly two million pregnant women are infected with *T. pallidum* each year. Approximately 50% of women with untreated syphilis have been transmitting the infection to their newborn child, resulting in profound adverse outcomes including an estimated 440,000 perinatal deaths each year [87]. In Hungary, there was no congenital syphilis from 1978 until 1994. Both in 1994 and 1996, three early cases of congenital syphilis were observed, all in foreigners and from pregnancies without prenatal care. Since 1994, however, almost every year a case has been observed in Hungary. In 2007 and 2008, one and two cases were observed respectively [88]. In a next three-year period, four connatal syphilis cases were confirmed in Hungary. In Switzerland, in contrast to international guidelines, screening for syphilis in pregnancy is not generally recommended, but within the Swiss population, infectious syphilis cases in women of childbearing age increased substantially from 2006 to 2009 [89]. In Hungary, the performance of screening examinations within prenatal care is compulsory in order to prevent the development of syphilis, but only in the first trimester of pregnancy.

RPR test used for screening has the advantage of being inexpensive, widely available, and necessary for determining the efficacy of treatment. Limitations of this non-treponemal test include the lack of sensitivity in primary and late syphilis and the possibility of a prozone reaction or false-positive results [90]. Prozone reactions occur in 1–2% of patients with secondary syphilis [91], when antibody is in excess and blocks the normal antibody–antigen reaction. Dilution of the serum sample exhibiting this prozone reaction is adequate to obtain a readily detectable reaction.

Treponemal tests like EIA or TPPA are technically more difficult to perform and more expensive, but they remain reactive for years with or without treatment. Due to the recommendation of Centers for Disease Control and Prevention (CDC), Binnicker et al. have suggested to use a reverse syphilis screening algorithm, in which sera are screened using an automated treponemal test (e.g. EIA) [92]. Samples that are reactive by EIA are then tested by RPR to assess disease and treatment status and provide a supplemental marker of infection. It is also recommended that sera reactive by EIA but nonreactive by RPR be analyzed by the TPPA assay [93]. Several antigens that elicit high antibody titers during *T. pallidum* infection and are not cross-reactive with serum from patients with other common spirochetal diseases have been identified [94]. TPPA and EIA tests were developed using these recombinant antigens. While false-positive results can occur also

with treponemal tests [44], we have used these two tests from different manufacturers, as screening and confirming methods.

From the 241 mothers with positive syphilis screening tests, 217 were successfully treated before pregnancy. The next 13 pregnant women diagnosed with syphilis during pregnancy opted for treatment. The rest of 11 mothers were diagnosed with syphilis only at delivery. All four connatal syphilis cases were from mothers without undergoing prenatal care and syphilis screening. The success rate for mother-to-child transmission intervention (number of successful interventions/number of syphilis positive women who received intervention) was 100% in our cases. In the program supported by the Shenzen local government, the success rate was lower, 99.1%, because of mothers who refused treatment or had late diagnosis and treatment [95]. Due to the better compliance of the patients, the performance of compulsory screening examinations within prenatal care is a good prevention against the development of connatal syphilis in Hungary. Rawstron et al. determined maternal IgM status to be better indicator for a risk of connatal syphilis than a maternal RPR titer ≥1:16. However, they described that neither a titer ≥1:16 nor TP IgM reactivity identified all mothers who delivered infected infants; they found babies with congenital syphilis whose mothers had negative TP IgM and RPR titers ≤1:8 [45]. In our study, the mother of the twins with connatal syphilis had only an RPR titer of 1:2. None of the mothers of the infected children was IgM negative in our cases. The infant of the untreated mother, who had an RPR titer of 1:64, but was negative for IgM, was uninfected.

Infected infants can produce IgM in utero after 3 months [90]. Previous studies using either ELISA or *T. pallidum* IgM Western Blot have similarly found that IgM antibodies cannot be detected in all babies with congenital syphilis [96][97]. Serodiagnosis of congenital syphilis is difficult because of the transfer of the IgG antibodies from mother to fetus. Fetus produces IgM antibodies (rheumatoid factor= RF), against maternal IgG [95]. IgG–RF complex reacts in IgM immunoblot, or maternal IgG compete with fetal IgM for the Ag-binding position, resulting in false-positive or false-negative tests, respectively. To eliminate maternal IgG and IgG–RF complexes from the sera of newborns, Mastsorb reagent was used before IgM immunoblot tests. In previous studies with IgM immunoblot test, more reactivity was observed to the 47-kDa antigen than to antigens of 45 kDa or lower [98][99][100]. All our infants IgM-reactive sera demonstrated also this reactivity. We found reactivity to antigens of 45, 17, and 15 kDa only in one case. Our observations confirm that antenatal syphilis screening with the

parallel use of treponemal (EIA, TPPA) and nontreponemal (RPR) tests facilitates treatment during pregnancy. Successful treatment offsets vertical transmission. The use of IgM immunoblot examination allows the identification and treatment of high-risk newborns.

6.3 Measurement of the antimicrobial activities of various antiseptics against *C. trachomatis*

We tested the cytotoxic effects and antichlamydial effects of various antiseptics that can be used intravaginally. The cytotoxicity tests revealed that iodine and borax did not influence the viability of the HeLa cervical epithelial cell lines at the tested concentration range, while the povidone-iodine and chlorhexidine showed a concentration-dependent toxicity after 48 hours of incubation, the time of the developmental cycle of *C. trachomatis*. In the case of these two antiseptics we used the maximum non-toxic concentration for further tests of antichlamydial capacity. We used a preincubation of the chlamydial elementary bodies with the antiseptics, to mimic the effect of these compounds on the extracellular infectious form of *C. trachomatis.* However it cannot be excluded that these antiseptics can be transported into the host cells and may have an effect on the intracellular development. Further studies needed to measure the intracellular effects of the antiseptics.

Instead of measuring the chlamydial growth inhibition by immunofluorescent staining of the so-called chlamydial inclusions, we used the recently developed qPCR based chlamydial DNA accumulation measurement as a readout of chlamydial replication. Our qPCR data showed that the chlorhexidine and povidone-iodine had an antichlamydial effect, while the iodne aqueous solution and borax did not possess any antichlamydial activity in the tested concentration range. Chlorhexidine had the lowest antichlamydial MIC, but the MIC was close to the that the maximum non-toxic concentration, therefore the therapeutic index is low, at least beased on this in vitro data. Our data indicates that, chlorhexidine could be an effective antichlamydial agent in vivo, but may be applied as a short term rinsing, rather than as a long-term vaginal gel. On the other hand, the povidone-iodine had a MIC of 97 μg/ml, while its maximum non-toxic concentration was 390 μg/ml, suggesting that this antiseptic can be applied long-term intravaginally. Also, it should be noted that the longer presence of povidone-iodine and its longer interaction with the chlamydial elementary bodies might result in an even lower MIC compared to the MIC we measured after the one hour coincubation. Since the qPCR

was performed on cell lysates as templates and not on purified DNA, there is a possibility, that the applied antiseptics had a direct inhibition of the DNA polymerase. As we mentioned it in the results section, we performed control experiments to exclude this possibility. Our results indicated that the antiseptics did not inhibit the qPCR and the observed Chlamydia growth inhibition of povidone-iodine and chlorhexidine could not be due to the inhibition of the qPCR itself.

Also, to validate the qPCR based growth measurements, we used the immunofluorescence based ChlamyCount growth measurement system to measure the growth-reducing effects of the antiseptics. ChlamyCount data validated the qPCR measurements in the case of povidone-iodine and chlorhexidine, although the extent of detected growth reduction was lower than that determined by qPCR. The reason could be that the dynamic range of our immunofluorescent ChlamyCount method was about 2 $\log_{10}$ [77], while the qPCR method's dynamic range was ~5 $\log_{10}$ [75]. Different from the qPCR results, the ChlamyCount method also showed a limited chlamydial growth inhibition in the case of iodine aqueous solution and borax. The fact that the chlamydial DNA synthesis remained constant after the application of these latter two compounds but the inclusion numbers slightly decreased, might indicate that small portion of the iodine aqueous solution and borax treated chlamydial EBs become persistent, maintaining the chlamydial DNA synthesis [101][102], but formed smaller/ less intense inclusions that was not detected by the ChlamyCount method.

Altogether, our results showed, that povidone-iodine had the widest antichlamydial therapeutic index and could maintain an antichlamydial effect when used intravaginally. Intravaginal povidone-iodine has already been used as a preoperative antiseptic to reduce post-surgery endometritis after cesarean sections [103] and histerectomy [104]. Povidone-iodine also can be attractive in bacterial vaginosis treatment because it has no significant antimicrobial activity against lactobacilli [105][106]. Since *C. trachomatis* infection could be linked to bacterial vaginosis, povidone-iodine may treat/ limit these two clinical entities at the same time. Also it is worth to note, that povidone-iodine and chlorhexidine has been used intravaginally at a significantly higher concentrations than we used in our in vitro tests [107][108][109]. Since the maximum in vivo tolerable/ non-toxic concentrations of these compounds are higher than in our in vitro toxicity assay, this could result in an even higher antichlamydial effect in vivo.

7. Novel results of the thesis

1. We showed that the prevalence of *U. urealyticum/ parvum* species is high in sexually active female and male populations in Hungary.

2. We revealed that in the Hungarian population the age distribution was different between the Ureaplasma positive females and males.

3. We performed a large-scale antibiotic resitance screen of Ureaplasma isolates in Hungary. We showed that the erythromycin and clindamycin resistances were extremely high, but the cross-resistance between the two macrolides, the older erythromycin and the newer azithromycin was infrequent. Every fourth strain was resistant to ofloxacin as representative of fluoroquinolons. Doxycycline proved to be the most effective agent with 97.3% susceptibility, followed by tetracycline and azithromycin. Because of the extremely high ratio of erythromycin and clindamycin resistant strains, the present guideline to treat ureaplsma infection have to be change by replacing erythromycin with azithromycin. Moreover, administering erythromycin has the danger to further select the resistant strains.
4. We studied the incidence of gestational and connatal syphilis in the Hungarian population. We concluded, that maternal IgM immunoblot results identify mothers at risk of delivering babies with connatal syphilis better than the height of maternal RPR titer. Our data of Hungarian newborns, supports the previous results in connatal syphilis serology. In newborns, IgM test which depends on the infant's response has more specificity in diagnosing connatal syphilis than IgG that can transfer across the placenta.

5. We tested the antichlamydial effect of four vaginal antiseptics. We showed that povidone-iodine and chlorhexidine had a marked antichlamydial activity when applied at concentrations that was not toxic to the host epithelial cells. The therapeutical index of povidone-iodine was higher than that of chlorhexidine, indicating a higher clinical safety in vivo.

8. Acknowledgements

Special thanks to Professor Dr. Sarolta Kárpáti, former director and chairwoman of the Department of Dermatology, Venereology and Dermatooncology, Semmelweis University, Budapest, Hungary, to admit me, and to Professor Emeritus Dr. Ferenc Rozgonyi, former leader of the National STD Microbiology Diagnostic Laboratory, Department of Dermatology, Venereology and Dermatooncology, Semmelweis University, Budapest, to govern me in the *Ureaplasma* studies, and to the co-authors of my publications for involving me in these studies.

I am grateful for the help of Dr. Dezső Virok, Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary to direct the Chlamydia experiments and to overview my PhD studies. The skilful technical assistance of the laboratory staffs in both Budapest and Szeged is also highly appreciated. I also highly appreciate the help of Ágnes Tölgyesi from the library of Kálmán Pándy Békés County Municipal Hospital, Gyula in thesis preparation.

Thanking for the helping hand from Professor Dr György Bártfai and Dr Gábor Németh Director of the Department of Obstetrics & Gynecology at Szeged University Clinics and also special thanks for being persistent & attentive within the professional cooperation

Special thanks to Dr Tamás Major for the helping hand given within the professional cooperation.

I am also very grateful for the patience, encouragement and continuous help of my wife and our children, which were absolutely necessary to prepare this work.

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2017

Párducz L., Rozgonyi F.: A kamaszok veszélyeztetettsége a XXI. században (Chlamydia, Mycoplasma és Ureaplasma fertőzés jelentősége**).**
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Publications of the thesis

I.

Ureaplasmas: from commensal flora to serious infections

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The two existing human pathogen *Ureaplasma* species containing 14 serovars are associated with a variety of maladies. Colonizing the human urogenital tract, ureaplasmas cause infections mainly in these organs. However, in the special population of immunosuppressed patients or preterm and very low birth weight newborns such serious *Ureaplasma* infections such as meningitis or pericarditis have been described. Because they lack a cell wall, ureaplasmas are very susceptible to drying and other adverse environmental conditions; therefore, careful attention has to be given to specimen collection and transportation. Commercially available culture based tests made the laboratory diagnosis of *Ureaplasma* species much easier. PCR methods provide further facilities to get faster diagnosis, simultaneously with serovar identification, quantitation of *Ureaplasma* or detection of other sexually transmitted infectious pathogens. Although fluoroquinolone, macrolide, and tetracycline resistant strains are known, antibiotic treatment of *Ureaplasma* infections is not problematic for now. The mechanism of antibiotic resistance has not been entirely understood till now. Biofilm-forming ability of *Ureaplasmas* can be important in chronic infections and antibiotic resistance. The basic microbiology of *Ureaplasma* species, such as clinical manifestations, the diagnostic opportunities, and the therapeutic options are reviewed in the current article.

Reviews in Medical Microbiology 2011, **22**:73–83

Keywords: antibiotic resistance, characterization, clinical features, diagnostic methods, treatment, *Ureaplasma parvum*, *Ureaplasma urealyticum*

Introduction

Ureaplasmas, originally called 'T-mycoplasmas', were first described by Shepard in 1954. Since then these organisms have been studied thoroughly, but it seems likely that our knowledge of ureaplasmas is still insufficient, especially in the field of their antibiotic resistance, ability of biofilm formation, and their role in chronic and mixed infections. Studying human ureaplasmas is of importance, because of their widespread pathogenicity.

Classification

Ureaplasmas are eubacteria in the class *Mollicutes*. For a long time, *Ureaplasma urealyticum* was the only known species in human *Ureaplasma* infections. On the basis of 16S rRNA sequences, this species was subdivided into two separate species (instead of the previous biovar 1, biovar 2 classification): *Ureaplasma parvum* containing serovars 1, 3, 6, and 14, and *U. urealyticum* containing serovars 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13 [1,2].

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Received: 28 May 2011; accepted: 28 May 2011.

DOI:10.1097/MRM.0b013e328349477d

ISSN 0954-139X © 2011 Wolters Kluwer Health I Lippincott Williams & Wilkins

Morphology, biochemistry

Ureaplasmas are the smallest self-replicating cells and the simplest free-living bacteria [1]. They originate from clostridial-like Gram-positive bacteria by gene deletion [3]. Ureaplasmas lack cell walls. This prevents them from staining by *Gram* stain. Lacking a cell wall also explains the pleomorph morphology of ureaplasmas and their extreme susceptibility to dehydration. These bacteria exist like parasites within the eukaryotic cells of their host, in many animals and also in humans. Ureaplasmas are highly dependent on the nutrient components of their host organism. This parasite-like feature of ureaplasmas is based on their high metabolic flexibility and the lack of many genome regulatory elements [4]. Ureaplasmas have limited biosynthetic abilities: hydrolyzing urea and adhering to human mucosal surfaces [1]. They generate ATP by hydrolysis of urea by urease. This property is unique among *Mollicute* bacteria. *U. urealyticum* uses three predicted enzymatic components for energy production: urease, an ammonia/ammonium transporter, and an FOF1-ATPase. The urease enzyme of *U. urealyticum,* whose activity is 30–180-fold higher than that reported in other bacteria, has a main role in the energy generating process. Because of the very limited biosynthetic capacity of ureaplasmas, they must import more nutrients for growth than other bacteria. Twenty-eight different *U. urealyticum* transporters have been identified representing nine transporter families. Despite this, ureaplasmas lack transport systems for bases, nucleotides, nickel, urea, etc. Thirty-three hypothetical U. urealyticum proteins are described, which have five or more predicted transmembrane regions. Maybe the lacking transporters are among these hypothetical proteins. *U. urealyticum* has six closely related iron transporters. These apparently arose through gene duplication. It is assumed that ureaplasmas have a respiration system that is not present in other bacteria with small genome size [5].

The genome of *U. parvum* is 760 Mb, whereas the genome of *U. urealyticum* is 840–1140 Mb [4]. The *U. parvum* serovar 3 genome is among the smallest known genomes with 751, 717 base pairs [2]. One hundred and seventy-five genes unique to *U. parvum* have been identified. These unique genes represent 35% of the *U. parvum* genome. Four hundred and seven out of the 613 *U. parvum* genes were found in the *U. urealyticum* genome, meaning that 75% of the functional genome of *U. parvum* was detected in *U. urealyticum*. The minimal functional core of *U. parvum* is 538 genes. Most of the unique *U. parvum* strain specific genes have unknown function. Four hypervariable regions (UU32eUU33, UU145eUU 170, UU440eUU447 and UU527eUU529) have been also identified in the genome. Ninety-three percent of the variable gene pool is located in these regions [4]. The G + C nucleotide content of the genome is only 25.5%. The G + C content of individual genes may predict their necessity in *Ureaplasma* survival [5]. Data obtained from the sequencing of the complete genomes of all *Ureaplasma* serovars are already available.

Urease is also responsible for the pathogenic potential of ureaplasmas. Seven genes were detected within the urease gene cluster. *ure*A, *ure*B, and *ure*C encode the structural subunits; *ure*E, *ure*F, *ure*G, and a truncated *ure*D gene encode accessory proteins. These genes are well conserved amongst the serovars, but variations between the two human *Ureaplasma* spp. exist and are used in the identification process to separate them [6].

Virulence factors

While *Ureaplasma* is a commensal bacterium in urogenital tract of healthy asymptomatic adults, it is considered of low virulence. However, they can cause occasionally severe invasive infections. IgA protease, adhesins, urease, phospholipases A1, A2, and C, hemolysins, and hydrogen peroxide production were proposed as virulence factors of ureaplasmas [2,7].

With the aid of cell-surface adhesin proteins, ureaplasmas adhere to a variety of human cells including urethral epithelial cells, spermatozoa, or erythrocytes. They bind spontaneously to neutrophils and activate the first component of complement system directly. The receptors for ureaplasmal cell-surface adhesin proteins–similar to *Mycoplasma pneumoniae* or other mycoplasmas–suggested to be sialyl residues and/or sulfated compounds. The cytadherence proteins are not organized into a demonstratable attachment organelle. The adhesin proteins of ureaplasmas have not been completely characterized [3].

Ureaplasma spp., due to their urease activity, are connected with urinary stone formation. Secretory products of ureaplasmas such as ammonia can produce a local cytotoxic effect [3]. IgA proteases degrade mucosal IgA and therefore promote the *Ureaplasma* colonization of mucosal surfaces. With the aid of phospholipase A and C, ureaplasmas may initiate preterm labor. Arachidonic acid liberation and altered prostaglandin synthesis, as well known triggers of preterm labor, are the consequences of phospholipase activity of ureaplasmas. The activities of phospholipase A2 are serotype specific, while the activities of phospholipase A1 and C are similar. *U. parvum* has two hemolysins. They are encoded by the *hlyC* and *hlyA* genes. Hemolysin *A* may function as a virulence factor in *Ureaplasma* spp. Hemolysin *C* has an orthologue in *M. pneumoniae* [8]. It appears that many serotypes are invasive. Host factors and antigen variability may be more important in invasive *Ureaplasma* infections than the serotypes [9].

Although phenotypic production of IgA protease and phospholipases by ureaplasmas is described, the encoding genes have not been revealed thus far [3]. Correlation

between the presence of virulence factor genes in the *Ureaplasma* genome and the incidence of human disease has not been proved. The UU145eUU170 fragment in the *U. parvum* genome is likely to be a putative pathogenicity island (PAI). The genes of this PAI determine the virulence of this microorganism and the clinical outcome of *U. parvum* infection is likely to be associated also with this PAI. The combination of variable *Ureaplasma* strain specific genes with generally known virulence factor 'genes' can explain how these bacteria initiate the pathological processes on the urogenital mucosal surface. The pathogenic role of different *Ureaplasma* serovars has to be examined in the future [4,10].

U. urealyticum and *U. parvum* are able to form biofilms *in vitro*. Biofilms consist of aggregated bacterial cells and extracellular matrices of biopolymeric substances. Biofilm infections are very difficult to treat with antibiotics and disinfectants. The host immune system is ineffective at clearing the biofilm infection, while white blood cells are unable to access the bacteria. Biofilms provide a survival advantage to bacteria resulting in persistent infections. Antibiotics are also usually ineffective, because of the reduced permeability of biofilms. Biofilms contribute to the pathogenesis of chronic infections. In the case of chronic bacterial prostatitis or acute necrotizing chorioamnionitis, biofilm-forming, epithelium-adhering bacteria were demonstrated by scanning electron microscopy. Biofilms can be the cause of persistent inflammation and are partly responsible for frequent antimicrobial treatment failures. It is supposed that ureaplasmas are able to form biofilms using a minimal, stochastically regulated genetic system [11].

Antigens

The ureaplasmal multiple-banded antigen is the predominant antigen recognized in human ureaplasmal infections. Multiple-banded antigen contains both serovar-specific and cross-reactive epitopes. It has highly variable size. Antigen size variation may be a mechanism through which the organism evades host defenses [2]. Multiple-banded antigen of *Ureaplasma* spp. is immunogenic, and it may stimulate the host inflammatory response. Each *Ureaplasma* serovar contains multiple multiple-banded antigen genes. Some serovars contain multiple copies of the same type of multiple-banded antigen gene. The variations in the multiple-banded antigen genes are used to separate *U. urealyticum* and *U. parvum* species, and to distinguish the serovars from each other to some extent [3].

Epidemiology

Ureaplasmas are the most common bacteria in the human urogenital tract [12]. Their prevalence in the lower urogenital tract of healthy adults is high, up to 80% [1]. The detection rate is higher for women. Screening of asymptomatic men showed that 11% of them were colonized with human ureaplasmas [13]. Up to 50% of men with *Ureaplasma* show a urogenital bacterial invasion. *Ureaplasma* was detected in children (5%), in sexually inactive women during reproductive age (40%), in sexually active women (60–80%), and also in postmenopausal women (25%). In pregnancy, the detection rate can be as high as 82% and 24% in the puerperium. These fluctuations in prevalence may reflect estrogen dependence. Women with a genital infection, several sexual partners, low socioeconomic status, and smokers have a higher rate of colonization. There is also dependence on the ethnicity [10,12,14]. *U. parvum* is the most common isolated species from clinical specimens [15]. Both species may occur simultaneously. *U. parvum* is the predominant species in newborn serum and liquor samples detected by PCR [2].

A high incidence (52%) of *U. urealyticum* was observed also in nonsexually active young females with vaginal discharge [16]. The incidence of genital ureaplasma infections is twice as much in infertile couples than in fertile couples. The colonization rate of women who had an abortion was shown to be 20% higher in comparison with uninfected women. Women with habitual abortion had 50% higher colonization rates than uninfected pregnant women [12].

Ureaplasmas can be detected in the endotracheal specimens of newborn infants in up to 40% within 30 min to 24 h after birth. In preterm infants *Ureaplasma* spp. can be present for long periods in the lower respiratory tract. In full-term infants, the *Ureaplasma* colonization is transient and declines beyond 3 months of age [8].

Immunology

In neonatal *Ureaplasma* infection, the levels of TNF-α, IL-1β, IL-8, and monocyte chemoattractant protein-1 are consistently increased [1,2]. Human macrophages exposed to *Ureaplasma* antigen produce TNF-α and IL-6 and release vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1). *Ureaplasma* stimulates also nitric oxide production and up-regulation of iNOS, and nuclear factor kappa B (NF-κB) activation. The Triton X-114 detergent extracted lipoproteins from certain *Ureaplasma* serovars activate NF-κB through TLR2 and TLR4 cooperatively with TLR1 and TLR6 [2]. Ureaplasmas can activate directly the first component of complement [3]. It is suggested but not proved that immunity to invasive infection by ureaplasmas is type specific [8].

Clinical manifestation

Ureaplasmas are sexually transmitted diseases. The way of transmission can be orogenital (can be isolated from pharynx) and anogenital also; however, the clinical role of the anal infection is not yet clear [17].

Vertical transmission and colonization may happen during delivery from infected mother to the newborn child. Until pubertation, colonization decreases and only after beginning sexual activity–related to the number of partners–increases again [18].

The pathogenic role of ureaplasmas are proven in nongonorrheal and nonchlamydial urethritis (NGNCU) and in bacterial vaginosis. Prostatitis, epididymitis, and sexually acquired reactive arthritis (SARA) are suspected complications also, but further investigations are needed to demonstrate that the correlations were proven in cervicitis and in pelvic inflammatory disease (PID) development [19].

Urogenital mycoplasmas, including ureaplasmas can be pathogenic agents in the following diseases: infertility, prostatitis, epididymitis, orchitis, spermatocystitis, pyelonephritis, cystitis, nongonorrheal urethritis (NGU), urinary calculi, PIDs, chronic plasma cell endometritis, postpartum endometritis, chorioamnionitis, spontaneous abortion, septic abortion, postpartum fever, premature birth, and stillbirth [3,12,14].

U. urealyticum can be observed in 10–20% of NGU [19]. Because of their special metabolism and IgA protease activity, they have urogenital predisposition. In case of symptoms of NGNCU, they can be isolated over 10^3 cfu/ml concentration. Furthermore, ureaplasmas can be isolated also from symptom free patients. In male patients, the symptoms of urethritis are correlated with the concentration of ureaplasmas, but no similar findings can be set in female patients [20].

Nonurogenital *Ureaplasma* infections are extremely rare in the adult population. Among these, postoperative mediastinitis with pericarditis, post-transplant pericarditis, spontaneous bacterial pericarditis with tamponade have been described. *Ureaplasma* spp. caused meningitis in immunosuppressed and transplant patients. Intrarenal abscesses in transplanted kidneys, pleural fluid in lung transplants, suppurative arthritis, subcutaneous abscesses, and osteomyelitis also have been reported [7,9,21]. A rapid loosening of a hip prosthesis due to *U. urealyticum* infection was also described [22]. *Ureaplasma* spp. are the most common bacteria isolated from infected joints in persons with hypogammaglobulinemia. Apart from joint involvement (recurrent destructive oligoarticular arthritis), subcutaneous abscesses, persistent urethritis, and urethrocystitis/cystitis in hypogammaglobulinemic patients have been associated with ureaplasmal infection [8,13,23].

The degree of *Ureaplasma* colonization strongly correlates with an adverse effect on pregnancy outcome [6]. Ureaplasmas can invade the amniotic fluid early in pregnancy and can persist in the amniotic fluid subclinically for several weeks (even with intact fetal membranes). The bacteria then initiate an intense inflammatory reaction that may be followed by preterm labor. *Ureaplasma* spp. are among the most common organisms that can be isolated from amniotic fluid and inflamed placentas [13]. Isolation of *Ureaplasma* spp. but not *M. hominis* from the chorioamnion has been consistently associated with histological chorioamnionitis. Ureaplasmas are inversely related to birth weight, independently from the duration of labor, rupture of fetal membranes or presence of other bacteria. Preterm labor cannot be predicted reliably from the vaginal carriage of *Ureaplasma* spp., but there is an association between their presence in the amniotic fluid or placenta and the time of labor [8]. Although *Ureaplasmas* can be detected in endometrial tissue of healthy, nonpregnant women, they may be present at the time of implantation. Therefore, they might be involved in early pregnancy losses. Ureaplasmas have causal role in spontaneous abortion [1].

Although coexistence of ureaplasmas with bacterial vaginosis was demonstrated, the exact role and significance of *M. hominis* and *Ureaplasma* spp. in bacterial vaginosis remains uncertain [3,8,14]. Correlation between *Ureaplasma* spp. and *U. parvum* serovars and vaginal flora changes were investigated. The presence of *U. parvum* serovar 6 was significantly correlated with normal vaginal flora, whereas *U. urealyticum* and *U. parvum* serovar 3/14 were correlated with the absence of lactobacilli [10].

Ureaplasma spp. play an important role in both female and male infertility [8].

Ureaplasma spp. can be transmitted from an infected female to the fetus or neonate by at least three different routes: ascending intrauterine infection, hematogenous route through placental infection through the umbilical vessels, and through passage of an infected maternal birth canal. The range of vertical transmission of *Ureaplasma* spp. was shown to be 18–88%. It varies inversely with gestational age. The in-utero *Ureaplasma* infection is common. Neonates may also be colonized initially at the time of delivery. After birth by sectio cesarea, *Ureaplasma* colonization of neonates does not occur. Intrauterine infection with *Ureaplasma* spp. can result in chorioamnionitis, dissemination to fetal organs, and congenital pneumonia with sepsis. These consequences of the intrauterine Ureaplasma infection can be observed even in newborns [3,8,24].

Perinatal *Ureaplasma* infection or persistent, but not transient *Ureaplasma* colonization of the airways in very low birth weight (VLBW) infants appears to promote the inflammatory cascade in the lung (just as they do in the

amniotic fluid) and may impair alveolar development directly or in conjunction with oxidant and ventilator-induced lung injury. Therefore, *Ureaplasma* contributes to subsequent development of bronchopulmonary dysplasia (BPD) [1]. Relationship of specific serovars and the development of BPD is as yet undetermined. The low TLR2 and TLR4 expression in early gestation may increase the susceptibility of the fetal lung to *Ureaplasma* infection and delay clearance. Postnatal exposure to mechanical ventilation, oxygen, and other infections may stimulate pulmonary TLR expression and enhance *Ureaplasma* mediated inflammatory signaling [2]. The infection promotes a proinflammatory cytokine cascade in the respiratory tract and apparently blocks expression of the regulatory cytokines (IL-6 and/or IL-10) [3]. It was reported that 26% of neonates who were culture-positive for *Ureaplasma* spp. in the lower respiratory tract had *Ureaplasma* bacteremia. Ureaplasmal bacteremia may accompany severe pneumonia and meningitis in neonates. *Ureaplasma* spp. are among the most commonly isolated bacteria from cerebrospinal fluid of VLBW infants. According to a study, serum *Ureaplasma*-positive infants had a 2.3-fold increased risk of intraventicular hemorrhage. A number of other infectious conditions, such as fatal nonimmune hydrops fetalis or brain abscesses, have been associated with ureaplasmas [3,8]. *Ureaplasma* spp. can cause meningitis in preterm and also in full-term neonates. Clinically significant ureaplasmal infections rarely occur in infants born after 34 weeks of gestation [8]. *Ureaplasma* infection may be present as wheezing, pneumonitis, pertussis-like syndrome, and different forms of arthritis in older children [25].

It is reasonable to suggest that mycoplasmas and ureaplasmas may increase the development of AIDS in HIV-1-infected patients, but this has not been proved yet [26].

The basis for these diverse clinical outcomes is not yet understood. Because of the frequency with which *U. urealyticum* and *U. parvum* occur in healthy asymptomatic individuals, it is suggested that only certain subgroups of the species are disease-associated [10].

It is assumed that novel associations between human ureaplasmas and other human diseases will be identified in the near fututre.

During a 26-month-long period, 4154 samples were sent from our STD outpatient service for *Ureaplasma* culture. Two thousand and forty samples were collected from male patients. The vast majority of these samples were obtained from urethra. Only 15 samples were glans swab, ejaculatum, or urine. None of these last ones showed positive culture results. Three percent of the urethra samples turned out to be *Ureaplasma* positive by culture. According to these data, NGU is the main symptom in male patients. In case of balanitis, glans swab was not a suitable sample for *Ureaplasma* culture.

Two thousand and fourteen samples were collected from female patients. Two thousand and forty-seven samples were cervix swabs and 7% of these were *Ureaplasma* positive by culture. Fifty-nine urethra and eight urine samples from females were examined. Eleven percent of the cervix specimens were *Ureaplasma* positive, whereas all the urine samples were negative. It can be stated that cervix swab is the most suitable sample for *Ureaplasma* culturing in female patients. Altogether 247 *Ureaplasma* isolates were identified in the studied period (unpublished data).

Diagnostic

Because of lacking cell wall, ureaplasmas are extremely susceptible to drying and other adverse environmental conditions. Therefore, careful attention has to be given to specimen collection. The inoculation of transport medium is recommended at bedside whenever possible. Proper transportation conditions are needed to get viable cells in the diagnostic laboratory [8].

Ureaplasma spp. can be detected by several laboratory methods. Culture is the reference standard for detection of *Ureaplasma* spp. Specialized media: Shepard's 10B broth, A7, and A8 agar can be used for culturing. Culture media are completed with antibacterial agents (broad-spectrum semisynthetic penicillin) in order to minimize bacterial overgrowth, and with pH indicator, such as phenol red, in order to detect *Ureaplasma* growth. Broths that have changed color should be subcultured within a short time into a fresh broth and onto agar plates, while the culture can lose viability rapidly (within a few hours). A7 agar is used traditionally for *Ureaplasma* isolation. Microscopic examination of the colonies is neccessary. Agar plates have to be incubated in an atmosphere of room air supplemented with 5–10% CO_2 or in an anerobic environment of 95% N_2 plus 5% CO_2 for the best growth. Colonies typically grow in 2–5 days. The morphology of the colonies is granular, brown, 15–60 μm in diameter. According to the colonial morphology and urease production, genus level identification is possible. Cultures cannot be considered negative until 7 days of no growth. A8 agar does not contain manganese salts that inhibit some *Ureaplasma* serotypes; therefore, it has become the preferred growth medium in many laboratories. Commercially available diagnostic kits such as Mycoplasma Duo (Bio-Rad) and Mycoplasma IST 2 (bioMérieux) are simplified alternatives to conventional culture. These rapid diagnostic kits contain selective and differential liquid media. Growths of organisms are detectable after 24–48 h of incubation by biochemical activity caused color change. Specifically, they test the urea hydrolysis of *Ureaplasma* spp. or the arginine hydrolysis of *Mycoplasma hominis* via a phenol red pH indicator that detects ammonia liberation. These

rapid culture techniques have relatively similar sensitivities, specificities, and positive predictive values as traditional culture methods [7,27]. Culture provides only an initial positive or negative result. Subculture and molecular techniques are necessary to determine species type and the serovar [13]. *Ureaplasma* cultures can be overgrown by other microorganisms such as *Proteus* spp. and yeasts, spoiling the sensitivity of culture method. Inhibitory substances present in a clinical specimen also affect negatively the result of culturing [6].

Nucleic acid amplification tests have also been developed for *Ureaplasma* spp. The first PCR method for detection of human ureaplasmas in clinical samples was published in 1992 by Lee *et al.* [28]. Since then PCR methods have been increasingly used in the diagnosis of *Ureaplasma* infections and in the study of this pathogen. PCR methods have advantages over traditional culture methods: organism identification can occur even if the numbers of bacteria are low, viability is not necessary, and rapid identification within 24 h is possible [29]. Subtyping of isolates can be performed faster with PCR methods (24–48 h after sample collection) than with culture methods [13]. According to multiple studies, the detection of ureaplasmas in female genitourinary specimens, including cervical swab, amniotic fluid, and vaginal specimens by PCR is comparable or superior to that by culture. (Reported sensitivities for PCR detection ranged from 90–100%, whereas contemporaneous culture yielded detection sensitivities of 40–91%.) The PCR techniques have been used as a diagnostic tool for rapid detection of ureaplasmal infection in neonates with lower respiratory infections. The possible role of ureaplasmas in BPD has been examined also by PCR [8]. Because of the high sensitivity of PCR, false positive results can occur, principally due to intersample cross-contamination. Gene targets for PCR assays used to detect ureaplasmas and to define species and subtypes have included the subunits of urease gene, 16S rRNA genes and the multiple-banded antigen gene [30]. Heterogeneity of the multiple-banded antigen gene can be the basis of serotype identification [13]. Sophisticated nucleic acid amplification tests are necessary to discriminate between the two *Ureaplasma* spp. This is the reason of the lack of species determination in most studies untill the past few years [1,2]. Recently, multiplex real-time PCR assays have been developed and focused on quantitative detection of the two species separately. It can also be used to discriminate among all of the serovars [3,31,32]. Real-time TaqMan PCR assays have been developed that allow rapid, specific, sensitive, and quantitative detection with a 100 times greater sensitivity than conventional PCR, using the same primer sets and cycling conditions [15]. Convenient differentiation of *U. parvum* and *U. urealyticum* is also possible with real-time TaqMan PCR assays. Discrimination between harmless commensal colonization and clinically significant *Ureaplasma* infection can de done by the application of quantitative PCR techniques and PCR serotyping [13].

PFGE is a valuable tool for characterization of genetic relatedness of different *Ureaplasma* isolates, but it cannot be considered as a gold standard for genotyping of ureaplasmas. Other more rapid and less technically demanding methods (PCR-based assays of 16S ribosomal RNA, 16S-23S ribosomal RNA spacer regions, genes for urease subunits or multiple-banded antigen) are used to distinguish species, serovars, and subtypes within serovars. Although pulsed field gel electrophoresis (PFGE) is suitable for differentiating *U. parvum* from *U. urealyticum*, the four serovars of *U. parvum* and the serovars of *U. urealyticum* except 7, 11 and 4, 12 [33].

Multiplex PCR assay for simultaneous detection of *Trichomonas vaginalis, M. hominis*, and *U. urealyticum* has been developed [34]. Multiplex PCR-based microarray for detecting *Neisseria gonorrheae, Chlamydia trachomatis* and *U. urealyticum* simultaneously and separately in the presence of the internal control is also available [35]. A fluorescence polarization assay has been developed for simultaneous detection of *U. urealyticum*, *U. parvum*, *M. hominis*, and *Mycoplasma genitalium*. The fluorescence polarization assay (based on the asymmetric PCR coupled with hybridization-fluorescence polarization assay) can detect coinfection more effectively than the sequence method. Fluorescence polarization assay might serve as an alternative for the detection of multiple genitourinary infections [36].

Serological tests (enzyme immunoassay, microimmunofluorescence) for *Ureaplasma* spp. have also been developed and are mainly used in research [8].

Methods for antimicrobial susceptibility testing by microbroth, agar dilution, and agar gradient diffusion have been utilized [8]. Broth microdilution is the most practical, widely used method [3]. The inoculum size, the pH of media, and the incubation time alter the minimum inhibitory concentration (MIC) dramatically, therefore, standardized antimicrobial susceptibility testing methods are needed [37,38]. Methodological guidelines and quality control parameters have been developed by the Clinical and Laboratory Standards Institute Subcommittee [3].

SIR Mycoplasma (Bio-Rad) is a liquid medium for antibiotic sensitivity testing. It contains the following antibiotics at two different concentrations: doxycycline, tetracycline, azithromycin, josamycin, erythromycin, ofloxacin, and the following ones at a single concentration: clindamycin and pristinamycin. The growth of ureaplasmas is objectively measured by the metabolic activity (hydrolysis of urea). The breakpoints for the antimicrobials tested are as follows in mg/L: tetracycline $S \leq 4$, $R \geq 8$; doxycycline $S \leq 4$, $R \geq 8$; azithromycin

$S \leq 2$, $R \geq 4$; erythromycin $S \leq 1$, $R \geq 4$; josamycin $S \leq 2$, $R \geq 8$; ofloxacin $S \leq 1$, $R \geq 4$; clindamycin $S < 2$, $R > 2$; and pristinamycin $S < 2$, $R > 2$.

Mycoplasma IST-2 (bioMérieux) kit contains strips that give information on the presence or absence of *M. hominis* and *U. urealyticum*, give an estimate of the density of each organism [$>10^4$ colony-forming unit (CFU)/ml], and provide additional information on antibiotic susceptibility to erythromycin, clarithromycin, azithromycin, doxycycline, tetracycline, ciprofloxacin, ofloxacin, josamycin, and pristinamycin. The development of red color indicates the growth of bacteria and the resistance to a given antimicrobial agent. The breakpoints for the antimicrobials tested (according to the guidelines of CLSI) are as follows in mg/L: tetracycline $S \leq 4$, $R \geq 8$; doxycycline $S \leq 4$, $R \geq 8$; clarithromycin $S \leq 1$, $R \geq 4$; azithromycin $S \leq 0.12$, $R \geq 4$; erythromycin $S \leq 1$, $R \geq 4$; josamycin $S \leq 2$, $R \geq 8$; ciprofloxacin $S \leq 1$, $R \geq 2$; and ofloxacin $S \leq 1$, $R \geq 4$ [10,14].

SIR Mycoplasma kit has the advantage that the CFU of the inoculum used for antibiotic susceptibility testing can be chosen according to the density of bacteria (below or up to 10^4 CFU) in the specimen. Another advantage of this kit is that the antibiotic susceptibility of *M. hominis* and *U. urealyticum* can be tested separately, even if both bacteria are present in the clinical sample. With Mycoplasma IST-2 kit, just the common effective antibiotics can be determined in double *Mycoplasma* and *Ureaplasma* infections.

The biofilm-forming property of *Ureaplasma* isolates should be examined routinely, at least in patients with recurring symptoms. By the commercially available antibiotic susceptibiliy testing kits, only the planktonic *Ureaplasma* cells are tested and the sessile cells are out of observation. Therefore, it can occur that a *Ureaplasma* isolate is determined as susceptible to an antibiotic; however, the biofilm-forming, sessile form of the bacteria is resistant. This can result in therapeutic failure.

Therapy

Ureaplasma spp. are originally susceptible to bacteriostatic agents such as protein synthesis-inhibiting macrolides and tetracyclines as well as bactericidal agents including fluoroquinolones [37]. These drugs are the major antibiotics in treatment of *Ureaplasma* infections. Azithromycin (1 g single dose per os) or doxycycline (2×100 mg daily for 7 days per os) are the first choice drugs. Resistance to all three antibiotic classes has been described in clinical *Ureaplasma* isolates [14,39].

Macrolides are often used as first-line therapies for *U. urealyticum* infections. This should be changed in the near future, while recent epidemiological investigations have revealed substantial resistance [40]. Strains isolated recently from our patients showed 85% erythromycin resistance (unpublished data). New macrolides are the most promising antibiotics for use in neonatal ureaplasmal and mycoplasmal infections. Traditionally, erythromycin has been the most commonly used antibiotic in infants against *Ureaplasma* spp. The eradication of ureaplasmas with erythromycin from the respiratory tract of neonates was shown to be effective in variable rates. In-vitro studies with azithromycin show good inhibitory activity against ureaplasmas. Azithromycin has combined antimicrobial and anti-inflammatory properties, in addition, the special pharmacokinetic properties allow higher intracellular and tissue concentration, better tolerance, fewer adverse effects, and fewer drug interactions when compared with erythromycin. Therefore, azithromycin is an attractive agent for use in premature infants with ureaplasmal infections, but it has not been studied thoroughly in that population up to now. Timing of treatment may be crucial, while early treatment and eradication of ureaplasmas may be necessary to interrupt the inflammatory cascade generated by them and therefore, the development of BPD can be prevented. At present, data obtained from clinical trials are insufficient to determine whether antibiotic treatment of *Ureaplasma* in neonates has any influence on the development of BPD and its comorbidities [1,3,8].

Eradicating *Ureaplasma* spp. from the genital tract of pregnant women and the lung of a fetus or newborn may be difficult. The effect of macrolide antibiotics can be altered by mutations in 23S rRNA, coinfection with *M. hominis* or protective biofilm formation [2]. In the case of biofilm-forming *Ureaplasma* infection, clarithromycin is the drug of choice as it can penetrate the biofilm and then the bacterial membrane to reach the ribosome in order to inhibit biofilm synthesis [11]. Due to potential toxicities, fluoroquinolones are not usually recommended for use in neonates or pregnant women [3].

Instead of macrolides, doxycycline is the most commonly used antibiotic in the treatment of NGU. In case of tertracycline resistance, fluoroquinolones and doxycycline seem to be the most suitable treatment alternatives. Josamycin and pristinamycin are also therapeutic options, even as first choice drugs when empirical therapy is required [14,41].

Experience in treating invasive *Ureaplasma* infections is limited.

According to our own experiences, in case of *U. urealyticum* and *Chlamydia trachomatis* coinfections, azithromycin is the drug of choice as it is effective against both agents. Azithromycin is also suitable for the treatment of a single *U. urealyticum* infection.

Antibiotic resistance

As ureaplasmas lack cell wall peptidoglycan, they are not affected by beta-lactams or glycopeptides. They are not susceptible to sulfonamides or trimethoprim as they do not synthesize folic acid. Rifampicin is also inactive against them. They are intrinsically resistant to lincosamides except in high concentrations. Linezolid is not active against *Ureaplasma* spp. [11]. The extent of acquired resistance varies geographically. The antimicrobial therapy policies and the prior antimicrobial exposure in different populations determine the antibiotic resistance rates [14].

Fluoroquinolones are active against ureaplasmas, but resistance has been reported [3]. It is likely that the widespread use of fluoroquinolones for treatment of respiratory and urogenital infections promoted the appearance and the spread of quinolone resistance in *Ureaplasma* spp. Mutations in the *gyrA* and *parC* genes of the DNA gyrase/topoisomerase IV complex occurred in the presence of antimicrobial selective pressure [42]. Amino acid substitution in the type II topoisomerase proteins was proposed to be the reason of fluoroquinolone resistance. The most commonly noted substitutions were as follows: Ser83Leu in ParC protein, Asp112Glu in GyrA protein along with Ala125Thr, and Ala136Thr in ParC protein (triple substitution). All are within the quinolone resistance determining regions. It was proved that the latter triple mutation is not related to quinolone resistance. It is a species-specific polymorphism, which is found in all *Ureaplasma* serovars. This fact highlights the importance of species determination of *Ureaplasma* isolates. The Gln100Arg amino acid transitions in GyrA could potentially be responsible for the ciprofloxacin MIC of 128 mg/L [39]. The Ser83Asn and Asp87Tyr or Val in ParC (corresponding to changes at amino acid positions 80 and 84 in *Escherichia coli* ParC) were commonly observed in fluoroquinolone-resistant *Mycoplasma* and *Ureaplasma* spp. strains [43]. Naturally occurring fluoroquinolone resistance in *Ureaplasma* spp. from the USA was first reported in 2005. Till 2009, a total of 33 fluoroquinolone-resistant *Ureaplasma* isolates have been described [42]. Seventy-five percent of the fluoroquinolone-resistant strains identified to date were *U. urealyticum*, even though this species is isolated only in ~20% of all *Ureaplasma* clinical isolates. The ParC Ser83Leu (or S80L in *E. coli*) mutation was found in 58% of the fluoroquinolone-resistant *Ureaplasma* strains. This mutation is a strong candidate for fluoroquinolone resistance. Homologous mutations are identified in many other fluoroquinolone-resistant bacteria, such as *Streptococcus pneumoniae* and *Staphylococcus aureus* [38,39]. In case of Ser83Leu, a point mutation leads to an amino acid substitution. The substitution is two amino acids downstream from the proposed active site of the ParC protein [37,39,42]. The proposed Gram-positive origin of ureaplasmas may be responsible for the higher incidence of mutations within the topoisomerase IV proteins [39]. The drug resistance rates in *U. urealyticum* strains isolated from Turkish pregnant women were 92.6% to ciprofloxacin and 85.2% to ofloxacin. Resistance to josamycin was not observed, although some strains had intermediate resistance [14]. Strains isolated from our Hungarian patients showed 21% resistance to ofloxacin and 10% resistance to josamycin (unpublished data). The mechanism of resistance for many fluoroquinolone resistant strains is still unknown. Site-directed mutagenesis methods can be useful to confirm the reported mutations whether they fully account for the fluoroquinolone resistant phenotype in ureaplasmas [42].

U. urealyticum was considered as an intrinsically sensitive species to macrolides. Recently, resistance to macrolides has been widespread in *Ureaplasma* spp. The phenotype of acquired macrolide resistance mainly to roxithromycin and azithromycin was detected [44]. Strains isolated from our Hungarian patients showed 85% resistance to erythromycin and 12% resistance to azithromycin (unpublished data). The molecular characteristics of resistance to macrolides are well understood in bacteria, but are still undefined in *U. urealyticum*. The only reported mechanisms accounted for acquired resistance to macrolides in *U. urealyticum* up to now are the transition mutations in 23S rRNA [40]. The C2243N (T or C) transition in the 23S rRNA sequence might be associated with the acquired resistance to roxithromycin and azithromycin. This mutation has an influence on the tertiary structure of 23S rRNA and the binding of roxithromycin and azithromycin to 23S rRNA [44]. High-level erythromycin resistance is extremely uncommon in ureaplasmas. At the ribosomal level, no mechanism for macrolide resistance has been verified in ureaplasmas. Erythromycin MIC range was found to be from 0.125 to 8 mg/l with a MIC90 of 2 mg/l. MIC90 values for the newer macrolides, azithromycin and clarithromycin, were 1 mg/l and 0.063 mg/l, respectively. These data were obtained from over 300 clinical isolates of *Ureaplasma* spp. [8]. A mutation of the erythromycin-binding site around nucleotide position 2067 (2058 in *E. coli*) near the peptidyl transferase loop in domain V of one of the 23S rRNA operons was found consistently in erythromycin resistant *Ureaplasma* strains. Additional mutations of the associated L4 or L22 protein was found occasionally. Selected macrolide resistant mutants of *U. parvum* were associated with a variety of mutations in 23S rRNA and in L4 and L22 ribosomal proteins [45]. A deletion of two adjacent amino acids in the L4 protein was detected for the highly resistant strain (MIC 64 mg/l). Other resistance mechanisms, such as DNA methylation, expression of antibiotic modifying proteins, drug efflux via ion channels may be also responsible for the increased tolerance to erythromycin for ureaplasmas [38]. To detect the presence of a putative efflux phenotype, the susceptibilities of erythromycin resistant *Ureaplasma* mutants were determined in the presence and lack of

reserpine. No significant difference in MIC values was detected between the two groups, the putative efflux mechanism was not proved [45]. *U. urealyticum* harboured the *erm*B, *msr*A, *msr*B, and *msr*D genes. These genes confer resistance to macrolides and (or) lincosamides. The *erm*B gene, closely associated with the int-Tn gene, may be located in transposon in *U. urealyticum*. The *erm*B gene (a subtype of the *erm* gene) encodes a protein that can post-transcriptionally methylate the binding sites of macrolides, the 23S rRNA in ribosome of bacteria. This results in a resistant phenotype, while affecting macrolide binding to the 23S rRNA. The *msr* genes are common active efflux genes. They confer low level resistance to 14-, 15-membered macrolides (M phenotype) or 14-, 15-membered macrolides and streptogramin B (MS phenotype) in many bacteria. *msr* genes, mainly the *msr*B and *msr*D genes, are also commonly observed molecular mechanisms of resistance to macrolides in *U. urealyticum*. The *erm*B and *msr* genes can be located in plasmids, transposons, or in the pathogenicity islands of enteric pathogens. The resistance genes might have been transmitted by these mobile genetic elements from enteric bacteria to other bacteria species, probably to *U. urealyticum*, too. *Erm*B gene may be a part of a transposon in *U. urealyticum*. The three *msr* gene subtypes were not associated with the int-Tn gene, so they were not suggested to locate on transposon in *U. urealyticum* [40]. It is possible that *U. urealyticum* isolates have inherently increased tolerance to erythromycin, compared with *U. parvum* isolates [38].

Tetracyclines are generally effective against *Ureaplasma* spp., but resistance is becoming more common. Acquisition of the *tetM*-transferable genetic element that mediates tetracycline resistance was first described in *Ureaplasma* by Roberts and Kenny in 1986 [46]. Currently, this is the only mechanism of tetracycline resistance described in ureaplasmas. Tetracycline resistance in ureaplasmas has been reported to occur in approximately 10% of clinical isolates [8]. Strains isolated from our Hungarian patients showed 5% resistance to tetracycline and 4% resistance to doxycycline (unpublished data). *Ureaplasma* strains resistant to tetracycline with a functional TetM protein were detected to be additionally resistant to doxycycline. Screening for the *tetM* gene could detect tetracycline-resistant strains, but a false-positive strain was already found. This fact is a note of caution for the use of PCR screening for antibiotic resistance [37,38]. Forty-five percent of *Ureaplasma* strains, isolated primarily from adults, were tetracycline-resistant in the USA [3].

The sessile culture form of *Ureaplasma* which forms biofilm shows reduced antibiotic susceptibility, compared with the planktonic culture form. The major differences between the two types of cell growth were observed in case of erythromycin and telithromycin. These antibiotics are more active against planktonic cultures. The most active antibiotic against biofilm-forming *Ureaplasma* is clarithromycin. According to a study, all examined *Ureaplasma* strains were fully susceptible to clarithromycin, independent of the type of culture [11].

All of the doxycycline resistant *Ureaplasma* strains isolated from our Hungarian patients were resistant simultaneously to at least one of the other tested antibiotics. The same was observed in case of azithromycin, but at a lower ratio. These facts suggest that ex juvantibus administration of any of them (however, they are the first choice drugs) may select cross-resistant strains for both antibiotics (unpublished data).

Further research is needed regarding resistance mechanisms, biofilm formation, and biofilm induced antibiotic resistances in human *Ureaplasma* spp.

Prophylaxis

PCR testing of amniotic fluid in second trimester can help to identify women at risk for preterm labor and delivery because of *Ureaplasma* infection [8]. Real-time PCR assays could also be used to quantitatively screen vaginal swabs for *U. parvum* and *U. urealyticum* as risk factors in pregnant women [6]. Early antimicrobial treatment of infants based on the result of susceptibility testing could play a role in interrupting the *Ureaplasma* caused inflammatory cascade, hereby improving respiratory and other outcomes. Additional studies using azithromycin as an adjunctive therapy in preterm labor are now warranted [3].

Conclusion

Studies on ureaplasmas have clearly proven that these bacteria may be not only commensals in the bacterial flora of the human female genital tract but they can cause a variety of local and generalized infections both in females, sexually active males, fetuses, and newborns, depending on the general health condition and the immunological status of an individual. This has to be kept in mind for medical staff working in both the medical practice and the microbiological diagnostic services. Both primary and secondary immunodefficient individuals such as VLBW newborns and transplant patients are at high risk of generalized life-threatening ureaplasmal infections. Our knowledge is insufficient at present on the pathogenesis and pathomechanism of different ureaplasmal infections; therefore, further examinations are needed. Particularly the exact understanding of pathogenity of different *Ureaplasma* serovars is essential.

Because of the growing incidence of resistance to a panel of antimicrobial agents in *Ureaplasma* strains and their ability to form biofilm, treatment of supposed *Ureaplasma* infection has to be based on prior cultivation and

antibiotic sensitivity testing. Besides these examinations, strict consideration of intrinsic resistance is mandatory.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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II.

Az *Ureaplasma urealyticum* és a *Mycoplasma hominis* antibiotikum-érzékenysége és gyakorisága szexuálisan aktív egyének genitális mintáiban

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Az *Ureaplasma urealyticum* és *Mycolasma hominis* fontos szerepet tölt be az urogenitális fertőzések kiváltásában. *Célok:* A szerzők a Semmelweis Egyetem, Bőr-, Nemikórtani és Bőronkológiai Klinika STD-ambulanciáján 2008. május 1. és 2010. július 31. között vizsgált betegek genitális mintáiban az *Ureaplasma urealyticum* és *Mycoplasma hominis* gyakoriságának és antibiotikum-érzékenységének elemzését tűzték ki célul. *Módszerek:* Férfiaknál az urethrából, nőknél a cervixből és urethrából vattapálcával (Biolab) vett mintákat Urea-Myco DUO kit (Bio-Rad) segítségével tenyésztették, majd a pozitívnak bizonyult minták esetén a rezisztenciát U9 levesben SIR Mycoplasma kittel (Bio-Rad) határozták meg. *Eredmények:* A vizsgált 4154 beteg genitális mintájából 247 esetben (6%) *Ureaplasma urealyticum* és 26 esetben (0,63%) *Mycoplasma hominis* tenyészett ki. Mind az *Ureaplasma uraelyticum*, mind a *Mycoplasma hominis* törzsek többsége (75%, illetve 77%) cervixből, kisebb hányada (25%, illetve 23%) férfi és női urethramintából származott. Az *Ureaplasma urealyticum*- és a *Mycoplasma homonis*-pozitívak a 16–60 év között minden korcsoportban megtalálhatók voltak, de legnagyobb arányban a 21–40 évesek között fordultak elő. A legtöbb *U. urealyticum* törzs megőrizte érzékenységét tetracyclin (95%), doxycyclin (96%), azithromycin (88%) és josamycin (90%) iránt, míg a törzsek 21%-a ofloxacin, 85%-a erythromycin-, 79%-a clindamycinrezisztens volt. Az *Ureaplasma urealyticum* törzsek 77%-a erythromycinre és clindamycinre együttesen is rezisztens volt, ami jelzi, hogy az ex iuvantibus adott két antibiotikum mindegyike szelektálhatja a keresztrezisztenciával bíró törzseket is. A *Mycoplasma hominis* törzsek között a clindamycin-, doxycyclin-, ofloxacin- és tetracyclinrezisztens törzsek aránya 4–12% volt. *Következtetések:* Az összes vizsgált antibiotikumra mindössze egyetlen *Ureaplasma urealyticum* törzs bizonyult érzékenynek, ezért a tenyésztés vagy a kórokozó kimutatása molekuláris genetikai módszerrel (PCR) nem elégséges a biztosan sikeres terápiához, ahhoz az antibiotikum-érzékenység meghatározása is szükséges. A szerzők felhívják a figyelmet a hazai *U. urealyticum* nagymértékű erythromycin-, clindamycin- és ofloxacinrezisztenciájára. Magyarországon ez az első ilyen klinikai mikrobiológiai közlemény. Orv. Hetil., 2011, *152,* 1698–1702.

Kulcsszavak: *U. urealyticum, M. hominis* férfi, nő, gyakoriság, antibiotikum-érzékenység, -rezisztencia

Frequency and antibiotic resistance of *Ureaplasma urealyticum* and *Mycoplasma hominis* in genital samples of sexually active individuals

Ureaplasma urealyticum and *Mycoplasma hominis* have important role among the causative agents of sexually transmitted diseases. *Aim:* The aim of the study was to determine the frequency and antibiotic resistance of *Ureaplasma urealyticum* and *Mycoplasma hominis* in genital samples obtained from patients examined in the Sexually Transmitted Diseases Centre of the Department of Dermatology, Venerology and Dermatooncology, Semmelweis University, Budapest between May 1, 2008 and July 31, 2010. *Patients and methods:* Samples were taken from the

urethra in men and from the cervix and urethra in women by universal swab (Biolab®) into Urea-Myco DUO kit (Bio-Rad®) and were incubated for 48 hours at 37 C°. Antibiotic sensitivity of positive samples was determined in U9 bouillon using SIR Mycoplasma kit (Bio-Rad®). *Results:* Samples for 4154 patients aged 16-60 years were examined. In 247/4154 samples (6%) *U. urealyticum* and in 26/4154 samples (0.63%) *M. hominis* was isolated from the genital tract. Most *U. urealyticum* and *M. hominis* strains (75% and 77%, respectively) were cultured from cervix, while the remaining 25%, and 23% from the male and female urethra, respectively. *U. urealyticum* and *M. hominis* were most commonly detected in patients aged between 21 and 40 years. The majority of *U. urealyticum* strains were sensitive to tetracycline (94%), doxycycline (95%), azithromycin (88%) and josamycin (90%), but were resistant to ofloxacin (21%), erythromycin (85%) and clindamycin (79%). Seventy-seven percent of the *U. urealyticum* strains were simultaneously resistant to erythromycin and clindamycin, suggesting that ex iuvantibus therapies may select cross-resistant strains to both antibiotics. The resistance of *M. hominis* to clindamycin, doxycycline, ofloxacin and tetracycline varied between 4% and 12 %. *Conclusions:* Because none of the strains was sensitive to all examined antibiotics, the antibiotic sensitivity of *U. urealyticum* and *M. hominis* strains should be determined. The high rate of ofloxacin, erythromycin and clindamycin resistance should be considered in the therapy of *U. urealyticum* infections in Hungary. This is the firstsuch a clinical microbiological study in this topic in Hungary.
Orv. Hetil., 2011, *152,* 1698–1702.

Keywords: *Ureaplasma urealyticum, Mycoplasma hominis,* men, women, frequency, antibiotic resistance

(Beérkezett: 2011. augusztus 9.; elfogadva: 2011. augusztus 30.)

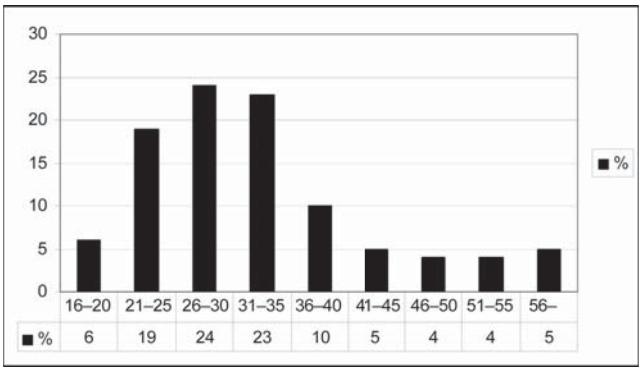
A Mollicutesek osztályának több fajáról igazolták, hogy az emberi szervezetben képesek kolonizációt és megbetegedést okozni; a genitális traktus fertőzéseinek egy részéért a *Mycoplasma hominis, Mycoplasma genitalium,* valamint az *Ureaplasma* speciesek – *Ureaplasma urealyticum, Ureaplasma parvum* – felelősek. A pubertást követően mind az *U. urealyticum,* mind a *M. hominis* kimutatható a szexuálisan aktív populáció jelentős hányadában, az *U. urealyticum* fordul elő gyakrabban [1, 2, 3].

Az urogenitális rendszerben okozhatnak akut és krónikus gyulladásos kórképeket, nőkben vaginitist, salpingitist, kismedencei gyulladást, vetélés utáni lázat, valamint gyermekágyi lázat, urethralis szindrómát és habituális abortuszt, chorioamnionitist, valamint következményes koraszülést; újszülöttekben neonatalis fertőzéseket, mint pneumonia, szepszis [1]. Férfiaknál létrejöhet akut és krónikus húgycsőgyulladás – elsősorban az *U. urealyticum* és a *M. genitalium* szerepe igazolt –, prostatitis, krónikus prostatitis, here- és mellékheregyulladás, valamint oligoasthenospermia [2]. Mindkét nemben okozhatnak vesekövet, pyelonephritist, Reiter-szindrómát, valamint szexuális úton szerzett reaktív arthritist [1, 2].

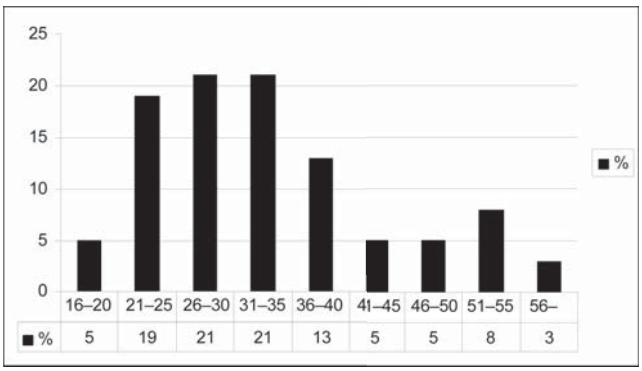
Vizsgálataink célja az volt, hogy megállapítsuk, milyen gyakori az *U. urealyticum* és *M. hominis* hordozás és fertőzés szexuálisan aktív személyekben és milyen a baktérium antibiotikum-rezisztencia spektruma.

Módszer

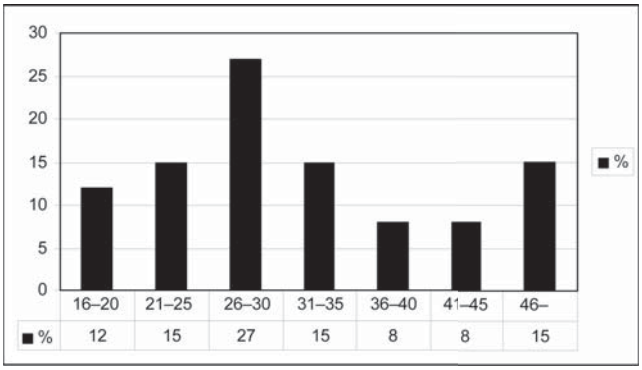
A mintákat cervix/urethra vattás, rugalmas pálcával (Biolab) vettük; a feldolgozáshoz és tenyésztéshez a Mycoplasma Duo kitet (Bio-Rad) használtuk; az inkubálás 37 °C-on 48 órán keresztül, emelt CO_2 tenziójú közegben történt. Az antibiotikum-érzékenység meghatározása U9 levesben történt a SIR Mycoplasma kit (Bio-Rad) segítségével, a tenyésztéssel azonos körülmények között [2].



1. ábra A kitenyészett *Ureaplasma urealyticum* törzsek korcsoport szerinti eloszlása nőkben (185 = 100%)



2. ábra A kitenyészett *Ureaplasma urealyticum* törzsek korcsoport szerinti eloszlása férfiakban (62 = 100%)



3. ábra | A kitenyészett *Mycoplasma hominis* törzsek korcsoport szerinti eloszlása (26 = 100%)

Eredmények

2008. május 1. és 2010. július 31. között a Semmelweis Egyetem, Bőr-, Nemikórtani és Bőronkológiai Klinika STD-ambulanciájára érkező betegek genitális mintáit vizsgáltuk *U. urealyticum* és *M. hominis* fertőzésre vagy hordozásra. Összesen 4154 minta érkezett *(1. táblázat)*, ezen belül 2040 származott férfitól, 2114 nőtől. A férfiak 3%-ából, a nők 9%-ából sikerült *U. urealyticumot* izolálni. A férfiak 0,2%-ából, míg a nők 1%-ából tudtunk *M. hominist* kitenyészteni. A férfiaknál kizárólag a húgycsőből, a nőknél a húgycsőből és a cervixből tenyészett ki *U. urealyticum*. A férfiaknál csak a húgycsőből, míg a nőknél csak a cervixből származó minták tartalmaztak *M. hominist*.

Mind a férfiak, mind a nők esetén minden vizsgált életkorban előfordul a kórokozó *(1–3. ábra)*, mindkét nemben 21–35 éves korcsoportokban tenyészett ki az *U. urealyticum* és a *M. hominis* legnagyobb arányban.

Az *U. urealyticum*-, valamint a *M. hominis*-pozitív egyének között mindkét nemben 40% fölött volt a tünetmentesek aránya, a nők esetén a betegek 48%-a colpitisben, 3%-a urethritisben, 5%-a egyéb betegségben szenvedett. Az *U. urealyticum*- és *M. hominis*-pozitív férfiak esetén a beérkezett minták 36%-a urethritisben, 13%-a balanitisben, 6%-a egyéb betegségben szenvedő betegtől származott.

A kitenyészett *U. urealyticum* és *M. hominis* törzseknél antibiotikumrezisztencia-vizsgálatot végeztünk, majd a kapott adatokat összesítettük *(2. és 3. táblázat)*.

A *2. táblázatból* látható, hogy *U. urealyticum* esetén megjelentek tetracyclin-, doxycyclin-, ofloxacin- és azithromycinrezisztens törzsek, valamint kimagasló az erythromycin- és clindamycinrezisztencia.

1. táblázat | *Ureaplasma urealyticum* és *Mycoplasma hominis* kimutatására beérkező minták és kitenyészett törzsek aránya (%)

Minta	Férfiak (n)	*U. urealyticum*-pozitívak (%)	*M. hominis*-pozitívak (%)	Nők (n)	*U. urealyticum*-pozitívak (%)	*M. hominis*-pozitívak (%)
Húgycső	2025	3	0,2	59	*7*	–
Cervix	–	–	–	2047	11	1
Glans	2	0	–	–	–	–
Ejakulátum	*7*	0	–	–	–	–
Vizelet	6	0	–	8	–	–

2. táblázat | A kitenyészett *Ureaplasma urealyticum* törzsek rezisztenciamegoszlása (247 = 100%)

Antibiotikum	Érzékeny törzsek száma	Érzékeny törzsek százaléka	Rezisztens törzsek száma	Rezisztens törzsek százaléka
Ofloxacin	195	*79*	52	*21*
Tetracyclin	234	*95*	13	*5*
Doxycyclin	238	*96*	9	*4*
Clindamycin	52	*21*	195	*79*
Erythromycin	36	*15*	211	*85*
Azithromycin	218	*88*	29	*12*

3. táblázat | A kitenyészett *Mycoplasma hominis* törzsek rezisztenciamegoszlása (26 = 100%)

Antibiotikum	Érzékeny törzsek száma	Érzékeny törzsek százaléka	Rezisztens törzsek száma	Rezisztens törzsek százaléka
Ofloxacin	24	*92*	2	*8*
Tetracyclin	23	*88*	3	*12*
Doxycyclin	25	*96*	1	*4*
Clindamycin	25	*96*	1	*4*

A továbbiakban megvizsgáltuk az Egészségügyi Minisztérium szexuálisan terjedő betegségekre vonatkozó szakmai irányelvében szereplő antibiotikumokra rezisztens törzseknél, hogy mely más antibiotikumra mutatnak még keresztrezisztenciát. Vizsgálataink alapján minden doxycyclinrezisztens törzs, valamint az ofloxacinrezisztens törzsek 96%-a mutat keresztrezisztenciát erythromycinre; a doxycyclinrezisztens izolátumok 89%-a, az azithromycinrezisztensek 79%-a, az ofloxacinrezisztensek 96%-a, az erythromycinrezisztensek 90%-a bizonyult rezisztensnek clindamycinre is. Az erythromycin-clindamycin keresztrezisztencia a kitenyészett izolátumok 77%-ában figyelhető meg; ez konstitutív rezisztencia, mivel az általunk alkalmazott módszerrel az indukált rezisztenciát nem tudtuk vizsgálni.

A kitenyészett *M. hominis* törzsek esetén is elvégeztük az *U. urealyticum*nál leírt antibiotikum-érzékenységi vizsgálatot. A *3. táblázatból* leolvasható, hogy a tetracyclin esetén 12%, a protokollban szereplő többi antibiotikum esetén 10% alatt marad a rezisztens törzsek aránya. A makrolidekkel szemben a *M. hominis* törzsek természetes rezisztenciával rendelkeznek, ezért nem szerepelnek erre vonatkozó adatok a táblázatban.

Megbeszélés

Vizsgálataink alapján elmondható, hogy az *U. urealyticum* és a *M. hominis* mindkét nemben előforduló kórokozó. Minden, szexuálisan aktív korcsoportban ki tudtuk mutatni a baktériumokat, férfiak és nők esetében is 21–35 éves korosztályból származott a legtöbb izolátum. A tünetek tekintetében mindkét nemben az *U. urealyticum*- és a *M. hominis*-pozitívak között egyaránt 40% felett volt a tünetmentes hordozók aránya.

Rezisztenciavizsgálatainkat összevetve a nemzetközi szakirodalomban közölt adatokkal, egyes antibiotikumok tekintetében – mint a makrolidek, clindamycin és tetracyclin – hasonló tendenciát észleltünk, másoknál, mint a fluorokinolonok, eltérést tapasztaltunk.

A tetracyclin- és doxycylinrezisztencia tekintetében a szakirodalomban található adatokhoz hasonló eredményeket kaptunk. A rezisztencia hátterében eddig csak a *tetM* gént írták le, amely egy, a bakteriális riboszómához kötődő védő fehérjét termel [4, 5].

Az erythromycin és clindamycin esetén kiemelkedően magas a rezisztencia, valamint a keresztrezisztencia, akárcsak külföldön [6, 7]. Az alkalmazott vizsgálómódszer csak a konstitutív keresztrezisztenciát mutatja ki. A makrolidek tekintetében elsősorban az azithromycin és josamycin a leginkább hatékony, de már megjelentek ezen makrolidekre is rezisztens törzsek [6, 8, 9]. A makrolidrezisztencia hátterében több mechanizmus állhat; aktív efflux (*msrD, msrB* gének); 23S rRNS mutációja vagy metilációja (*erm* gén). Az aktív efflux hátterében álló *msrD,* valamint *msrB* gének a lincosamidoktól is védik a baktériumot; míg a 23S rRNS szerkezetében történő változások – metiláció; *erm* gén által vagy mutáció – nem okoznak lincosamidrezisztenciát [8, 9].

A vizsgálórendszerben az ofloxacin által képviselt fluorokinolonokkal szembeni rezisztenciát összevetve más országokkal, vizsgálatunk alapján jelentősen kedvezőbb a helyzet Magyarországon [6, 10, 11]. A fluorokinolonrezisztencia hátterében a giráz, valamint a topoizomeráz enzimeket védő fehérjék állnak, amelyek termelését a *gyrA, parC, parE* gének kódolják [12, 13].

Az elsőként választandó antibiotikum *U. urealyticum,* valamint *M. hominis* fertőzésben a doxycyclin. Azonban terhességben, terhesség gyanúja esetén, valamint doxycyclinallergia esetén, illetve, ha egyéb okból kifolyólag a doxycyclin alkalmazása kontraindikált, *U. urealyticum* fertőzésben az azithromycin, *M. homonis* esetén a clindamycin az első választandó gyógyszer [14]. Abban az esetben, ha *Chlamydia trachomatis* és *U. urealyticum* együttes fertőzést diagnosztizálunk, azithromycin a választandó szer a kezeléshez, mert erre mindkét baktériumfaj jelentősen megőrizte érzékenységét [15]. Biofilmképző *U. urealyticum* törzsek esetében a clarithromycin az első választandó szer, mivel képes a biofilmen áthatolni és képződését megakadályozni. Minden kitenyészett *U. urealyticum* törzsnél ajánlatos a biofilmképzési és a biofilm képződését gátló teszt elvégzése [16].

U. urealyticum, valamint *M. hominis* fertőzés gyanúja esetén, az első választandó antibiotikumokkal történő kezelés kudarca esetén mindenképpen javasoljuk a rezisztenciavizsgálat elvégzését.

Legkorszerűbben viszont az jár el, aki még a terápia megkezdése előtt elvégezteti a tenyésztést és antibiotikum-érzékenységi vizsgálatot *Ureaplasma/Mycoplasmára*.

Összefoglalva: Vizsgálatunk során – 2008. május–2010. július között – klinikánk STD-ambulanciájáról érkező betegek genitális mintáiból 247 esetben sikerült *U. urealyticumot*, 26 esetben *M. hominist* izolálni, valamint antibiotikum-érzékenységet meghatározni. A kitenyészett *U. ureplasma* és *M. hominis* törzsek több mint 50%-a panaszokkal rendelkező személyekből származott.

A vizsgálatunk során minden általunk vizsgált antibiotikumra érzékeny törzset nem találtunk, ezért van jelentős szerepe a kezelés megkezdése előtt a tenyésztésnek, valamint az antibiotikumérzékenység-meghatározásnak.

Köszönetnyilvánítás

A szerzők köszönetüket fejezik ki *Győriné Bencze Ildikónak* és *Vörös Elvirának* kiváló technikai munkájukért.

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„Valójában sem az önbecsülés, sem az emberszeretet nem csorbul, midőn világossá tesszük, hogy az elképzelés és az arra való törekvés szervi folyamatok eredménye.”

(Wilhelm Griesinger)

III.

Eur J Clin Microbiol Infect Dis (2013) 32:1423–1426
DOI 10.1007/s10096-013-1892-y

ARTICLE

Incidence and antibiotic susceptibility of genital mycoplasmas in sexually active individuals in Hungary

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Received: 26 February 2013 / Accepted: 29 April 2013 / Published online: 18 May 2013

Abstract The aim of this study was to examine the incidence and antibiotic sensitivity of *Ureaplasma urealyticum* and *Mycoplasma hominis* strains cultured from the genital discharges of sexually active individuals who attended our STD outpatient service. Samples were taken with universal swab (Biolab®, Budapest, Hungary) into the Urea-Myco DUO kit (Bio-Rad®, Budapest, Hungary) and incubated in ambient air for 48 h at 37 °C. The determination of antibiotic sensitivity was performed in U9 and arginin broth using the SIR Mycoplasma kit (Bio-Rad®, Budapest, Hungary) under the same conditions. Between 01.05.2008 and 31.12.2011, 373/4,466 (8.35 %) genito-urethral samples with *U. urealyticum* and 41/4,466 (0.91 %) genito-urethral samples with *M. hominis* infection were diagnosed in sexually active individuals in the National STD Center, Semmelweis University. *U. urealyticum* was isolated in 12.54 % in the cervix and 4.1 % in the male urethra, while *M. hominis* was isolated in 1.33 % in the cervix and 0.51 % in the male urethra. The affected age group was between 21 and 60 years old. *U. urealyticum* strains were sensitive to tetracycline (95.9 %), doxycycline (97.32 %), and azithromycin (85.79 %), and resistant to erythromycin (81.23 %), clindamycin (75.06 %), and ofloxacin (25.2 %). Cross-resistance occurred in 38.71 % of patients to erythromycin and clindamycin. *M. hominis* strains were sensitive to clindamycin, ofloxacin, and doxycycline in more than 95 %, to tetracycline in 82.92 %, and no cross-resistance was detected among the antibiotics. Our study confirms that the continuously changing antibiotic resistance of ureaplasmas and mycoplasmas should be followed at least in a few centers in every country, so as to determine the best local therapy options for sexually transmitted infection (STI) patients.

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Introduction

Some species in the class of Mollicutes have been proved to colonize and induce disease in the genital tract, from the *Mycoplasma* genus, most commonly *Mycoplasma hominis*, *Mycoplasma genitalium*, and *Mycoplasma fermentans*, as well as *Ureaplasma* spp., such as *Ureaplasma urealyticum* containing serovars 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13, and *Ureaplasma parvum* containing serovars 1, 3, 6, and 14. After puberty, colonization of the urogenital tracts by genital mycoplasmas occurs as a result of sexual activity in both sexes. The frequency of colonization increases with the number of partners [1–3].

However, genital mycoplasmas are isolated from cervicovaginal and urethral specimens of healthy patients in more than 50 %. They are found relatively frequently on the genital mucosa of sexually active people with manifest disease, of which *U. urealyticum* and *M. hominis* can be detected, with *U. urealyticum* being commonly present. The production of IgA protease, adhesins, urease, phospholipase A1, A2, and C, hemolysins, and hydrogen peroxide are proposed as virulence factors in ureaplasmas [4]. By the contributing effects of these virulence factors, genital mycoplasmas can cause acute and chronic inflammations in the urogenital tract. Furthermore,

they are etiological factors of salpingitis and pelvic inflammatory disease, postpartum sepsis, and urethral syndrome. Their occurrence in expectant mothers provides a reservoir for vertical transmission to the fetus and induce chorioamnionitis, habitual abortion, as well as consequential premature birth and neonatal infections, such as pneumonia and sepsis [1]. In male patients, because of their attachment to urethral epithelial cells and spermatozoa, they also cause acute or chronic urethritis—primarily, the role of *U. urealyticum* and *M. genitalium* has been proved—acute and chronic prostatitis, epididymitis, epididymoorchitis, as well as oligoasthenospermia [3, 5, 6]. Mycoplasmas might contribute to renal calculi formation, pyelonephritis, Reiter's syndrome, and sexually acquired reactive arthritis (SARA) in both sexes [1].

The aim of our study was to determine the incidence and antibiotic resistance spectrum of *U. urealyticum* and *M. hominis* infections in sexually active individuals.

Methods

Samples were taken with universal flexible cotton swabs (Biolab®, Budapest, Hungary) from patients with non-gonococcal, non-chlamydial (NGNC) urethritis or other genito-urethral complaints and from symptom-free promiscuous patients who presented between 1st May 2008 and 31st December 2011. Our study is retrospective, but previously, the samples were evaluated periodically. The clinical picture was defined by complete physical examination according to the patient complaints. Samples were taken from the urethra, urine, glans and ejaculate of male patients, and from the cervix, urethra, and urine of female patients. If more than one sample was collected from a single patient, we collected urethral samples first, followed by ejaculate and urine samples. Samples were cultured immediately in ambient air for 48 h at 37 °C using the Mycoplasma DUO kit (Bio-Rad®, Budapest, Hungary), according to the facts that the STD Center and the microbiologic laboratory is located in the same building and the samples are collected two times per day. This kit allows the differential titration of two *Mycoplasma* species that may be present in the same patient. A change in color from yellow to red without clouding the medium indicated the presence of mycoplasmas ($\geq 10^4$ CCU/ml). The determination of antibiotic sensitivity was performed in U9 or arginine broth (SIR Mycoplasma kit, Bio-Rad®, Budapest, Hungary) for *U. urealyticum* or *M. hominis* under the same conditions. Doxycycline (4 mg/L and 8 mg/L), tetracycline (4 mg/L and 8 mg/L), azithromycin (2 mg/L and 4 mg/L), josamycin (2 mg/L and 8 mg/L), erythromycin (1 mg/L and 4 mg/L), clindamycin (2 mg/L), pristinamycin (2 mg/L), and ofloxacin (1 mg/L and 4 mg/L) resistances were tested in a liquid-medium antibiogram. The growth of *Mycoplasma* was objectively measured by their metabolic activity, releasing ammonia and making the medium turn alkaline, which resulted in a color change. If the microorganism was sensitive to the tested antibiotic, its metabolism was inhibited and the medium remained yellow.

Results

We examined *U. urealyticum* and *M. hominis* infection of genital samples from sexually active patients admitted to our STD Center between 1 May 2008 and 31 December 2011. A total of 4,466 samples were examined (Table 1). Out of the 4,466 samples, 2,157 were from male patients and 2,309 were from female patients. Previous microbiological examinations excluded *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections.

U. urealyticum was isolated from 4.07 % of the male samples and 12.34 % of the female samples, and *M. hominis* was isolated from 0.5 % and 1.29 % of the male and female samples, respectively.

Among the *U. urealyticum*- and *M. hominis*-positive patients, the age group of 26–35 years was predominant.

The ratio of symptom-free persons among *U. urealyticum*-positive as well as *M. hominis*-positive individuals was above 40 % in both sexes. The dominant clinical symptoms in females were vaginal discharge (23.53 %), genital pruritus

Table 1 Distribution of specimens cultured for *Ureaplasma urealyticum* and *Mycoplasma hominis* strains between May 2008 and December 2011 and incidence of infections (%)

Samples	Men	*U. urealyticum* positive	*M. hominis* positive	Women	*U. urealyticum* positive	*M. hominis* positive
Urethra	2142	88 (4.1 %)	11 (0.51 %)	61	4 (6.5 %)	0
Cervix	–	–	–	2,240	281 (12.54 %)	30 (1.33 %)
Glans	2	0	0	–	–	–
Ejaculate	7	0	0	–	–	–
Urine	6	0	0	8	0	0
Subtotal	2,157	88 (4.07 %)	11 (0.5 %)	2,309	285 (12.34 %)	30 (1.29 %)
Total	4,466					

Table 2 Distribution of antibiotic resistance of 373 *Ureaplasma urealyticum* strains cultured from genital samples between May 2008 and December 2011 in Hungary

Antibiotic	No. of strains tested	Sensitive		Moderately sensitive		Resistant	
		No.	%	No.	%	No.	%
Erythromycin	373	47	12.56	23	6.1	303	81.23
Azithromycin	373	320	85.79	17	4.55	36	9.65
Clindamycin	373	85	22.78	8	2.1	280	75.06
Ofloxacin	373	264	71.58	15	4.2	94	25.2
Tetracycline	373	358	95.9	0	0	15	4.02
Doxycycline	373	363	97.32	1	0.27	9	2.41

(9.19 %), and colpitis (11.76 %). The dominant clinical symptoms in men were urethritis (35.48 %), balanitis (13.97 %), and urethral discharge (7.52 %).

Table 2 shows the antibiotic resistance of *U. urealyticum* strains that were sensitive to tetracycline (95.9 %), doxycycline (97.32 %), and azithromycin (85.79 %). Resistance to several antibiotics was detected among the *U. urealyticum* strains, as they were resistant to ofloxacin (25.2 %), erythromycin (81.23 %), and clindamycin (75.06 %). The antibiotic cross-resistance of *U. urealyticum* strains was also examined and 77.55 % of the ofloxacin-resistant strains demonstrated cross-resistance to erythromycin. Every ofloxacin-resistant strain showed resistance to doxycycline as well. Every ofloxacin- or erythromycin-resistant and 72.22 % of azithromycin-resistant isolates proved to be also resistant to clindamycin. Erythromycin–clindamycin cross-resistance was shown in 38.71 % of all strains; this is constitutive resistance, since the antibiotic sensitivity test used in this study was unable to recognize the inducible clindamycin resistance.

Table 3 shows that more than 95 % of the *M. hominis* strains were susceptible to ofloxacin, clindamycin, and doxycycline, and approximately 82.92 % of them were susceptible to tetracycline. Mycoplasmas possess inherent resistance to macrolides, therefore, there were no relating examinations carried out.

Discussion

Our results confirm that *U. urealyticum* and *M. hominis* are relatively commonly detected in the cervix and urethra of sexually transmitted infection (STI) patients and high-risk individuals. The bacteria were present with the highest rate in the age group 26–35 years in both men and women. The proof of causality of genital mycoplasmas is still lacking, but our results show that the ratio of carriers with symptoms was approximately 30 % in both sexes among *U. urealyticum*- and *M. hominis*-positive patients.

Comparing our resistance results with those published recently [7, 8], similar tendencies were detected regarding certain macrolides, clindamycin and tetracycline, particularly the very high erythromycin and constitutive clindamycin resistance and cross-resistance, while we found deviations in the rate of fluoroquinolone resistance [8]. Regarding macrolides, primarily azithromycin and josamycin were the most effective, but we isolated resistant strains as well [8, 9]. There can be more than one mechanism in the background of macrolide resistance, e.g., active efflux (*msrD*, *msrB* genes) or 23S rRNS mutation or metilation (*erm* gene). The *msrD* and *msrB* genes—also responsible for active efflux—protect the bacteria from lincosamides too, while the changes in the 23S rRNS structure—metilation by *erm* gene or mutation—do not result in lincosamide resistance [10].

Antibiotic resistance is the most common reason for treatment failure in genital *Mycoplasma* infections. In mycoplasmas, the *tetM* gene is responsible for tetracycline resistance, producing a ribosome-protective protein [11, 12].

Fluoroquinolone resistance develops in the presence of gyrase and topoisomerase enzyme-protecting proteins (*gyrA*, *parC*, *parE*). The fluoroquinolone resistance as indicated by ofloxacin studies show a significantly favorable situation in the Hungarian population compared to in other nations [7, 10, 13–15].

Table 3 Antibiotic sensitivity of 41 *Mycoplasma hominis* strains cultured from genital samples between May 2008 and December 2011 in Hungary

Antibiotic	No. of strains tested	Sensitive		Moderately sensitive		Resistant	
		No.	%	No.	%	No.	%
Clindamycin	41	39	95.12	0	0	2	4.88
Ofloxacin	41	39	95.12	0	0	2	4.88
Tetracycline	41	34	82.92	2	4.8	5	12.19
Doxycycline	41	40	97.56	0	0	1	2.43

Doxycycline, the first drug of choice for *U. urealyticum* and *M. hominis* infections, showed the least resistance. In case of pregnancy, infancy, and doxycycline allergy or in any other cases where it is contraindicated, the medication to be chosen is azithromycin for *U. urealyticum* and clindamycin for *M. hominis* infections [16]. If a simultaneous infection of *C. trachomatis* and *U. urealyticum* is found, azithromycin should be chosen, because both bacterial strains are susceptible to it [17]. For biofilm-producing *U. urealyticum* strains, clarithromycin is the best medication, which penetrates the biofilm, inhibiting its formation.

As in many other countries, there are different opinions as to whether genital mycoplasmal infections should be treated or not. In our study, approximately 60 % of the patients had clinically relevant symptoms due to mycoplasmas, and they received adequate antibiotic treatment. Our study confirms that the continuously changing antibiotic resistance of ureaplasmas and mycoplasmas should be followed at least in a few centers in every country, so as to determine the best local therapy options for STI patients. Moreover, because of the role of genital mycoplasmas in severe neonatal infections and adverse outcomes during virtually every state of pregnancy, it would be recommended to prevent bacterial transmission from the mother to the fetus by the early identification and eradication of these microorganisms with antibiotics administered before conception, even in the case of asymptomatic colonization. Furthermore, it is advisable to perform tests regarding biofilm formation and determine minimal biofilm-inhibiting concentrations of antibiotics for all *U. urealyticum* strains [18].

In Hungary, our study indicates that, in genital *U. urealyticum* and *M. hominis* infections, doxycycline is highly recommended. If doxycycline is contraindicated, azithromycin is the drug of choice.

Acknowledgment The skillful technical assistance of Ildikó Bencze Győriné and Elvira Vörös is highly appreciated.

Conflict of interest There are no competing interests and no funding.

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IV.

European Journal of Microbiology and Immunology 2 (2012) 2, pp. 157–160
DOI: 10.1556/EuJMI.2.2012.2.9

THE IMPORTANCE OF IgM POSITIVITY IN LABORATORY DIAGNOSIS OF GESTATIONAL AND CONGENITAL SYPHILIS

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Received: March 19, 2012; Accepted: April 3, 2012

From January 1, 2009 through December 31, 2011, from 33,753 blood samples for syphilis screening, *Treponema pallidum* infections were confirmed in 241 pregnant women at the Department of Dermatology, Venerology, and Dermatooncology of Semmelweis University Budapest. In this period, four children born to inadequately or untreated women were confirmed to have connatal syphilis. The height of rapid plasma reagin (RPR) titer was measured to determine the stage of the infection and to examine the success of the antilues therapy. The diagnosis of maternal syphilis infection was confirmed with enzyme linked immunosorbent assay (ELISA), *T. pallidum* particle agglutination (TPPA), and IgG and IgM immunoblots. Maternal IgM immunoblot results identify mothers at risk of delivering babies with connatal syphilis better than the height of maternal RPR titer. The standard serological tests are less useful in newborns because of IgG transfer across the placenta. IgM test which depends on the infant's response has more specificity in diagnosing connatal syphilis.

Keywords: maternal IgM, fetal IgM, immunoblot, RPR, connatal syphilis

Introduction

Although risk of fetal infection is much higher during early maternal syphilis (the first year of infection) than during later stages, *Treponema pallidum* can be transmitted from the bloodstream of the infected woman to her developing fetus at any time during pregnancy [1]. Current Hungarian guidelines suggest that all pregnant women should be tested for *T. pallidum* infection in the first trimester, but some laboratory uses only rapid plasma reagin (RPR), a nontreponemal test for screening. Nontreponemal tests become positive only 6 weeks after exposure [2], in secondary syphilis they can show a false-negative result because of prozone effect [3], and despite that they are reactive in the early latent stage, their reactivity decreases with increasing latency [4]. Treponemal tests ELISA or TPPA can be used for screening, in all stages with or without signs and symptoms of syphilis [2]. Rawstron et al. determined maternal IgM status to be more helpful in identifying babies at high risk of congenital infection than a maternal RPR titer ≥1:16 [5]. Our study was undertaken to evaluate *T. pallidum* IgM status in 241 mothers with syphilis to determine if positive tests indicate a risk of connatal syphilis better than a reactive rapid plasma reagin test.

Because standard serological tests for syphilis detect both immunoglobulin IgG and IgM, including transplacentally acquired maternal IgG, they cannot be used to provide a laboratory diagnosis of congenital syphilis when a single serum sample is tested. In this study, *T. pallidum* IgM immunoblots were evaluated for the identification of babies with congenital syphilis.

Materials and methods

We used rapid plasma reagin (RPR) (Omega Diagnostics, UK), *T. pallidum* particle agglutination (TPPA) test (Fujirebio Inc., Japan), and enzyme immunosorbent assay (Syphilis II-EIA) test (BioRad, France) to investigate 241 maternal serum samples and 242 serum samples of the babies born to this mothers. IgM immunoblots (MAST, UK) were prepared from all the 483 serum samples, but the IgM–IgG complex and the maternal IgG were evaluated with Mastsorb (MAST, UK) from the infants' serum. IgG immunoblots (MAST, UK) were prepared from the 241 maternal serum samples. One of the infants had symptoms like neurosyphilis; this diagnosis was confirmed with Venereal Disease Research Laboratory (VDRL) test (Omega Diagnostics, UK) and TPPA in the cerebrospinal fluid of this infant.

Results

Positive syphilis serology was noted in 241 pregnant women of the 33,753 delivering serum samples at the Department of Dermatology, Venerology, and Dermatooncology of Semmelweis University, Budapest, Hungary from January 1, 2009 through December 31, 2011 *(Table 1)*. In

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ISSN 2062-509X / $ 20.00 © 2012 Akadémiai Kiadó, Budapest

this three-year period, 230 of the mothers were served with an adequate prenatal care. A total of 217 cared mothers had already been adequately treated for syphilis before pregnancy; they were advised to repeat therapy, according to the Hungarian guidelines [6]. These women had RPR titer of 0–1:16; results of TPPA, EIA, and IgG immunoblot tests were positive, but IgM immunoblots results were negative. Children born to these mothers had RPR titers of 0–1:8, equal or less than those of their mothers. All TPPA and EIA reactions were positive in these children, but only IgM immunoblots were negative. These infants were uninfected, without any symptoms of connatal syphilis at birth. They received careful follow-up examinations and serological testing at 0, 3, 6, 9, and 12 months after birth.

RPR titer was negative in two mothers who acquired syphilis stage I during their pregnancy; the diagnose was confirmed with positive TPPA, EIA, and IgM immunoblot positive results and with negative IgG immunoblot results. They were immediately treated with intramuscular benzathine penicillin. None of the two children presented clinical or laboratory evidence of infection; no positive serological test, including IgM immunoblot, was found in these two infants. Nine pregnant women received the diagnosis of syphilis latent recens during their pregnancy; RPR titers in this group of mothers were 1:16 and 1:32 in five and four cases, respectively. Except for the IgM immunoblot, all the treponemal tests were positive in these mothers. After adequate treatment, the RPR titers decreased fourfold. Their infants were born with the same serological results as the mothers, but they were uninfected and showed no symptoms. Syphilis latens tarda was the diagnosis of two pregnant mothers; RPR titers were 0, results of TPPA, EIA, and IgG immunoblot tests were positive, but those of IgM immunoblot test were negative. After adequate treatment during pregnancy, the infants of these mothers had the same serological results as their mothers, but none of them was infected.

Finally, 11 women, having received inadequate prenatal care, classified as latent syphilis patients, were first found to have reactive test results for syphilis at delivery. Seven mothers had the diagnosis of syphilis latens tarda, with negative RPR and IgM immunoblot results, but with positive TPPA, EIA, and IgG immunoblot tests. At delivery, the RPR test results of their infants were negative, but TPPA and EIA were positive. None of these children had a positive IgM immunoblot result.

The other four mothers had the syphilis latens recens diagnosis at delivery, with an RPR titer at least 1:2 to a maximum of 1:256. TPPA, EIA, and IgG immunoblot tests were all positive. One mother with an RPR titer of 1:64 has already had IgM negative immunoblot result. Her baby had the same serological results and had no clinical symptoms of syphilis, after adequate treatment the follow-up examinations and serological testing at 0, 3, 6, 9, and 12 months of birth confirmed the absence of connatal syphilis infection.

The last three women were found to have positive IgM results together with the other reactive syphilis tests at delivery. The first of them, an intravenous drug user, has had an RPR titer only of 1:2, but one of her twin sons has died immediately after birth. The RPR titer of this 'A' infant was 1:8. RPR titer of 'B' infant was 1:2; his treponemal tests, including IgM immunoblot, were all positive. Clinical symptom of seizures suggested the existence of neurosyphilis in this child. Lumbar puncture was performed to obtain cerebrospinal fluid for VDRL and TPPA tests. VDRL was negative, but TPPA was positive of a 1:80 titer. The second mother–child pair had the same RPR titer of 1:256; all treponemal tests, including IgM immunoblot, were positive in both of them, but the infant had no symptoms of connatal syphilis. The last mother had an RPR titer of 1:64; TPPA, EIA, and IgG and IgM immunoblots were positive. The premature daughter had RPR titer of 1:64 and positive results of TPPA, EIA, and IgM immuno-

Table 1. Date and staging of maternal syphilis diagnosis

Before pregnancy	During pregnancy			At delivery	
Succesfully treated	Syphilis I	Syphilis latens recens	Syphilis latens tarda	Syphilis latens recens	Syphilis latens tarda
$n=217$	$n=2$	$n=9$	$n=2$	$n=4$	$n=7$

n = Number of the mothers with the identified diagnosis

Table 2. Serological results of mother–child pairs from cases when maternal syphilis latens recens diagnosis was established only at delivery

	Maternal RPR titer	Maternal IgM	RPR titer of newborn	IgM of newborn	Diagnosis of newborn
Mother 1	1:64	Negative	1:64	Negative	Uninfected
Mother 2	1:2	Positive	Twin A: 1:8	Positive	Connatal syphilis
			Twin B: 1:2	Positive	Connatal syphilis
Mother 3	1:256	Positive	1:256	Positive	Connatal syphilis
Mother 4	1:64	Positive	1:64	Positive	Connatal syphilis

blot. Prematurity was the only non-specific clinical manifestation of connatal syphilis in this case *(Table 2)*.

Discussion

Globally, nearly two million pregnant women are infected with syphilis each year. Approximately 50% of women with untreated syphilis have been transmitting the infection to their newborn child, resulting in profound adverse outcomes including an estimated 440,000 perinatal deaths each year [7]. In Hungary, there was no congenital syphilis from 1978 until 1994. Both in 1994 and 1996, three early cases of congenital syphilis were observed, all in foreigners and from pregnancies without prenatal care. Since 1994, however, almost every year a case has been observed in Hungary. In 2007 and 2008, we revealed one and two cases, respectively [8]. In a three-year period from 2009 to 2011, we confirmed four connatal syphilis cases in Hungary. In Switzerland, in contrast to international guidelines, screening for syphilis in pregnancy is not generally recommended, but within the Swiss population, infectious syphilis cases in women of childbearing age increased substantially from 2006 to 2009 [9]. In Hungary, the performance of screening examinations within prenatal care is compulsory in order to prevent the development of syphilis, but only in the first trimester of pregnancy.

RPR test used for screening has the advantage of being inexpensive, widely available, and necessary for determining the efficacy of treatment. Limitations of this nontreponemal test include the lack of sensitivity in primary and late syphilis and the possibility of a prozone reaction or false-positive results [4]. Prozone reactions occur in 1–2% of patients with secondary syphilis [10], when antibody is in excess and blocks the normal antibody–antigen reaction. Dilution of the serum sample exhibiting this prozone reaction is adequate to obtain a readily detectable reaction. Some Hungarian laboratory uses only RPR test from undiluted serum samples, so they may detect a false-negative result in prenatal screening.

Treponemal tests like EIA or TPPA are technically more difficult to perform and more expensive, but they remain reactive for years with or without treatment. Due to the recommendation of Centers for Disease Control and Prevention (CDC), Binnicker et al. suggest to use a reverse syphilis screening algorithm, in which sera are screened using an automated treponemal test (e.g. EIA) [11]. Samples that are reactive by EIA are then tested by RPR to assess disease and treatment status and provide a supplemental marker of infection recommended that sera testing reactive by EIA but nonreactive by RPR be analyzed by the TP-PA assay [12]. Several antigens that elicit high antibody titers during *T. pallidum* infection and are not cross-reactive with serum from patients with other common spirochetal diseases have been identified [13], TPPA and EIA tests have explored the use of these recombinant antigens. While false-positive results can occur also with treponemal tests [2], we used these two tests from different manufacturers, as screening and confirming methods.

From the 241 mothers with positive syphilis screening tests, 217 were successfully treated before pregnancy. The next 13 pregnant women diagnosed with syphilis during pregnancy opted for treatment. The rest of 11 mothers were diagnosed with syphilis only at delivery. All our four connatal syphilis cases were from mothers without undergoing prenatal care and syphilis screening. The success rate for mother-to-child transmission intervention (number of successful interventions/number of syphilis positive women who received intervention) was 100% in our cases. In the program supported by the Shenzen local government, the success rate was lower, 99.1%, because of mothers who refused treatment or had late diagnosis and treatment [14]. Due to the better compliance of the patients, the performance of compulsory screening examinations within prenatal care is a good prevention against the development of connatal syphilis in Hungary.

Rawstron et al. determined maternal IgM status to be better indicator for a risk of connatal syphilis than a maternal RPR titer $\geq$ 1:16. However, they described that neither a titer $\geq$ 1:16 nor TP IgM reactivity identified all mothers who delivered infected infants; they found babies with congenital syphilis whose mothers had negative TP IgM and titers $\leq$ 1:8 [5]. In our study, the mother of the twins with connatal syphilis had only an RPR titer of 1:2. None of the mothers of the infected children was IgM negative in our cases. The infant of the untreated mother, who had an RPR titer of 1:64, but was negative for IgM, was uninfected.

Infected infants can produce IgM *in utero* after 3 months [4]. Previous studies using either ELISA or *T. pallidum* IgM WB have similarly found that IgM antibodies cannot be detected in all babies with congenital syphilis [15, 16]. Serodiagnosis of congenital syphilis is difficult because of the transfer of the IgG antibodies from mother to fetus. Fetus produces IgM antibodies (rheumatoid factor), against maternal IgG [14]. IgG–RF complex reacts in IgM immunoblot, or maternal IgG compete with fetal IgM for the Ag-binding position, resulting in false-positive or false-negative tests, respectively. To eliminate maternal IgG and IgG–RF complexes from the sera of newborns, Mastsorb reagent was used before IgM immunoblot tests. In previous studies with IgM immunoblot test, more reactivity was observed to the 47-kDa antigen than to antigens of 45 kDa or lower [17–19]. All our infants IgM-reactive sera demonstrated also this reactivity. We found reactivity to antigens of 45, 17, and 15 kDa only in one case.

Our observations confirmed that antenatal syphilis screening with the parallel use of treponemal (EIA, TPPA) and nontreponemal (RPR) tests facilitates treatment during pregnancy. Successful treatment offsets vertical transmission. The use of IgM immunoblot examination allowed the identification and treatment of high-risk newborns.

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V.

Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Impact of antiseptics on *Chlamydia trachomatis* growth

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Significance and Impact of the Study: We measured the antichlamydial effects of various antiseptics. These antiseptics are being used for the treatment of bacterial vaginosis, but their effect on the bacterial vaginosis-related sexually transmitted infections, particularly the most frequent *Chlamydia trachomatis* (*C. trachomatis*) infections has not been investigated. We showed that povidone-iodine (Betadine) inhibited the chlamydial growth in concentrations that was not toxic to the epithelial cells. We concluded that due to its additional antichlamydial effect, povidone-iodine could be a preferable antiseptic in bacterial vaginosis treatment.

Keywords
antiseptic, Betadine, Chlamydia, chlorhexidine, growth, povidone-iodine, quantitative PCR.

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2016/0725: received 4 April 2016, revised 30 June 2016 and accepted 19 July 2016

doi:10.1111/lam.12625

Abstract

Bacterial vaginosis is a frequent dysbiosis, where the normal lactobacillus-dominated flora is replaced by an anaerob/aerob polymicrobial flora. Bacterial vaginosis increases the risk of acquiring sexually transmitted infections (STI) including the most frequent *Chlamydia trachomatis* infections. Intravaginal antiseptics are part of the bacterial vaginosis treatment, and ideally they should also inhibit the bacterial vaginosis-related STI. Therefore, we tested the antichlamydial activity of four antiseptics: iodine aqueous solution, povidone-iodine, chlorhexidine and borax. First, we measured the impact of antiseptics on the viability of the HeLa cervical epithelial cells, and calculated the maximum nontoxic concentrations. Next, we infected the cells with *C. trachomatis* preincubated for 1 h with the particular antiseptic. The chlamydial growth was measured by direct quantitative PCR (qPCR) of the infected cells. The minimal inhibitory concentrations (MIC) of chlorhexidine and povidone-iodine were 3·91 and 97 μg ml^{-1} respectively; however, the MIC of chlorhexidine was close to its maximum nontoxic concentration. The iodine aqueous solution and the borax showed no antichlamydial activity. Our *in vitro* studies showed that chlorhexidine and particularly povidone-iodine are potentially able to limit the bacterial vaginosis-related *C. trachomatis* infection.

Introduction

Chlamydia trachomatis is an obligate intracellular bacterium with a cell tropism to epithelial cells of the conjunctiva and the urogenital tract. The bacterium is particularly capable of establishing persistent infections and chronic inflammation. The local inflammation could lead to tarsus and oviduct fibrosis potentially leading to blindness and infertility. Sexually transmitted infections (STI) caused by the urogenital tract pathogens, *C. trachomatis* serovars D-K and L1-L3 are the most frequent STIs in the world (CDC Sexually Transmitted Disease Surveillance 2013; http://www.cdc.gov/std/stats13/std-trends-508.pdf). While antiseptics are not part of the antichlamydial chemotherapy, they have various intravaginal applications including the prevention of postoperative infections before caesarean section (Memon *et al.* 2011; Haas *et al.* 2013), trans-vaginal ultrasound-guided ovum pick-up (Bhandari *et al.* 2015), surgical treatment of HPV-generated cervical lesions (Gerli *et al.* 2012) and other invasive procedures (Gornall *et al.* 1999; Velasco *et al.* 2009), and prevention of early-onset neonatal group B streptococcal infection during labour (Ohlsson *et al.* 2014).

Besides these applications, antiseptics are being used to prevent/treat prechlamydial states such as bacterial vaginosis. Bacterial vaginosis is a state where the normal lactobacillus flora of the vagina is disappearing and is replaced by a polybacterial flora. Several studies showed that bacterial vaginosis is a significant risk factor of acquiring STIs including chlamydial STIs with a relative risk of 2·0–3·4 (Wiesenfeld *et al.* 2003; Aghaizu *et al.* 2014; Abbai *et al.* 2015). The connection between bacterial vaginosis and chlamydia infection is not clear, but one of the possibilities is the increased indole production of the bacterial vaginosis-related bacteria (Aiyar *et al.* 2014). Urogenital *C. trachomatis* serovars likely propagate in a tryptophan-deprived environment due to the interferon-gamma-induced indole 2,3-dioxygenase, but the bacterium can survive by producing tryptophan from the exogenous indole. The treatment of bacterial vaginosis is complex including the antibiotics metronidazole, clindamycin; the antiseptics benzydamine, chlorhexidine, hydrogen peroxide, povidone-iodine, borax (Reichman *et al.* 2009; Novakov Mikic and Budakov 2010; Verstraelen *et al.* 2012); acidification; prebiotics and recolonizing the cervicovaginal region with lactobacilli (Donders *et al.* 2014). Despite these treatments the long-term cure rate of bacterial vaginosis can be as low as 20–50% (Sobel *et al.* 2006; Mastromarino *et al.* 2013), therefore search for more effective therapies including effective antiseptic therapies is necessary. Importantly, an ideal antiseptic should treat the bacterial vaginosis and inhibit the underlying STIs, including the frequent chlamydial STI.

To test the antichlamydial activity of frequently used antiseptics, we infected HeLa cervical epithelial cells with *C. trachomatis* in the presence of iodine aqueous solution, povidone-iodine, chlorhexidine and borax. For chlamydial growth measurement we used the recently published direct qPCR-based approach (Eszik *et al.* 2016).

Results and discussion

Cell viability of HeLa cells incubated with antiseptics

First, we tested the long-term impact of the antiseptics on the viability of the HeLa human cervical epithelial cells. HeLa cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (MTT assay) after 48 h of incubation with the particular antiseptic dissolved in cell culture medium (Fig. 1a–d). Maximum cytotoxicity was observed at concentrations of =>1562 μg ml^{-1} for povidone-iodine, while the viability reached its maximum at concentration of =<390 μg ml^{-1}. We considered this value the maximum nontoxic concentration. Maximum cytotoxicity was observed at concentrations of =>8 μg ml^{-1} for chlorhexidine, its maximum nontoxic concentration was 2 μg ml^{-1}. Maximum cytotoxicity was observed at concentrations of 800 μg ml^{-1} for iodine aqueous solution, its maximum nontoxic concentration was 200 μg ml^{-1}. Maximum cytotoxicity was observed at concentrations of 800 μg ml^{-1} for borax, its maximum nontoxic concentration was 25 μg ml^{-1}, however, the viability reached 80% at 400 μg ml^{-1}.

Direct qPCR measurement of the impact of antiseptics on *Chlamydia trachomatis* growth

The direct qPCR method was used to determine the antichlamydial activity of antiseptics (Fig. 2a–d). HeLa cells were infected with *C. trachomatis* multiplicity of infection 8 (MOI 8) after preincubation (37°C, 1 h) with serial 1 : 2 dilutions of povidone-iodine, chlorhexidine, iodine aqueous solution and borax starting with the maximum nontoxic concentrations. The antiseptics' minimal inhibitory concentrations (MIC) were calculated as it was described before (Eszik *et al.* 2016): briefly, the chlamydial DNA concentrations (threshold cycle (C_t) values) measured in the three parallel wells of a given antiseptic concentration were compared with the C_t values measured in the three parallel wells of the highest antiseptic concentration (we considered it as the inoculum) using Student's *t*-test. The lowest antiseptic concentration, where the C_t values did not change significantly compared with the inoculum was considered the minimum inhibitory concentration (MIC) value. The MIC value of povidone-iodine was 97 μg ml^{-1}, the MIC value of chlorhexidine was approximately 4 μg ml^{-1}. The iodine aqueous solution and the borax did not show antichlamydial activity in the tested concentration range. The difference of antichlamydial activity of iodine and povidone-iodine was striking, considering that the microbiologically active compound was the iodine in both substances. Povidone-iodine is an iodophor compound where the free iodine is complexed with the amphipathic polyvinyl pyrrolidone polymer. There are several differences between iodine aqueous solution and povidone-iodine. Povidone-iodine is more stable, releases the iodine more slowly and importantly could bring the iodine close to membranes due to its amphipathic nature. The increased membrane solubility could be one of the reasons of the higher antichlamydial activity. *Chlamydia trachomatis* is a Gram-negative bacterium, with an outer membrane containing a variety of proteins possibly involved in attachment/entry such as MOMP, polymorphic outer membrane proteins B-H, and other proteins involved in the early manipulation of the host cells, such as the type III secretion system ring protein PulD/YscC (Birkelund *et al.* 2009; Liu *et al.* 2010). Interfering with the functions of these proteins

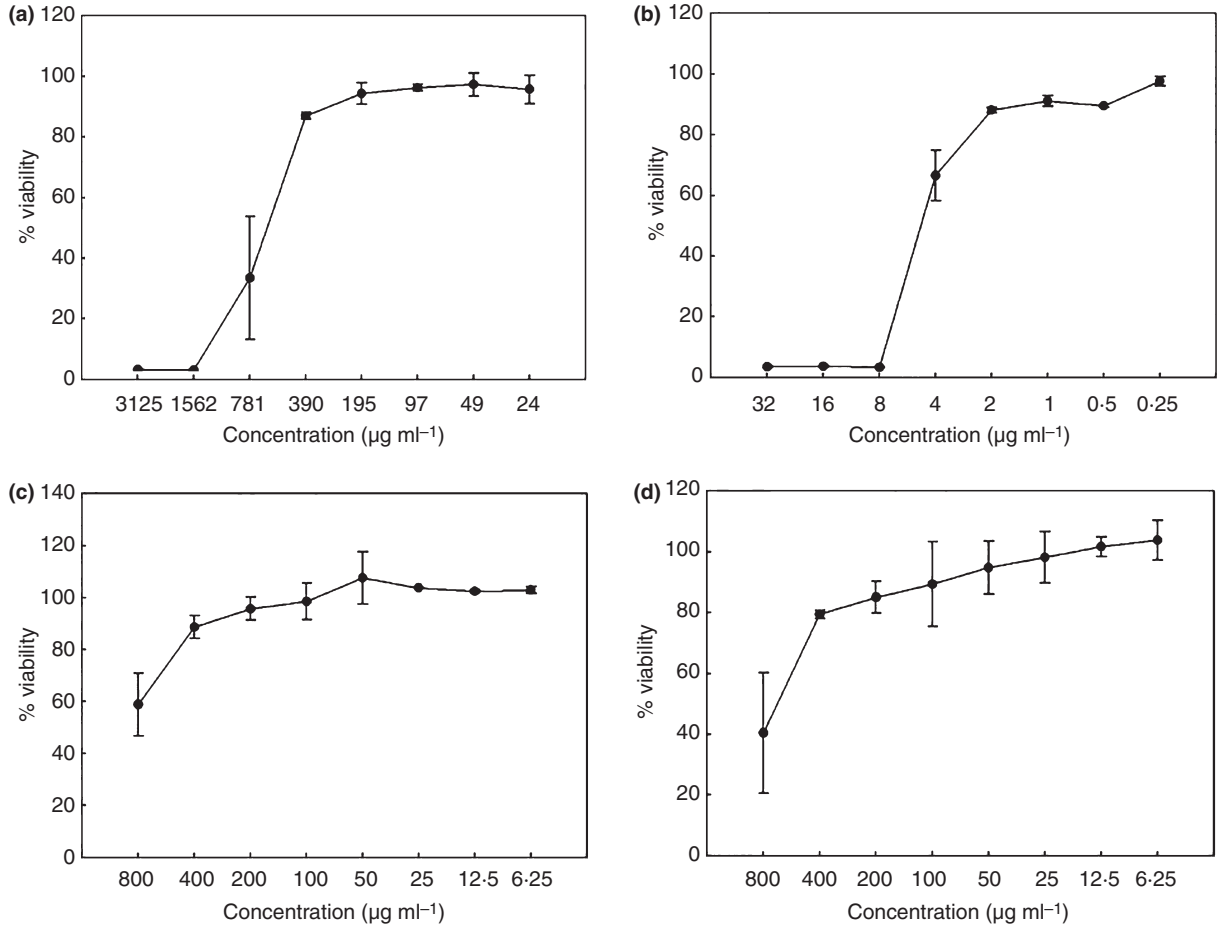

Figure 1 MTT cell viability assay of HeLa cells incubated for 48 h with (a) povidone-iodine, (b) chlorhexidine, (c) iodine aqueous solution and (d) borax dissolved in cell culture medium. Viabilities of the treated cells were compared to the untreated controls. MTT assay was performed as described in the Materials and methods. Three parallel measurements were performed for each antiseptic concentration. Data are means ± standard deviations.

directly or by destabilizing the outer membrane could lead to decreased infectivity. The measured MIC of the chlorhexidine was lower than that of the povidone-iodine; however, its MIC was close to the 4 μg ml^{-1} concentration that decreased the viability of the epithelial cells after 48 h incubation. These data indicate, that the chlorhexidine could be an effective antichlamydial agent, but should be applied as a short-term rinsing, rather than as a long-term vaginal gel. On the other hand, the povidone-iodine had a MIC of 97 μg ml^{-1}, while its maximum nontoxic concentration was 390 μg ml^{-1}, suggesting that this antiseptic can be applied long-term intravaginally. Also, it should be noted that the longer presence of povidone-iodine and its longer interaction with the chlamydial elementary bodies might result in an even lower MIC measured after the 1 h coincubation.

Estimation of the qPCR inhibitory activity of the antiseptics

Since the growth-related chlamydial DNA synthesis was measured by a qPCR method, we tested whether the applied antiseptics had a direct inhibitory impact on the qPCR. This effect could appear as a false-positive antichlamydial activity. We mixed cell lysates of HeLa cells infected with untreated *C. trachomatis* with cell lysates from uninfected HeLa cells treated with the maximum concentration of antiseptic applied for the Chlamydia inhibition. If there was no PCR inhibition then the C_t level of the 1 : 1 mixture (basically a twofold dilution of the chlamydial DNA) of the infected and uninfected but antiseptic containing cell lysates would have been approx. 1 cycle higher (50% less chlamydial DNA concentration) than the *C. trachomatis* infected cells lysate's alone. The

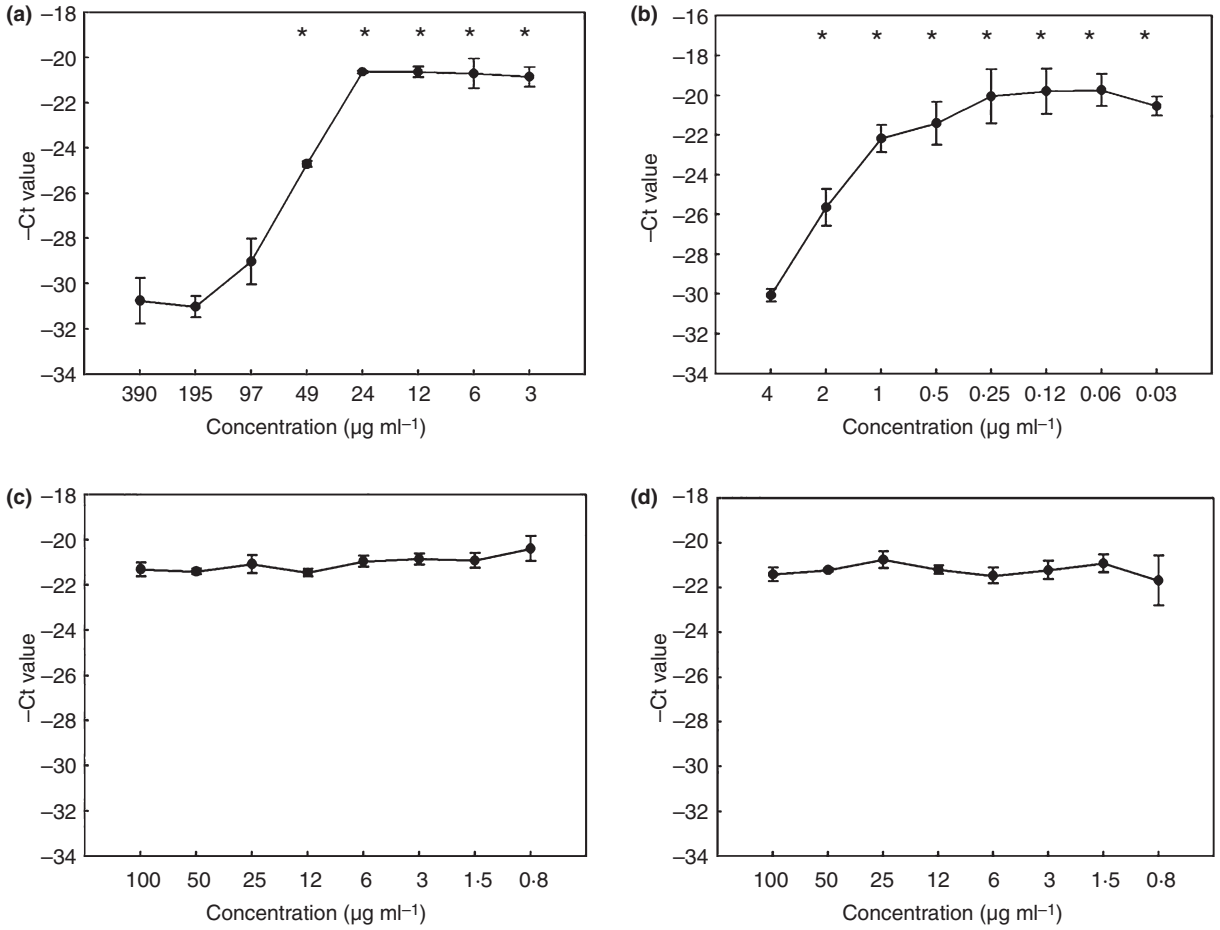

Figure 2 Measurement of antichlamydial activity of the antiseptics by direct qPCR. HeLa cells were infected with *Chlamydia trachomatis* (MOI 8) preincubated with various concentrations of (a) povidone-iodine, (b) chlorhexidine, (c) iodine aqueous solution and (d) borax for 1 h 37°C. Each infection at a particular antiseptic concentration was performed in three parallel wells. At 48 h post infection, the cells were lysed and the chlamydial DNA concentration was measured by direct qPCR. Data are the average $-C_t$ values ± standard deviations. *: the C_t values are significantly different ($P < 0{\cdot}05$) from the values measured in the presence of the highest concentration of antiseptics using Student's *t*-test.

C_t levels of the povidone-iodine, chlorhexidine, iodine aqueous solution and borax mixtures were 1·33, 0·79, 0·85 and 1·28 cycles higher than the untreated *C. trachomatis*-infected cell lysate's (Fig. 3). These results indicate that the antiseptics did not inhibit markedly the qPCR and the observed Chlamydia growth inhibition of povidone-iodine and chlorhexidine could not be due to the inhibition of the qPCR itself.

ChlamyCount immunofluorescent measurement of the impact of antiseptics on *Chlamydia trachomatis* growth

To validate the qPCR results with an independent chamber slide infection system method (Bogdanov *et al.* 2014), we performed *C. trachomatis* infections (MOI 8) in the presence of the antiseptics with the highest concentrations used for qPCR. Infected and control cells were fixed 48 h post infection, and the chlamydial inclusions were labelled with an Alexa-647-labelled anti-chlamydia LPS antibody. The slide was scanned with a DNA-chip scanner, and chlamydial inclusions were enumerated by the ChlamyCount software (Fig. 4). ChlamyCount inclusion number data showed that the povidone-iodine and chlorhexidine treatment decreased the chlamydial inclusion number approximately 94 and 94%, respectively, while the iodine aqueous solution and borax decreased 13 and 43%, respectively the number of chlamydial inclusions. These data validated the qPCR measurements in the case of povidone-iodine and chlorhexidine, although the extent of detected growth reduction was lower than that determined by qPCR. The reason could be that the dynamic range of our immunofluorescent ChlamyCount method

Figure 3 Estimation of the qPCR inhibition by the antiseptics. Cell lysates of HeLa cells infected with untreated *Chlamydiatrachomatis* (MOI 8) mixed with cell lysates from uninfected HeLa cells treated with SPG solution containing 390 μg ml^{-1} povidone-iodine, 4 μg ml^{-1} chlorhexidine, 100 μg ml^{-1} borax and 100 μg ml^{-1} iodine aqueous solution respectively. Untreated *C. trachomatis* (MOI 8)-infected cells are also processed (n = 3). Data are the average C_t values ± standard deviations.

was about 2 $\log_{10}$ (Bogdanov *et al.* 2014), while the qPCR method's dynamic range was approx. 5 $\log_{10}$ (Eszik *et al.* 2016). Different from the qPCR results, the ChlamyCount method also showed a limited chlamydial growth inhibition in the case of iodine aqueous solution and borax. The fact that the chlamydial DNA synthesis remained constant after the application of these latter two compounds, although the inclusion numbers slightly decreased, might indicate that a small portion of the iodine aqueous solution and borax-treated chlamydial EBs become persistent, maintaining the chlamydial DNA synthesis (Gérard *et al.* 2001; Belland *et al.* 2003), but formed smaller/less intense inclusions that was not detected by the ChlamyCount method.

In conclusion, our results showed that povidone-iodine had the widest antichlamydial therapeutic index and could maintain an antichlamydial effect when used intravaginally. Intravaginal povidone-iodine has already been used as a preoperative antiseptic to reduce postsurgery endometritis after caesarean sections (Asghania *et al.* 2011) and histerectomy (Sowapat *et al.* 2006) and as a treatment of bacterial vaginosis. Povidone-iodine also can be attractive in bacterial vaginosis treatment because it has no significant antimicrobial activity against lactobacilli (Sakakura *et al.* 1993; Wewalka *et al.* 2002). Since *C. trachomatis* infection could be linked to bacterial vaginosis, povidone-iodine may treat/limit these two clinical entities at the same time. Also it is worth to note, that povidone-iodine and chlorhexidine has been used intravaginally at a significantly higher concentrations than we used in our *in vitro* tests (van der Meijden *et al.* 1987; Onderdonk *et al.* 1992; Yu and Tak-Yin 1993). Since the maximum *in vivo* tolerable/nontoxic concentrations of these compounds are higher than in our *in vitro* toxicity assay, this could result in an even higher antichlamydial effect *in vivo*.

Materials and methods

Chlamydia strain propagation, HeLa cell culture

Chlamydia trachomatis serovar D strain (UW-3/CX, ATCC) was used, the strain was propagated and partially purified as described previously (Sabet *et al.* 1984). HeLa 229 cells (ATCC) were transferred into 96-well plates (Sarstedt, Nümbrecht, Germany) at a density of 6×10^4 cells per well in 100 μl of minimal essential medium (MEM) with Earle salts supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol l^{-1} L-glutamine, 1× MEM vitamins, 1× nonessential amino acids, 0·005% Na-pyruvate, 25 μg ml^{-1} gentamycin, 1 μg ml^{-1} fungisone. HeLa cells were incubated overnight at 37°C, 5% CO_2 to obtain a 90% confluent cell layer.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was performed to characterize the maximum nontoxic concentration of the antiseptics. HeLa cells were transferred into the wells of the 96-well plate (Sarstedt) at a density of 6×10^4 cells per well in 100 μl of culture medium (see above). The slides were incubated for 1 h at room temperature (RT) and then overnight at 37°C, 5% CO_2 to obtain a 90% confluent cell layer. Next day the medium was supplemented with the serial twofold dilutions of the antiseptics in three parallel wells for each concentration. After 48 h of incubation, 10 μl of the MTT (SIGMA, St. Louis, MO) labelling reagent (final concentration 0·5 mg ml^{-1}) was added to each well. The plate was incubated for 4 h at 37°C, 5% CO_2. After the incubation, 100 μl of the solubilization solution (10% SDS in 1 N HCl) was added into each well. The plate was allowed to stand overnight in the incubator at 37°C, 5% CO_2. Next day the optical density of the wells were measured by a microtitre plate reader (Labsystems Multiskan Ex 355; Thermo Fisher Scientific, Waltham, MA). The absorbance of the formazan product was measured at 540 nm. The average viability (OD 540) of three wells with untreated HeLa cells was considered the 100% viability. Viabilities of the treated cells were compared to the untreated controls as follows: Cell Viability (%) = (OD 540 of treated cells/OD 540 of untreated cells) × 100.

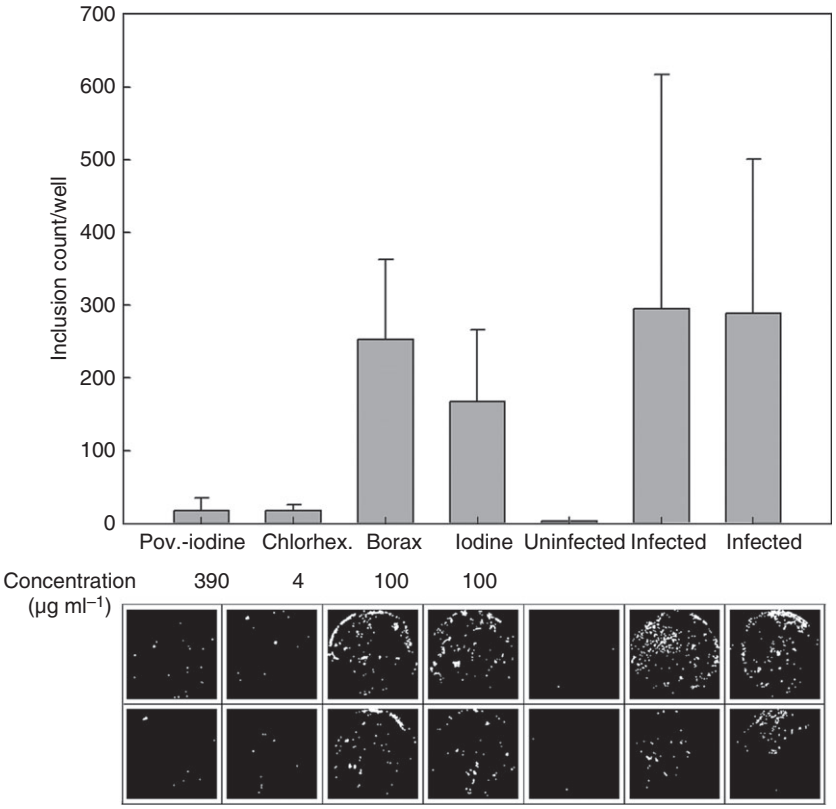

Figure 4 Measurement of antichlamydial activity of the antiseptics by immunofluorescent detection of the chlamydial inclusions. HeLa cells were infected with *Chlamydia trachomatis* (MOI 8) in the presence of the tested antiseptics. Four untreated *C. trachomatis* infected wells and uninfected wells were also included as controls. Each infection with a particular antiseptic was performed using parallel wells. The chlamydial inclusions were enumerated by the ChlamyCount software. The ChlamyCount processed well images and the counted inclusion numbers are shown. Data are means ± standard deviations.

Antiseptics used for *Chlamydia trachomatis* growth inhibition, *Chlamydia trachomatis* infection and DNA extraction

Iodine aqueous solution, povidone-iodine (Betadine, Egis, Budapest, Hungary), chlorhexidine-digluconate (Chlorhexamed; GlaxoSmithKline, Brentford, UK) and borax were diluted in sucrose-phosphate-glutamic acid buffer (SPG). Concentration ranges of 100–0·78 μg ml^{-1} for iodine aqueous solution and borax, 390–3 μg ml^{-1} for povidone-iodine and 4–0·003 μg ml^{-1} for chlorhexidine with two-fold dilutions were tested. Before infection, *C. trachomatis* elementary bodies were incubated in the antiseptics for 1 h, 37°C, 5% CO_2. HeLa cells were washed twice with 200 μl per well of phosphate-buffered saline (PBS), pH 7·4 and were infected at MOI 8 in 100 μl 0·5% (w/v) glucose medium for 60 min, RT. The cells were washed twice with PBS and culture medium containing 1 μg ml^{-1} cycloheximide was added. The plates were incubated at 37°C, 5% CO_2 for 48 h. The cells were washed with PBS twice, resuspended in 100 μl Milli-Q (MQ) water (Millipore, Billerica, MA), and subjected to two freeze–thaw cycles and mixing as it was described before (Eszik *et al.* 2016). The mixed lysates were used as a template in the qPCR. All reagents were purchased from SIGMA, unless otherwise indicated.

Direct quantitative PCR (qPCR)

Direct qPCR was performed as described before (Eszik *et al.* 2016) in a Bio-Rad CFX96 real-time system, using the SsoFast™ EvaGreen® qPCR Supermix (Bio-Rad, Hercules, CA) master mix and the *C. trachomatis* primer pairs *pykF*: 5′-GTTGCCAACGCCATTTACGATGGA-3′, 5′-TGCATGTACAGGATGGGCTCCTAA-3′. The PCR mixture consisted of 5 μl SsoFast™ EvaGreen® supermix, 1–1 μl forward and reverse primers (10 pmol each), 1 μl template and 2 μl MQ water to 10 μl final volume. After the 10 min at 95°C polymerase activation step, 40 PCR cycles of 20 s at 95°C and 1 min at 64°C were performed, with measurement of the fluorescence intensity at the end of the annealing–extension step. The qPCR ended with a melting curve analysis. Student's *t*-test has been used to

compare the statistical differences in C_t values between the two experimental conditions as it was described before (Eszik *et al.* 2016).

Immunofluorescent monitoring of *Chlamydia trachomatis* growth

Chlamydia trachomatis growth was evaluated by immunofluorescent staining as it was described before (Bogdanov *et al.* 2014). Briefly, semiconfluent layers of HeLa cells (6×10^4 cells per well) in chamber slides (Lab-Tek chamber slide system; Thermo Fisher Scientific) were infected with untreated *C. trachomatis* MOI 8 (preincubated in SPG buffer at 37°C for 1 h) or *C. trachomatis* preincubated with iodine aqueous solution (100 μg ml^{-1}), povidone-iodine (390 μg ml^{-1}), chlorhexidine (4 μg ml^{-1}) and borax (100 μg ml^{-1}) in SPG buffer at 37°C for 1 h. Before infection the wells were washed with 200 μl per well of PBS. After removal of the PBS solution, the treated and untreated chlamydiae were added and the cells were incubated at 37°C under 5% CO_2 for 1 h. After infection, the inocula were replaced with a culture medium containing 1 μg ml^{-1} cycloheximide and were incubated at 37°C, 5% CO_2 for 48 h. After removing the culture medium from the slides, the cells were washed twice with PBS (200 μl per well). After detaching the chamber structure from the slides, the cells were fixed with precooled 100% acetone at −20°C for 10 min. Anti-chlamydia LPS antibody (AbD Serotec, Oxford, United Kingdom) labelled with Alexa-647, was used at 1 : 200 dilution for detection of chlamydial inclusions. Following an incubation of 1 h at 37°C, the cells were washed three times with PBS for 7 min and at last with distilled water. Fluorescence signals were analysed with an Axon GenePix Personal 4100A DNA chip scanner and GenePix Pro (ver. 6.1) software (Molecular Devices, Sunnyvale, CA) using the Cy5 channel and a 5-μm resolution. Inclusion counts were determined by the ChlamyCount software as it was described before (Bogdanov *et al.* 2014).

Conflict of Interest

The authors declare no potential conflicts of interest.

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