High prevalence of fluoroquinolones resistance in *Ureaplasma* and *Mycoplasma* strains isolated from infertile women under initial evaluation in north-east Romania

MAREŞ MIHAI[1,2], NĂSTASĂ VALENTIN[1], GABRIELA ANTON[3], BLEOTU CORALIA[3], MIRON NORA[2], SOCOLOV DEMETRA[2]
[1] *University "Ion Ionescu de la Brad", 3 Aleea Sadoveanu, 700490-Iassy, Romania*
[2] *University of Medicine and Pharmacy "Gr. T. Popa", Iassy, Romania*
[3] *Stefan S. Nicolau Institute of Virology, 285 Mihai Bravu Ave., 030304-Bucharest, Romania*
Corresponding author: Mihai Mareş (mycomedica@gmail.com)

Abstract

This paper emphasizes the increasing prevalence of resistant strains of Mycoplasma hominis and Ureaplasma urealyticum to the fluoroquinolones in north-east Romania. The study was carried out on 534 infertile women under initial evaluation.

CIP resistance was higher in the case of MH (75%) compared with UU (53.76%). We observed the same situation for OFL but slightly decreasing, 30% for MH and 16.13% for UU. Double resistance CIP/OFL was observed only in 30% MH and 16.13% UU strain, but we must mention that 45% of MH and 37.63 % of UU show intermediate resistance to OFL, associated with high resistance to CIP (table 1). We mention that few strains were susceptible to CIP (5% for MH, respectively 11.83% for UU), or OFL (15% for MH, respectively 27.96% for UU).

The results of this study indicate that in our settings the MH is more likely resistant than UU to ciprofloxacin, the resistance rate being however very high for both species comparing with similar studies, probably due to the overuse of this antibiotic for the treatment of various infections. In our country, the extended use of these drugs must be reevaluated and limited due to their capability to easily induce resistance and to become ineffective to cure MH and UU infections.

Introduction

Infertility is clinically defined as the inability of a person or couple to conceive after one year of unprotected intercourse, or the inability of the female to carry a pregnancy to term. Nowadays it is estimated that this condition affects approximately 72.4 million women worldwide [1]. The childlessness has usually a lot of negative psychosocial consequences that may vary from fear, guilt, self-blame, marital stress, helplessness and depression to loss of social status, divorce or even violence-induced suicide [2].

The evaluation of an infertile couple comprise a broad panel of investigations – a detailed history, a complex physical examination and an exhaustive laboratory analyses, in order to identify the main cause of the patient's impossibility to contribute to a child conception [3]. Genital infections are common cause of infertility, often undiagnosed because of their non-specificity of clinical manifestations. Among the micro organisms involved in women infertility, the literature cites the following bacterial species: *Chlamydia trachomatis* (CT), *Ureaplasma urealyticum* (UU), *Mycoplasma hominis* (MH) and *Neisseria gonorrhoeae* (NG) [3]. A simple screening for these germs may improve early detection of the infection and permit to start an aetiological therapy in order to restore the reproductive status.

Both UU and MH are sexually transmitted bacterial pathogens undoubtedly implied in impairment of reproductive status, although numerous and often contradictory papers

concerning their real pathogenic potential have been published last years. Thus, UU is the most common bacteria of the human urogenital tract (with a detection rate of 67% in sexually active women and 50% in men respectively) that can cause lower pregnancy rates after *in vitro* fertilization [4, 5], higher abortion rate of spontaneous pregnancies [6], increasing of the risk of premature contractions and preterm delivery [7], puerperal endometritis [8], orchitis, epididymitis, spermatocystitis, prostatitis, urethritis [9], increased apoptosis in human spermatogens [10], impairment of semen parameters [11], less stable chromatin and DNA denaturation in spermatozoa [10]. MH is involved in the aetiology of salpingitis and pelvic inflammatory disease, but its occurrence in sexually active population is lower than UU, with an average of 10% [12, 13].

Fluoroquinolones, broad-spectrum antibiotics targeting bacterial type II topoisomerase (including gyrase and topoisomerase IV), have been used for treatment of urogenital infections caused by UU and MH and resistance has been already reported in clinical isolates [14]. Analysis of the gene sequence of topoisomerase IV and DNA gyrase suggested a role for the topoisomerase IV ParE subunit in fluoroquinolone-resistant UU [15].

This paper focuses on the estimation of the prevalence of fluoroquinolones resistant MH and UU strains isolated during a population-based study concerning women infertility in north-east Romania.

Material and methods

Patients. The study has consisted in a screening of 534 infertile women presented for initial evaluation in our outpatient clinic from May 2008 to September 2009. The median age of the patients enrolled in the study was 31 years (range 26-42).

Bacterial detection and antimicrobial susceptibility. In order to identify MH and UU and to evaluate the susceptibility of the strains to 9 antibiotics, including two fluoroquinolones – ciprofloxacin (CIP) and ofloxacin (OFL), the swab has been processed using Mycoplasma IST2 kits (bioMérieux, France) as indicated by the manufacturer. Briefly, the endocervical cotton swab included in the kit was inoculated in R1 transport medium, inhibiting most of the Gram-negative and Gram-positive bacteria. The combination of three antibiotics and one antifungal agent provides selectivity, ensuring that any contamining flora present in the specimen does not affect the test. The inoculated R1 medium was vortexed rapidly and 3 ml was added to the growth R2 medium, which contained 1 ml of lyophilized urea/arginine broth. After reconstitution and shaking, 55 µL was dispensed into each of the 22 test wells on the strip. Two drops of mineral oil were added to each well. The remainder of the R2 medium and the inoculated strip were then incubated at 37°C and observed for colour changes at 24 and 48 h. The condition is adapted to optimal growth of mycoplasma (pH, substrates and growth factors) and includes specific treatment substance (urea for UU and arginine for MH) and an indicator (phenol red) that allow in the case of positive cultures to display a color change in the stock, related to an increase in pH. A sample was considered positive if the estimated count of bacteria was greater than or equal to 10,000 colony forming units (CFU), which is recognized threshold for differentiating between colonization and infection. The susceptibility testing was performed with nine antibiotics effective against these described microorganisms, and with different concentrations, namely four macrolides: azithromycin (0.12 and 4 mg / L), erythromycin (1 and 4 mg / L), clarithromycin (1 and 4 mg / L) and josamycin (2 and 8 mg / L), two fluoroquinolones: ciprofloxacin (1 and 2 mg / L) and ofloxacin (1 and 4 mg / L), two tetracyclines: doxycycline (4 and 8 mg / L) and tetracycline (4 and 8 mg / L), and a streptogramin: pristinamycin (2 mg / L).

Confirmation of bacterial detection. The brush has been prepared for DNA isolation using the DNA-Sorb-A kit (Sacace Biotechnologies, Italy). All extracted DNA samples were processed in an Applied Biosystems 7300 Real Time PCR system (Applera, USA) using the *Mycoplasma hominis* Real-TM and *Ureaplasma urealyticum* Real-TM kits (Sacace Biotechnologies, Italy). The parameters of amplification were as follows: 95°C for 15 min, followed by 10 cycles of 95°C for 20 s, 65°C for 20 s and 72°C for 20 s, with a last stage of 35 cycles of 95°C for 25 s, 60°C for 50 s and 72°C for 15 s.

Results

In order to choose only the samples really containing MH and/or UU, the positive samples in Mycoplasma IST2 strips have been registered and confirmed using Real Time PCR assay. We chose this approach because of high sensitivity and specificity of the molecular method [16]. Thus, we have selected 40 positive samples for MH and 186 for UU, respectively. For these samples, the susceptibility profiles to CIP and OFL were analysed. The resistance percentage to CIP and OFL in MH and UU strains has a significant value. CIP resistance was higher in the case of MH (75%) compared with UU (53.76%). We observed the same situation for OFL but slightly decreasing, 30% for MH and 16.13% for UU. The graphical representation of data is shown in figure 1.
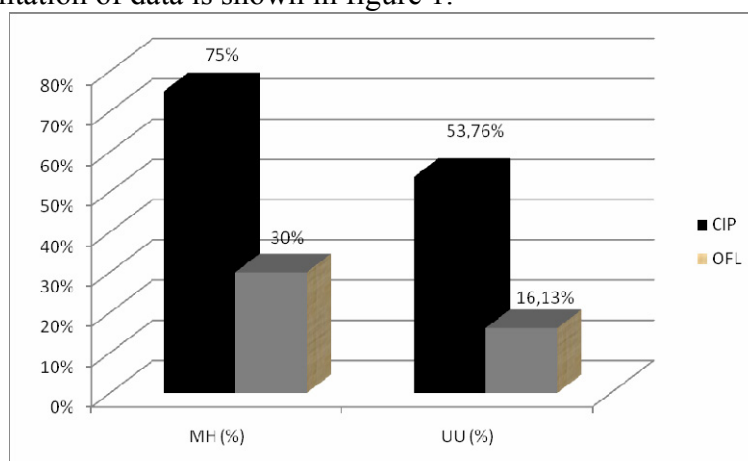

Figure 1. The prevalence of resistance to ciprofloxacin and ofloxacin in MH and UU isolated strains

Double resistance CIP/OFL was observed only in 30% MH and 16.13% UU strain, but we must mention that 45% of MH and 37.63 % of UU show intermediate resistance to OFL associated with high resistance to CIP (table 1). We must mention that few strains were susceptible to CIP (5% for MH, respectively 11.83% for UU), or OFL (15% for MH, respectively 27.96% for UU).

Table 1. Susceptibility profile for the confirmed MH and UU strains (Mycoplasma IST2 assessment).

Antimicrobial resistance status	MH (n)	UU (n)
Resistant to CIP / Resistant to OFL	12 (30%)	30 (16.13%)
Resistant to CIP / Intermediate to OFL	18 (45%)	70 (37.63%)
Intermediate to CIP / Intermediate to OFL	4 (10%)	34 (18.28%)
Intermediate to CIP / Susceptible to OFL	4 (10%)	30 (16.13%)
Susceptible to CIP / Susceptible to OFL	2 (5%)	22 (11.83%)
Total	40	186
MH: *Mycoplasma hominis*; UU: *Ureaplasma urealyticum*; CIP: ciprofloxacin; OFL: ofloxacin		

Discussion

The pattern of susceptibilities of mycoplasmas to antimicrobial agents is unique in that mycoplasmas do not have a cell wall that is the target for antibacterial agents like penicillin and the cephalosporins. Although human mycoplasmas and ureaplasmas are generally susceptible to tetracyclines and quinolones,resistance to quinolones such as ofloxacin, ciprofloxacin and sparfloxacin has been observed in clinical isolates [17]. The MH and UU isolated in Romania exhibited high resistance rates to ciprofloxacin and ofloxacin. Because the history of the patients reported a treatment with fluoroquinolones in the last 12 months (CIP in 57 cases, OFL in 19 cases, both in 14 cases and norfloxacin in two cases), it is likely that MH and UU isolates could become resistant to these antibiotics used for the treatment of other bacterial infections. Taking into consideration that the occurrence of post exposure cross-resistance to fluoroquinolones has been described in MH and UU strains, both *in vitro* and *in vivo*, after a target alteration located in the DNA gyrase and topoisomerase IV subunits, it seems that the same mechanism of resistance occurred in our strains too [18, 19]. Mutations may occur rapidly during fluoroquinolone therapy and this fact may be the most significant factor limiting the use of these antimicrobials [20, 21]. Moreover, in co-cultivation experiments of eukaryotic HeLa cells and MH it was shown that the subsequent addition of ciprofloxacin resulted in an increase of the micoplasma resistance to this antimicrobial agent and the genetic analysis revealed genomic rearrangements [22].

In the last decade, other studies reported increased rates of resistance to fluoroquinolones in MH and UU strains in China, Mexic and other countries, underlining the mutational mechanism of appearance [15, 23]. For Romania – where we can note the lack of other similar studies, the explanation of this high resistance percentage to fluoroquinolones occurred in MH and UU strains isolated from human patients, may be the frequent prescription of these drugs by the general practitioners for the treatment of urinary and respiratory tract infections, pneumonia, otitis or prostatitis, due to their reduced price and low percent of side reactions.

Conclusion

The results of this study indicate that in our settings the MH is more likely resistant than UU to ciprofloxacin, the resistance rate being however very high for both species comparing with similar studies, probably due to the overuse of this antibiotic for the treatment of various infections. In our country, the extended use of these drugs must be reevaluated and limited due to their capability to easily induce resistance and to become ineffective to cure MH and UU infections.

Acknowledgements

Transparency Declaration

References

1. J. BOIVIN, L. BUNTING, J.A. COLLINS, K.G. NYGREN, International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod,* 6, 1506-1512 (2007).
2. W. OMBELET, I. COOKE, S. DYER, G. SEROUR, P. DEVROEY, Infertility and the provision of infertility medical services in developing countries. *Hum Reprod Update,* 6, 605-621 (2008).
3. A.N. IMUDIA, L. DETTI, E.E. PUSCHECK, F.D. YELIAN, D.P. DIAMOND, The prevalence of Ureaplasma urealyticum, Mycoplasma hominis, Chlamydia trachomatis and Neisseria gonorrhoeae infections, and the rubella status of patients undergoing an initial infertility evaluation. *J Assist Reprod Genet,* 25, 43-46 (2008).
4. F. GUILLET-ROSSO, A. FARI, S. TAYLOR, R. FORMAN, J. BELAISCH-ALLART, J. TESTART, Systematic semen culture and its influence on IVF management. *Br J Obstet Gynaecol,* 94, 543-547 (1987).
5. J.M. MONTAGUT,S. LEPRETRE,J. DEGOY,M. ROUSSEAU,*Ureaplasma* in semen and IVF.*Hum Reprod,*6,727-729(1991).
6. G.G. DONDERS,B. VAN BULCK,J. CANDRON,L. LONDERS,A. VEREECKEN,B. SPITZ, Relationship of bacterial vaginosis and *Mycoplasma* to the risk of spontaneous abortion.*Am J Obstet Gynecol,*183,431-437 (2000).
7. S. GERBER, Y. VIAL, P. HOHLFELD, S.S. WITKIN, DETECTION of *Ureaplasma urealyticum* in second trimester amniotic fluid by polymerase chain reaction correlates with subsequent preterm labor and delivery. *J Infect Dis,* 187, 518-521 (2003).
8. C.M. WILLIAMS, D.M. OKADA, J.R. MARSHALL, A.W. CHOW, Clinical and microbiologic risk evaluation for post-cesarian section endometritis by multivariate discriminant analysis: role of intraoperative *Mycoplasma*, Aerobes and Anaerobes. *Am J Obstet Gynecol,* 156, 967-974 (1987).
9. T. VOLGMANN, R. OHLINGER, B. PANZIG, *Ureaplasma urealyticum*-harmless commensal or underestimated enemy of human reproduction? A review. *Arch Gynecol Obstet,* 273, 133-139 (2005).
10. M. REICHART, I. KAHANE, B. BARTOOV, *In vivo* and *in vitro* impairment of human and ram sperm nuclear chromatin integrity by sexually transmitted *Ureaplasma urealyticum* infection. *Biol Reprod,* 63, 1041-1048 (2000).
11. D.M. FOWLKES, J. MACLEOD, W.M.T. O'LEARY, Mycoplasmas and human infertility: correlation of infection with alterations in seminal parameters. *Fertil Steril,* 26, 1212-1218 (1975).
12. C.M. BEBEAR, Infections à mycoplasmes génitaux. In *Mycoplasmes et chlamydiae.* Ed. C Bébéar. Paris: Elsevier, pp 37-47 (2002).
13. K.B. WAITES, R.L. SCHELONKA, L. XIAO, P.L. GRIGSBY, M.J. NOVY, Congenital and opportunistic infections: *Ureaplasma* species and *Mycoplasma hominis. Seminars in Fetal & Neonatal Medicine,* 14, 190-199 (2009).
14. W. ZHANG, Y. WU, W. YIN, M. YU, STUDY of isolation of fluoroquinolone-resistant Ureaplasma urealyticum and identification of mutant sites. *Chinese Med. J.,* 115(10), 1573-1575 (2002).
15. X. XIE, J. ZHANG, Trends in the rates of resistance of Ureaplasma urealyticum to antibiotics and identification of the mutation site in the quinolone resistance-determining region in Chinese patients. *FEMS Microbiol Lett.,* 259(2), 181-186 (2006).
16. N. LUKI, P. LEBEL, M. BOUCHER, B. DORAY, J. TURGEON, R. BROUSSEAU, Comparison of polymerase chain reaction assay with culture for detection of genital mycoplasmas in perinatal infections. *Eur J Clin Microbiol Infect Dis,* 17, 255–263 (1998).
17. G. E. KENNY, F.D. CARTWRIGHT, SUSCEPTIBILITIES of *Mycoplasma hominis, M. pneumoniae*, and *Ureaplasma urealyticum* to GAR-936, Dalfopristin, dirithromycin, evernimicin, gatifloxacin, linezolid, moxifloxacin, quinupristin-dalfopristin, and telithromycin compared to their susceptibilities to reference macrolides, tetracyclines, mand quinolones. *Antimicrob Agents Chemother.,* 45(9), 2604–2608 (2001).
18. C.M. BEBEAR, H. RENAUDIN, A. CHARRON, M. CLERC, S. PEREYRE, C. BEBEAR, DNA gyrase and topoisomerase IV mutations in clinical isolates of *Ureaplasma* spp. and *Mycoplasma hominis* resistant to fluoroquinolones. *Antimicrob Agents Chemother,* 47, 3323-3325 (2003).
19. D. GRUSON, S. PEREYRE, H. RENAUDIN, A. CHARRON, C. BÉBÉAR, C.M. BÉBÉAR, *In vitro* development of resistance to six and four fluoroquinolones in *Mycoplasma pneumoniae* and *Mycoplasma hominis,* respectively. *Antimicrob Agents Chemother,* 49, 1190-1193 (2005).
20. C.M. OLIPHANT, G.M. GREEN, Quinolones: A comprehensive review. *American Family Physician,* 65, 455-464 (2002).
21. M.L. BEETON, V.J. CHALKER, N.C. MAXWELL, S. KOTECHA, O.B. SPILLER, Concurrent titration and determination of antibiotic resistance in *Ureaplasma* species with identification of novel point mutations in genes associated with resistance. *Antimicrob Agents Chemother,* 53, 2020-2027 (2009).
22. K.T. MOMYNALIEV, O.V. SMIRNOVA, V.G. LADYGINA, V.PH. ORLOVA, T.A. AKOPIAN V.M. GOVORUN, Development of Flouroquinolone (Ciprofloxacin) resistance in *Mycoplasma hominis* in the presence of HeLa cells. *Russian J. Genetics*, 38, 7 (2002).
23. R. FAGUNDO-SIERRA, A. SANCHEZ-SAINZ, J. PEREZ-JAUREGUI, Resistencia *in vitro* de aislamientos clinicos de *Mycoplasma hominis* y *Ureaplasma urealyticum* en Mexico. *Bioquimia,* 31, 124-131 (2006).