MOLECULAR-GENETIC CHARACTERISTICS OF M. HOMINIS CLINICAL ISOLATES EFFLUX SYSTEMS

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Abstract. The widespread occurrence of drug-resistant urogenital mycoplasmas and the high rate of their genetic variability make it possible to consider *Mycoplasma hominis* as a natural reservoir of antibiotic resistance determinants. Multiple drug resistance of many bacterial types is due to the active elimination of the antibiotic from the cell through various efflux systems. The genome structure of ten clinical isolates *Mycoplasma hominis* was studied using next-generation sequencing. The molecular genetic characterization family MATE proteins of Russian isolates *M. hominis* genes using bioinformatics analysis was obtained in the first. It has been established that proteins of the transport systems (ABC-transporters) and MATE play an important role in the formation of antibiotic resistance in urogenital mycoplasmas. The activity and functioning of transport systems are associated with the presence of mutations in the genes encoding regulatory proteins. Efflux systems should be considered as promising potential targets in the creation of new generation antibacterial drugs.

Keywords: *Mycoplasma hominis*, antibiotic resistance mechanisms, efflux system, ABC transporters, MATE.

List of Abbreviations

ABC transporters – ATP-binding cassette superfamily

BLAST – Basic Local Alignment Search Tool

CARD – Comprehensive Antibiotic Resistance Database

DNA – Deoxyribonucleic Acid

MATE – Multidrug and toxic compound extrusion family

MDR – Multiple drug resistance

MFS – Major Facilitator Superfamily

NCBI – National Center for Biotechnology Information

NGS – Next Generations Sequencing

PGAP – Prokaryotic Genome Annotation Pipeline

QRDR – Quinolone Resistance-Determining Region

RAST – Rapid Annotation Subsystem Technology

RND – Resistance Nodulation Division

SMR – Small Multidrug Resistance

UGT – Urogenital Tract

Introduction

Representative class Mollicutes *Mycoplasma hominis* is the smallest currently known prokaryote and refers to opportunistic microorganisms (Borchsenius *et al.*, 2016). At realizing its pathogenic properties, *Mycoplasma hominis* can cause inflammatory diseases of the urogenital tract (UGT), complications of pregnancy and childbirth, as well as infectious diseases of newborns (Belova & Nikonov, 2015; Moridi *et al.*, 2020). Treatment of urogenital infections associated with *M. hominis* is often difficult, due to the natural resistance of the bacterium to drugs that inhibit cell wall synthesis (Sidorenko & Tishkov, 2004). According to the literature, recently, there has been an increase in the number of *M. hominis* isolates resistant to fluoroquinolones, macrolides, tetracyclines, which are most often used in the treatment of inflammatory diseases (Chernova *et al.*, 2016). Long-term, asymptomatic persistence of *Mycoplasma hominis* isolates in the host organism, and uncontrolled use of antibiotics lead to selective pressure and the emergence of multi-resistant mycoplasma strains (Kolesnikova *et al.*, 2018).

The widespread occurrence of drug resistant urogenital mycoplasmas, the high rate of their genetic variability allows us to consider this microorganism as a natural source (reservoir) of antibiotic resistance determinants (Kolesnikova *et al.*, 2019). There are several mechanisms in the bacterial cell that provide antibiotic resistance, including the degradation of drugs, modification of the targets of antibiotic action, as well as the emergence of alternative pathways, and the elimination of antibiotics from the cell (Chernova *et al.*, 2016). Efflux systems, belonging to the families of ABC transporters (ATP Binding Cassette), RND (Resistance-Nodulation Division), MFS (Major Facilitator Superfamily), SMR (Small Multidrug Resistance), and MATE (Multidrug and Toxin Extrusion) play an important role in the emergence of multidrug resistance in many species of gram-positive and gram-negative bacteria (Fernandez &. Hancock, 2012; Sun *et al.*, 2014). Search and molecular characterization of the nature and characteristics of efflux systems of bacteria, including *Mycoplasma hominis*, is extremely difficult when using classical microbiological research methods. In scientific research, the advent and widespread use of modern molecular genetic methods, including high-throughput sequencing (NGS), have made it possible to deeply study the structural organization of bacterial genomes, including a detailed study of efflux systems.

Materials and Methods

The study subjects were ten M. hominis clinical isolates (M45, M57, MH1866, MH1817, MH529, MH1002, MH621, MH1019, MH1861, MH1991), found in epithelium scraped from the cervical canal of women suffering from inflammatory diseases of the urogenital tract. The women had provided a written informed consent to participate in the study. Detection, identification, determination of antibioticogram to seven antibacterial drugs (doxycycline, gentamycin, ofloxacin and ciprofloxacin, clindamycin, midecamycin and josamycin) mycoplasmas were carried out using commercial liquid differential diagnostic media produced by the Central Research Institute of Electrical Engineering of Rospotrebnadzor (registration number FSR 2008/03366). DNA study was isolated and purified sorption method using a commercial kit AmpliPrime DNA-sorb-V (2012/14019). The DNA concentration in the samples was determined using a Qubit fluorometer (Invitrogen, Austria). The DNA library for sequencing was prepared using the Nextera XT kit (Illumina). The quality of the DNA library was assessed on a QIAxel advanced system (Qiagen, Germany) using a QIAxcel DNA Fast Analysis Kit (15 - 3000 bp) for separating DNA fragments.

Whole genome sequencing was performed on a MiSeq sequencer (Illumina, USA) using the MiSeq reagent kit v2 (Illumina) for 500 cycles. The whole genome sequence of the M. hominis strain ATCC 23114 (GenBank FP236530.1) was chosen as a reference. Read archives and nucleotide sequences of whole genomes of 10 clinical isolates of *M. hominis* are available in the GenBank / EMBL / DDBJ database. The nucleotide sequences were aligned using the MiSeq sequencer firmware (Isis version 2.6.2.3).

Visualization and analysis of the obtained data were performed using the UGENE Unipro 1.31 software. Genome annotation was performed using a Rapid Annotation using Subsystem Technology (RAST) (Aziz *et al.*, 2008) and NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Sequence alignment was performed using the CLUSTALX 2.0 software (Thompson *et al.*, 1994). Sequence analysis of genes encoding ABC transporter proteins and MATE isolates *Mycoplasma hominis* carried out using BLAST algorithm and software package presented on the NCBI server. Comparison and Search antibiotic resistance was performed using reference DNA and protein sequences placed in a complex database (CARD) (Alcock *et al.*, 2020).

Homologous protein tertiary structure model MATEobtained using Phyre2 web portal for protein modeling server and UGENE Unipro 1.31 software. Phylogenetic trees were built with the neighbor-joining algorithm (Okonechnikov *et al.*, 2012) in the MEGA7.0 software 6.0 (Kumar *et al.*, 2016).

Results

The resistance profile of the studied strains *M. hominis* was determined: three strains (M45, M57, MH1866) were characterized by resistance to ciprofloxacin, three strains (MH1019, MH1861, MH1991) – to midecamycin, one strain MH1002 – to ofloxacin, strain MN529 was resistant to ciprofloxacin and midecamycin, isolate MH1817 was susceptible to all drugs included in the study. Molecular genetic characterization, the of the genome of all the strains under study was obtained using NGS sequencing. The whole-genome nucleotide sequences of *M. hominis* have been deposited in the international data base NCBI GenBank under the accession numbers

MRAY00000000 (*M. hominis* M45),
MRAX00000000 (*M. hominis* M57),
QNHJ00000000 (*M. hominis* MH621),
QOKO00000000 (*M. hominis* MH1866),
QOKP00000000 (*M. hominis* MH1991),
QNHH00000000 (*M. hominis* MH1019),
QOKQ00000000 (*M. hominis* MH1817),
QNHI00000000 (*M. hominis* MH1002),
QNHJ00000000 (*M. hominis* MH621),
QOKN00000000 (*M. hominis* MH1861),
QMJZ00000000 (*M. hominis* MH529).

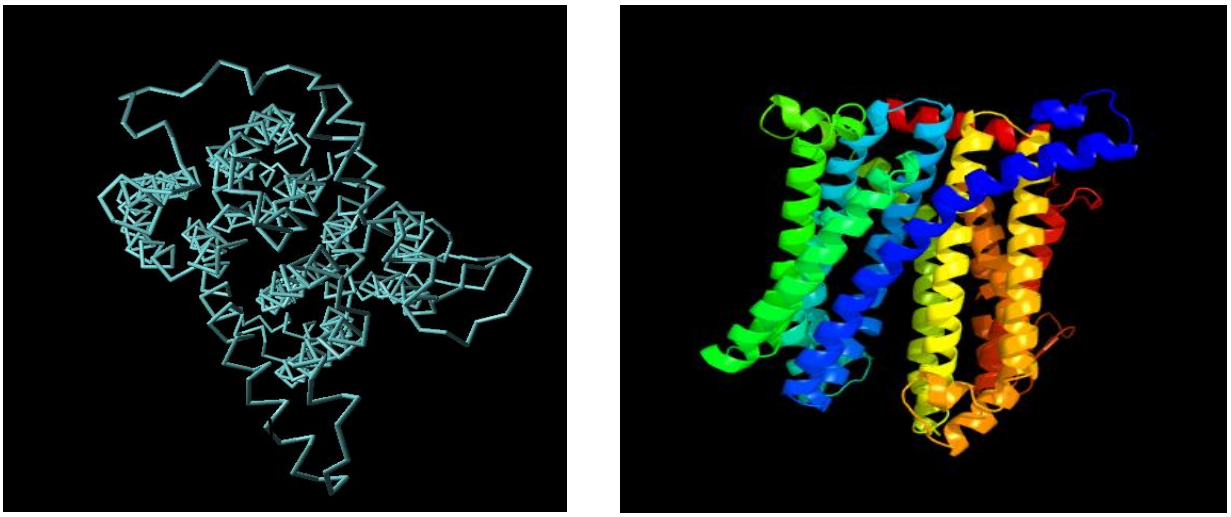
Bioinformatic analysis of the nucleotide sequence of the genome clinical isolates M. hominis have been allowed discover genes encoding efflux systems involved in membrane transport and active elimination of antibiotics from a bacterial cell, namely ABC transporters (ATP-binding cassette superfamily) and proteins of the MATE family (Multidrug and toxic compound extrusion family). An analysis the data acquired using the RAST server, showed that the ABC transporter system of the *M. hominis* isolates under study is represented by structural elements that transport oligopeptides through the bacterial cell membrane, determined by three copies of the oppB gene (encodes transport proteins – OopB permeases) and one copy of the oppC gene (encodes OopC permease).

Molecular genetic characteristics of the MATE protein family of Mycoplasma hominis clinical isolates. To in addition to ABC transporters, one copy of the gene encoding a protein homologous to proteins from the MATE family of multiple drug resistance was found in the genome of all *M. hominis* isolates under study (Multidrug and toxic compound extrusion family). The analysis of the profile of the gene encoding proteins of the MATE family revealed that in nine strains the length of its nucleotide sequence was 1809 nucleotides, and the amino acid sequence was 602 amino acids. The length of the gene encoding the MATE protein in the MH529 isolate was longer than in every analyzed *M. hominis* strain included and amounted to 1998 nucleotides and 665 amino acids. The tertiary structure (3D model) of the MATE protein of the clinical isolate MH529 is shown in Figure 1.

It was determined that the genes encoding proteins of the MATE family in the studied *M. hominis* isolates do not belong to the classic endogenous pumps of the active efflux system, such as AcrB in *E.coli (*Raherison *et al.*, 2005), MexB in *Pseudomonas aeruginosa*, QacA in *Staphylococcus aureus* (Kaatz *et al.*, 2005) and PmrA in *Streptococcus pneumonia (*Fernandez *et al.,2012)*. Mycoplasma genes encoding MATE are multicomponent and contain incomplete homologous sequences of two families: MATE_like superfamily (MATE_like 5, 8, 4, 14, 6, MATE_MeA_like, MATE_yoeA_like) and NorM superfamily (vmrA, NorM, matE) (Fig. 2).

Comparative analysis of gene sequence, family proteins encoding MATE ten *M. hominis* strains relative to the reference strain (GenBank number FP236530.1) has been revealed significant differences in the number of nucleotide substitutions (from 30 to 49) (Fig. 3). At the same time point mutations leading to a change in amino acid codons were much less common: from one in *M. hominis* M45 to four in every from analyzed strain *M. hominis* MH1817, *M. hominis* MH1019, *M. hominis* M57.

According to the CARD 2020 database, the genes encoding the MATE proteins of *M. hominis* are most similar to the genes responsible for multidrug resistance, namely: *cdeA Clostridioides difficille* (Dridi *et al.*, 2004), *mepA Staphylococcus aureus* (Kaatz *et al.*, 2006) and norM *Pseudomonas poae*. The mepA gene encodes a protein MepA, which is part of the MepRAB



A

Image of the tertiary structure of the MATE protein (UGENE Unipro 1.31)

B

Color Image N → C - Terminal End
Model dimensions (Å):X: 61.839 Y: 63.855 Z: 67.575

Fig. 1. Tertiary image of the protein structure of the MATE system in the clinical isolate *M. hominis* MH529. Images were obtained by the Phyre2 web portal for protein modeling server (A, B) and UGENE Unipro 1.31 software (A)

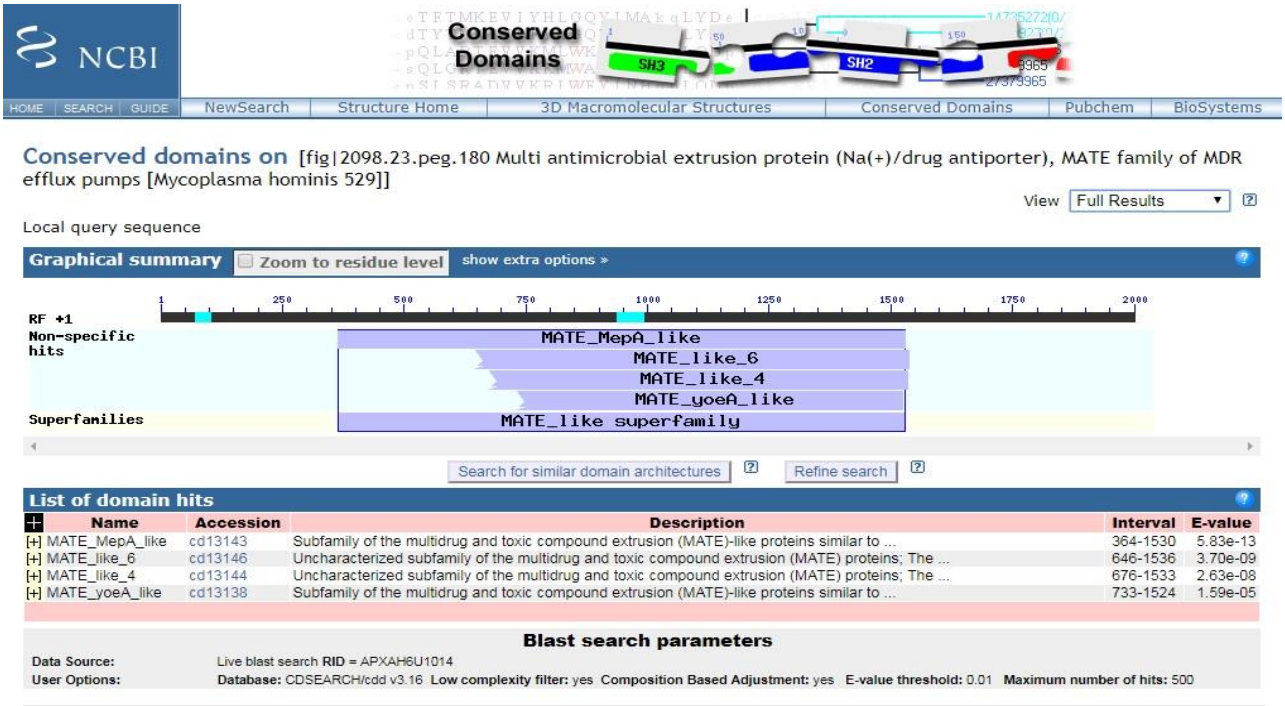


Fig. 2. Characterization of the structure of genes family proteins encoding MATE on the example of *M. hominis* clinical isolate MH529

Fig. 3. Comparative analysis of the sequence of genes, family proteins encoding MATE ten isolates *M. hominis* relative to the reference strain (GenBank number FP236530.1)

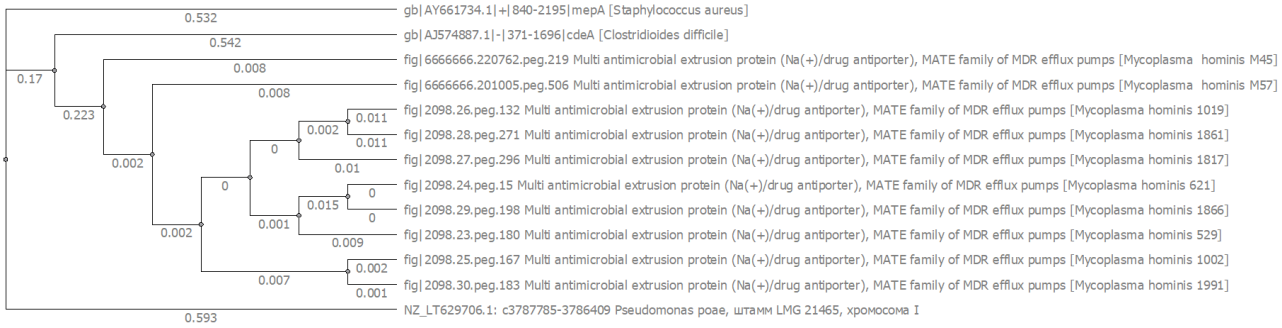


Fig. 4. The dendrogram of the genes' sequences annotated in GeneBank/NCBI encoding MATE family proteins of *M. hominis* strains (MH45, MH57, MH529, MH621, MH1002, MH1019, MH1817, MH1861, MH1866, MH1991) and *Pseudomonas poae*, *Clostridioides difficille*, *Staphylococcus aureus*. Phylogenetic tree was constructed by the UGENE Unipro software

cluster, it is involved in the clearance of drugs from the bacterial cell (Kaatz *et al.*, 2006).

In order to determine the evolutionary origin of the MATE system proteins of *M. hominis* isolates, a phylogenetic analysis of the relationship with the most homologous proteins re-sponsible for the multiple drug resistance of bacteria other species was carried out (Fig. 4).

The high level of homology of genes encoding MATE proteins in all studied *M. hominis* strains by phylogenetic analysis was indicated. The highest degree of gene homology is observed in pairs of clinical isolates that form single clusters with each other. *M. hominis* MH1002 – *M. hominis* MH1991, *M. hominis* MH1866 – *M. hominis* MH621 and *M. hominis* MH1019 – *M. hominis* MH1861.

However, the genes sequences in *M. hominis* M45 and *M. hominis* M57 stains are the most distant from the general studied genes group, they are isolated as a separate phylogenetic branch. The genes sequences encoding the MATE proteins in multiresistant *M. hominis* MH529 strain and sensitive to antibiotics *M. hominis* MH1817 strain do not form single clusters with other strains. They are located separately, but within the general group of the studied mycoplasmas.

Discussion

It is known that the efflux systems of ABC transporters are evolutionarily conservative, they are able to export a large number of biological compounds due to the energy of ATP hydrolysis (Raherison *et al.*, 2005). This property provides bacteria resistance not only to various classes of antibiotics, but also to disinfectants. Thus, proteins OopB and OopC, which are the part of the ABC transporter system in *M. hominis*, are involved not only in the transport of oligopeptides, but also in the removal of drugs from the bacterial cell. To date, the role of efflux in the development of resistance to fluoroquinolones by ABC transporters has been experimentally vindicated (Fernandez *et al.*, 2012; Sun *et al.*, 2014). The data of ciprofloxacin-resistance of *M. hominis* isolates M45 M57, MH1866, caused by mutational changes in genes encoding topoisomerases, were reported in a previously published study (Kolesnikova *et al.*, 2018).

We did not find mutations in the «hot» spots of the gyrA/gyrB and parC/parE genes in the *M. hominis* MH1002 isolate. Probably, the resistance to ofloxacin of the studied *M. hominis* strain MH1002 is associated with the active antibiotic elimination from the bacterial cell by work of ABC transporters.

It has been shown that the studied MATE efflux system genes of clinical mycoplasma isolates were identical to genes encoding proteins of the MATE family in representatives *Eubacterium* and *Clostridium (Eubacterium ventriosum* WP_005361628.1; *Clostridium tetani* E88 WP_017415026.1, *Clostridioides difficille* OGO77753.1) on 20–27%. The role of these proteins in the development of multidrug resistance is well established (Dridi *et al.*, 2004).

Proteins of the MATE family of *M. hominis* clinical isolates are similar to the integral membrane proteins – MepA of Staphylococcus aureus; they are involved in the export of metabolites across the cell membrane and also responsible for multidrug resistance (MDR) of various species bacteria. So, in the works of Chen et al. (2002) on the example of *Vibrio parahaemolyticus, Vibrio vulnificus* and *E. coli* had been proved, that the genes vmrA and NorM are Na^+-antitransporters of drugs in the MATE efflux system and associated with multiple drug resistance (Sun *et al.*, 2014).

As we have shown, the genes encoding proteins of the MATE family in nine Mycoplasma studied isolates were characterized by the same size: 1809 nucleotides. However, the length of the gene encoding the MATE protein in *M. hominis* isolate MH529, resistant to ciprofloxacin and midecamycin, was significantly longer and amounted to 1998 nucleotide bases. Changes in the QRDR region of the gyrA/gyrB and parC/parE genes in isolate MH529 was not detected, which probably indicates the active removal of antibiotics from the bacterial cell by efflux system MATE.

The gene encoding the protein of the MATE system in *M. hominis* isolate M45 is phylogenetically distant from similar genes of other studied mycoplasmas, it is closer to the gene *cdeA Clostridioides difficille*; its role in the development of fluoroquinolone resistance is well established (Dridi *et al.*, 2004). There is no information about the MATE efflux systems urogenital mycoplasmas characterization in the public domain. It is makes difficult to conduct in-depth comparative analysis. The wide representation mycoplasmas genome circulating in different countries of the world in the international GenBank/NCBI database will further allow assessing the structural organization of the main types efflux systems involved in the multi drug resistance emergence.

Conclusion

To sum up, detailed characteristic of genes encoding efflux system MATE Russian *M. hominis* isolates was obtained in first. It has been established that proteins of the efflux systems ABC - transporters and MATE play an essential role in the formation of antibiotic resistance in urogenital mycoplasmas. It has been shown that the activity and functioning of transport systems were associated with the presence of mutations in the genes encoding regulatory proteins. The efflux systems should be considered as promising potential targets in the creation of new generation antibacterial drugs.

Acknowledgements

The reported study was funded by State Task of the Federal Service for Surveillance on Customers Rights Protection and Human Wellbeing the Russian Federation № 121092300067-5.

References

ALCOCK B.P., RAPHENYA A.R., LAU T.TY., TSANG K.K., BOUCHARD M., EDALATMAND A., ... & MCARTHUR A.G. (2020): CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Journal Nucleic Acids Research* **8**(48), 517–525.

AZIZ R.K., BARTELS D., BEST A.A., DEJONGH M., DISZ T., EDWARDS R.A., … & ZAGNITKO O. (2008): The RAST server: rapid annotations using subsystems technology. *Journal BMC Genomics* **9**, 75.

BELOVA A.V. & NIKONOV A.P. (2015): Genital mycoplasmas (*U. parvum, U. urealyticum, M. hominis, M. genitalium*) in the structure of infectious complications in obstetrics, gynecology and perinatology. *Journal Almanac of Clinical Medicine* **39**, 140–150.

BORCHSENIUS S.N., CHERNOVA O.A., CHERNOV V.M. & VISHNYAKOV I.E. (2016): *Mycoplasmas in biology and medicine at the beginning of the 21st century.* St. Petersburg: Nauka, 333 pp.

CHEN J., MORITA Y., HUDA M.N., KURODA T., MIZUSHIMA T. & TSUCHIYA T. (2002): VmrA, a member of a novel class of Na (+)-coupled multidrug efflux pumps from Vibrio parahaemolyticus. *Journal Bacteriology* **184**(2), 572–576.

CHERNOVA O.A., MEDVEDEVA E.S., MOUZYKANTOV A.A., BARANOVA N.B. & CHERNOV V.M. (2016): Mycoplasmas and Their Antibiotic Resistance: The Problems and Prospects in Controlling Infections. *Journal ACTA NATURAE* **2**(29), 27–38.

DRIDI L., TANKOVIC J. & PETIT J-C. (2004): CdeA of Clostridium difficile, a new multidrug efflux transporter of the MATE family. *Journal Microbial Drug Resistance* **3**, 191–196.

FERNANDEZ L., ROBERT E. & HANCOCK W. (2012): Adaptive and Mutational Resistance: Role of Porins and Efflux Pumps in Drug Resistance. *Journal Clinical Microbiology Reviews* **4**, 661–681.

KAATZ G.W, MCALEESE F. & SEO S.M. (2005): Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel multidrug and toxin extrusion (MATE) transport protein. *Journal Antimicrobial Agents Chemotherapy* **5**, 1857–1864.

KAATZ G.W., DEMARCO C.E. & SEO S.M. (2006): MepR, a repressor of the *Staphylococcus aureus* MATE family multidrug efflux pump MepA, is a substrate-responsive regulatory protein. *Journal Antimicrobial Agents Chemotherapy* **4**, 1276–1281.

KOLESNIKOVA E.A., BRUSNIGINA N.F., MAKHOVA M.A. & ALEKSEEVA A.E. (2018): Whole genome sequencing of Mycoplasma hominis strains resistant to ciprofloxacin. *Journal Clinical Microbiology and Antimicrobial Chemotherapy* **1**, 68–72.

KOLESNIKOVA E.A., BRUSNIGINA N.F. & GRIGOR'EVA G.I. (2019): Antibiotic resistance mechanisms of urogenital mycoplasmas. *Journal Public health and habitat* **8**(317), 45–49.

KUMAR S., STECHER G. & TAMURA K. (2016): MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Journal Molecular Biology and Evolution* **7**, 1870–1874.

MORIDI K., HEMMATY M., AZIMIAN A., … & GHAZVINI K. (2020): Epidemiology of genital infections caused by *Mycoplasma hominis, M. genitalium* and *Ureaplasma urealyticum* in Iran; a systematic review and meta-analysis study (2000-2019). *Journal BMC Public Health* **20**(1), 1020–1025.

OKONECHNIKOV K., GOLOSOVA O. & FURSOV M. (2012): Unipro UGENE: a unified bioinformatics toolkit. *Journal Bioinformatics* **28**, 1166–1167.

RAHERISON S., GONZALEZ S., RENAUDIN H., … & BE´BE´AR C.M. (2005): Increased Expression of Two Multidrug Transporter-Like Genes Is Associated with Ethidium Bromide and Ciprofloxacin Resistance in *Mycoplasma hominis. Journal Antimicrobial Agents and Chemotherapy* **1**, 421–429.

SIDORENKO S.V. & TISHKOV V.I. (2004): Molecular basis of antibiotic resistance. *Journal Advances in biological chemistry* **2**, 263–306.

SUN J., DENG Z. & YAN A. (2014): Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. *Journal Biochemical and Biophysical Research Communications* **453**, 254–267.

THOMPSON J.D., HIGGINS D.G. & GIBSON T.J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Journal Nucleic Acids Research* **22**, 4673–4680.