UNIVERSITY OF BELGRADE

FACULTY OF BIOLOGY

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Genotyping, antibiotic resistance and biofilm formation of *Stenotrophomonas maltophilia* clinical isolates from Serbia

Doctoral Dissertation

Belgrade, 2017.

УНИВЕРЗИТЕТ У БЕОГРАДУ

БИОЛОШКИ ФАКУЛТЕТ

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Генотипизација, резистенција на антибиотике и фирмирање биофилма клиничких изолата *Stenotrophomonas maltophilia* из Србије

Докторска дисертација

Београд, 2017.

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Acknowledgment

Firstly, I would like to express my deeply gratitude to my supervisor professor Jelena Lozo for the continuous support of my PhD study and related research, for her patience, motivation and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis.

My sincere thanks also go to the Committee members Dr. Milan Kojić and professor Branko Jovčić, for their encouragements, help both in experimental as well as theoretical work and positive comments. In addition, I want to extend my gratitude and appreciation to all my colleagues in Laboratory for molecular microbiology in the Institute of molecular genetics and genetic engineering.

I wish to express my absolute thanks to my husband. He has been a true and great supporter and unconditionally loved me during my good and bad times. I could never have accomplished this dissertation without his love, support and understanding.

I am extremely grateful to my parents who raised me and taught me to study hard and to give priority in my life to the quest for knowledge.

ABSTRACT

Genotyping, antibiotic resistance and biofilm formation of *Stenotrophomonas maltophilia* clinical isolates from Serbia

Stenotrophomonas maltophilia is an environmental bacterium and an opportunistic pathogen usually associated with healthcare-associated infections, which has recently been recognized as a global multidrug resistant organism. The aim of this study was genotyping and physiological characterization of *S. maltophilia* collected during the routine health care at The Institute for Mother and Child Health Care of Serbia "Dr Vukan Čupić". It is the large, tertiary care pediatric hospital in Belgrade, Serbia, hosting the national reference cystic fibrosis (CF) center for pediatric and adult patients.

We characterized 88 *S. maltophilia* strains, 42 strains of cystic fibrosis (CF) and 46 strains of non-cystic fibrosis (non-CF) origin isolated from 2013 to 2015 in order to investigate their genetic relatedness and phenotypic traits. Genotyping was performed using sequencing of 16S rRNA gene, Pulse Field Gel Electrophoresis (PFGE) and Multi locus sequencing typing (MLST) analysis. Sensitivity to five relevant antimicrobial agents was determined, namely trimethoprim/sulfamethoxazole (TMP/SMX), chloramphenicol, ciprofloxacin, levofloxacin and tetracycline. Surface characteristics, motility, biofilm formation and adhesion to mucin were tested in all strains. In addition, influence of different factors (temperature, pH, agitation and CO_2) on biofilm formation, kinetics of selected biofilm producers and effect of TMP/SMX on formed biofilm were analyzed. Statistical approach was used to determine correlations between obtained results.

All analyzed clinical isolates belong to *S. maltophilia* species with identity ranging from 95% to 99% with *S. maltophilia* strains from the NCBI database what was confirmed by sequencing of PCR products of amplified 16S rRNA gene. PFGE analysis confirmed that most of the isolates were not genetically

related. Six new sequence types were determined and three already detected were found. Strains were uniformly sensitive to all tested antimicrobial agents. Swimming motility was observed in all strains, while none of the them exhibited swarming or twitching motility. Among strains able to adhere to mucin, no differences between CF and non-CF isolates were observed.

The majority of isolates (89.8%) were able to form biofilm with almost equal representation in both CF and non-CF strains. Analysis of biofilm formation in different growth conditions showed that changing of temperature and pH had the strongest effect on biofilm formation almost equally in group of cystic fibrosis (CF) and non-CF strains. TMP/SMX in concentration of 50 μg/ml reduced completely 24 h old biofilms while concentration of 25 μg/ml effects formed biofilms in a strain dependent manner. Among strains able to form strong biofilm CF isolates formed biofilm slower than non-CF isolates. Agitation conditions did not affect biofilm formation.

High genetic diversity among isolates implies the absence of clonal spread within the hospital. Positive correlation between motility, biofilm formation and adhesion to mucin was demonstrated. In addition, positive correlation between motility and strength of biofilm formed was demonstrated. Biofilm formation and motility were more pronounced among non-CF than CF isolates. However, a comparison of phenotypic characteristics of clinical isolates from CF and non-CF patients suggested that there was a difference between the two populations but not a CF phenotype.

Keywords: *Stenotrophomonas maltophilia*, healthcare-associated infections, trimethoprim–sulfamethoxazole, biofilm, PFGE, MLST

Scientific field: Molecular biology

Specific scientific field: Molecular microbiology

UDC: [579.253:[579.841:[574.58+616-78]]:[579.61:616-092](497.11)(043.3)

РЕЗИМЕ

Генотипизација, резистенција на антибиотике и формирање биофилма клиничких изолата *Stenotrophomonas maltophilia* из Србије

Stenotrophomonas maltophilia је срединска бактерија и опортунистички патоген који се најчешће повезује са нозокомијалним инфекцијама, а од скоро је сврстан у групу глобалних патогена који су вишеструко резистентни на антибиотике. Циљ ове докторске дисертације јесте гнеотипизација и фенотипска карактеризација колекције *S. maltophilia* која је сакупљена током свакодневне здравствене неге пацијената у Институт за здравствену заштиту мајке и детета „др Вукан Чупић". То је велика педијатријска болница у оквиру које се налази национални центар за лечење деце и одраслих оболелих од цистичне фиброзе.

У раду је окарактерисано 88 сојева *S. maltophilia* од којих 42 пореклом из пацијената оболелих од цистичне фиброзе (ЦФ) и 46 из пацијената који су имали друга оболења (нЦФ). Сојеви су скупљени у периоду од 2013. до 2015. године у циљу изучавања генетичке сродности и фенотипских особина. Секвенцирање PCR производа добијеног умножавањем гена за 16S рРНК, електофореза у пулсирајућем пољу (PFGE) и типизација секвенцирањем више локуса (MLST) су методе које су коришћене за генотипизацију. Анализирана је осетљивост на одабране релевантне антибиотике и то, триметоприм-сулфаметаксазол (ТМП/СМХ), хлорамфеникол, ципрофлоксацин, левофлоксацин и тетрациклин. Код свих сојева су анализиране површинске карактеристике, покретљивост, способност формирања биофилма и адхезије на муцин. Такође, анализиран је и утицај различитих фактора (температура, pH, шејкирање и CO_2) на формирање биофилма, кинетика формирања биофилма код одабраних сојева као и утицај ТМП/СМХ на формирани биофилм. Добијени резултати су статистички обрађени у циљу утврђивања корелација међу њима.

Секвенцирање PCR производа добијених умножавањем гена за 16S рРНК добијено је да сви анализирани клинички изолати припадају врсти *S. maltophilia* са идентичношћу од 95% до 99% са *S. maltophilia* из NCBI базе података. PFGE анализом је потврђено да међу изолатима постоји велика генетичка хетерогеност. MLST анализом је утврђено постојање шест нових и три претходно детектована типа секвенце. Сојеви су били сензитиви на све тестиране антимикробне агенсе. Такође, код свих сојева је детектована покретљивост пливањем, док ниједан није показивао друге типове покретљивости. Међу сојевима који су показали способност адхезије на муцин није уочена разлика између ЦФ и нЦФ изолата.

Већина изолата је показала способност формирања биофилма (89,8%) и то подједнако заступљени су били представници ЦФ и нЦФ групе. Анализом формирања биофилма у различитим условима раста показано је да промена температуре и pH најјаче утиче на формирање биофилма и то готово идентично на Цф и нЦФ сојеве. Применом ТМП/СМХ у коннцентрацији од 50 μg/ml долазило је до потпуне разградње 24 сата старог биофилма, док је приликом примене 25 μg/ml тај ефекат био зависан од соја. Код сојева који су формирали јак биофилм уочена је спорија кинетика формирања биофилма код ЦФ у односу на нЦФ сојеве. Шејкирање није утицало на формирање биофилма.

Велики генетички диверзитет међу изолатима указује да није дошло до клоналног ширења унутар болнице. Показана је позитивна коерлација између покретљивости, формирања биофилма и адхезије за мицин. Осим тога, позитивна корелација је показана између покретљивости и јачине формираног биофилма. Међутим, поређењем фенотипских карактеристика ЦФ и нЦФ клиничих изолата уочава се да постоје разлике разлике између ове две групе сојева, али не и ЦФ фенотип.

Ključne reči: *Stenotrophomonas maltophilia,* нозокомијална инфекција, триметоприм-сулфаметаксазол, biofilm, PFGE, MLST

Naučna oblast: Молекуларна биологија

Uža naučna oblast: Молекуларна микробиологија

UDK: [579.253:[579.841:[574.58+616-78]]:[579.61:616-092](497.11)(043.3)

CONTENTS

INTRODUCTION

1. INTRODUCTION

Stenotrophomonas maltophilia is an aerobic, non-sporulating, non-fermentative, motile, Gram-negative road shaped bacterium, found in various environmental sources, including water, soil, plant, animal and organic residues. In addition, it has been found in the hospital environment and homes. Detected in a wide range of ecosystems, its degradation capabilities enhanced research in possible biotechnological applications like plant growth promoting bacterium (PGPB) and biocontrol organism. In the last decade, general interest for S. *maltophilia* rose, since it was connected with a wide range of hospital-acquired infections, particularly pneumonia and bacteremia in debilitated, immunosuppressed patients, transplant recipients and in patients with cystic fibrosis. Although this organism is nonpathogenic in healthy individuals, it is considered as an important opportunistic pathogen increasingly associated with morbidity and mortality in susceptible people. In the last decade, it was classified in the group of emerging, Gram-negative, multiple drug resistant (MDR) organisms. *S. maltophilia* is intrinsically resistant to many broad-spectrum antibiotics including β-lactam antibiotics (including imipenem), quinolones, aminoglycosides, carbapenems, and tetracyclines. The variety of clinical patterns associated with *S. maltophilia* infection continues to increase. There are numerous virulence factors, which are associated with the pathogenicity of this bacterium such as adhesion capacity, biofilm formation, hydrophobicity, motility and synthesis of extracellular enzymes. Additionally, there is still a considerable doubt regarding the acquisition routes of *S. maltophilia* infection, although a number of sources in the hospital setting have been recognized, strains isolated from these sites vary from strains that obtained from clinical materials.

1. *Stenotrophomonas maltophilia* – classification, microbiology characteristics and identification

1.1. Classification

Taxonomy of the Genus *Stenotrophomonas*:

Domain Bacteria

Phylum Proteobacteria

Class Gammaproteobacteria

Order Xanthomonadales

Family Xanthomonadaceae

Genus *Stenotrophomonas* (Palleroni & Bradbury, 1993)

Species *Stenotrophomonas maltophilia*

The species *Stenotrophomonas maltophilia* was originally isolated in England from human pleural fluid in 1943, named as species *Bacterium booker*, genus *Alcaligenes* and it was reported as being non-motile and probably a skin contaminant. Later in 1961, after detail morphological, physiological and serological analysis it was reclassified as species *Pseudomonas maltophilia*, genus *Pseudomonas*. Analyzed strain was isolated in 1958 from an oropharyngeal swab from a patient with an oral carcinoma (Hugh and Ryschenkow, 1961). In parallel, *Pseudomonas melanogena* isolated from Japanese rice paddies in 1963 was later documented as *Pseudomonas maltophilia*. The use of DNA-rRNA hybridization techniques discovered the presence of five rRNA homology groups in the genus *Pseudomonas*, and the rRNA cistron analysis of the *P. maltophilia* ATCC13637 showed that it was similar to three *Xanthomonas* strains (Palleroni et al., 1973). This evidence was used by Swings and coauthors (1981) to suggest that *P. maltophilia* be reclassified in the genus *Xanthomonas*, species *X. maltophilia*. Furthermore, they mentioned several other factors to support this

observation. Some of them are guanine-cytosine (GC) content (*P. maltophilia*, 63 to 67.5%; *Xanthomonas*, 63 to 70%); enzymes comparison, particularly the lack of NADP-dehydrogenases; the rate of the same type of ubiquinones (*P. maltophilia* and *Xanthomonas* spp. both possess ubiquinones with eight isoprene units, whereas all other *Pseudomonas* strains possess nine units); and similar fatty acid and proteins composition. Additionally, studies of outer membrane esterase, established that *P. betle* and *P. hibiscicola* were synonyms of *X. maltophilia* (Debette et al., 1989, Singer et al., 1994). Later, Yang and coauthors (1993) using polyamine and fatty acid analysis, demonstrated that *X. maltophilia* possess profiles distinct from other species within the genus *Xanthomonas*. As well as, the possession of ubiquinones with eight isoprene units was shown unlimited to *X. maltophilia* and other members of the genus *Xanthomonas* (Oyiazu and Komagata, 1983). This was supported by another experiment with a *Xanthomonas* specific 16S rDNA sequence (Maes, 1993). Xanthomonades were recognized by the presence of a single 480 bp PCR fragment in which, *X. maltophilia* strains produced additional PCR fragments, leading to reinterpretation that *X. maltophilia* does not belong to the genus *Xanthomonas*. These findings, along with additional evidence, were used to reclassification of this bacterium *Xanthomonas maltophilia* in 1993 to create the new genus *Stenotrophomonas* with the sole member species *Stenotrophomonas maltophilia* (Palleroni & Bradbury, 1993). Drancourt and coauthors (1997) suggested a new species *Stenotrophomonas africana*, which is identical biochemically to *S. maltophilia* with some exceptions. However, the genotypic analysis revealed only 35% DNA homology between the two species. Today genus *Stenotrophomonas* contains not only *S. maltophila* but also other species: *S. nitritireducens, S. rhizophila, S. acidaminiphila, S. koreensis, S. dokdonensis. S. humi, S. terrae, S. chelatiphaga, S. ginsengisoli, S. daejeonensis, S. pavanii* and *S. tumulicola* (*http://www.bacterio.net/stenotrophomonas.html*).

The name *Stenotrophomonas* is from the Greek origin – *stenos* (sample), *trophos* (the one that is feed), and *monos* (unit). It describes the organism that can survive in the moisture surfaces that are rich with nutrients. Word *maltophila* in its root contains two words *maltum* (crops) and *philia* (affinity) (Conly and Shafran, 1996).

1.2. Microbiology characteristics

S. maltophilia is an aerobic, non-sporulating, non-fermentative, motile, Gram-negative, road shaped, bacterium, 0.7–1.8 × 0.4–0.7 μm in size. It is catalase positive, oxidase negative bacteria; although recent data point that, some *S. maltophilia* isolates are oxidase positive. Bacterial surface is covered with tin febrile structures (5 to 7 nm) and there are few polar flagella (40 to 50 nm), which are used for movement of bacteria (Brooke, 2012). Under microscope they can be seen as individual cells or in pares. On hard medium in Petrie dishes, they form smooth, round colonies with sharp edges, from white to light yellow color, while in MacConkey agar plates it produces dark pigment and form colonies with characteristic look. For the optimal growth for most of the strains from genus *Stenotrophomonas* amino acids, methionine and cysteine are necessary (Denton and Kerr, 1998). It does not grow at temperatures lower than 4°C or higher than 41°C and its optimal growth temperature is 35°C.

S. maltophilia is ubiquitous microorganism, thanks to its adaptability and resistance widely distributed in the environment. It is associated with aqueous sources both inside and outside the houses (lakes, rivers, water treatment and distribution systems, wastewater plants, sinkholes, tap water, bottled water). *S. maltophilia* was also isolated from soil, plant rhizosphere, animals. In addition, there was an increase in the isolation of *S. maltophilia* in hospital settings during last decade. It is opportunistic pathogen commonly associated with healthcare-associated infections in immunocompromised patients. *S. maltophilia* is the only representative of the genus *Stenotrophomonas* that is connected with human

infections, while other species are plant pathogens. In the last decade, it was classified in the group of emerging, Gram-negative, multiple drug resistant (MDR) organisms (Brook, 2014). In the hospital surroundings, it also colonizes suction tubing, catheters, hemodialysis water and other medical and non-medical equipment. Due to its low number, this bacterium usually led to colonization but not to infection of the humans. The most frequent place of colonization is respiratory tract of patients and in patient with hematological malignancy; a fecal content of *S. maltophilia* is increased (Denton and Kerr, 1998). From particular importance is its ability to form biofilm on different biotic and abiotic surfaces, which could lead to development of the infection (Nicodemo and Paez, 2007). Particularly sensitive are patients with cystic fibrosis, where it can colonize airways and cause chronic infections (Goncalves-Vidigal et al., 2011). However, the most common infections associated with *S. maltophilia* include respiratory tract infections, bacteremia, catheter-related infections and urinary tract infections, infections of skin and soft tissues, endocarditis, meningithis, intraabdominal infections, eye infections and others (Denton and Kerr, 1998; Nicodemo and Paez, 2007).

1.3. Identification

Isolates of *S. maltophilia* could be identified with standard microbiological methods, according their morphological characteristics, growth conditions, biochemical (pigment production, oxidase test, catalase tests, etc.) and physiological characteristics. However, for the precise identification it is necessary to use molecular biology techniques:

- Sequencing of the genes for 16S rRNA and 23S rRNA
- PFGE („Pulsed-Field Gel Electrophoresis")
- DDH („DNA-DNA hybridization")
- MLST („Multilocus Sequence Typing")
- AFLP („Amplified Fragment Length Polymorphism")

- RFLP („Restriction Fragment Length Polymorphism") *gyrB* gene and other.

For final molecular identification of *S. maltophilia* isolates, but this is also the usual practice for all other bacterial species, it is necessary to use two or more methods. In addition, *S. maltophilia* may be coupled with polymicrobial infections or can grow slower in the host, which make the isolation and identification of this bacterium more difficult. Scientists have mainly relied on PCR based method, e.g. sequencing of the 16S rRNA gene that is conceder as the most conserved gene of three rRNA molecules (5S, 16S and 23S). In addition, it has been suggested as an "evolutionary clock", which has led to the rebuilding of the tree of life (Woese, 1987). Sequencing of PCR products of amplified 16S rRNA gene was the main method on which scientist are rely on in the last two decades for the final identification and classification of bacteria. In addition, sequencing of intermediate region between 16S and 23S rRNA gene, was useful method for identification and determination the differences between some bacterial species or sequencing of the 23S rRNA gene alone, which was useful for *Streptococcus* identification (Clarridge, 2004).

For identification, both Gram-positive and Gram-negative bacteria very useful method is Pulse Field Gel Electrophoresis (PFGE). This method was described for the first time in 1984 as the method for analysis of genomic DNA of eukaryotic organisms, while today is one of the main methods for identification of different bacterial species (Tenover et al., 1997). It is based on separation of DNA fragments of large molecular weight, which are obtained after digestion of bacterial DNA with selected restriction enzymes. During PFGE, direction of electronic field is changed periodically (pulse) what enables large fragments to pass through gel and be separated. Obtain macrorestriction profiles is strain specific, sometimes species specific, and for genotyping of *S. maltophilia,* the genomic DNA was digested with *Xba*I enzyme (Tenover et al.,

1997). To examine local epidemiology infections with *S. maltophilia* the most frequently used methods are PFGE and RAPD – PCR, while for analysis of global epidemiology MLST is the method of choice (Kaiser et al., 2009).

Multilocus Sequencing Typing (MLST) are based on comparison of nucleotide sequences of seven housekeeping genes and for *S. maltophilia* those genes are *atpD* (H (+)-transporting two-sector ATPase), *gapA* (NAD-dependent glyceraldehyde-3-phosphate dehydrogenase), *guaA* (GMP synthase), *mutM* (DNA-formamidopyrimidine glycosylase), *nuoD* (NADH dehydrogenase), *ppsA* (pyruvate, water dikinase), *recA* (RecA protein). Each strain is specific with its sequence type (ST) which is representation of the unique combination of seven housekeeping genes. Due to high specificity and discriminatory potential this method is used more and more frequently, although expensiveness and complex methodology limit its everyday use in microbiological laboratories.

2. Clinical importance of *S. maltophilia*

2.1. Epidemiology

From the time when the genus *Stenotrophomonas* was, established bacteria from this genus were mainly associated with different plants, what is implemented in the name of the genus and species. Some of the species cause plant diseases while the other are typical endophyte which have PGP effect on plants or they could help them to survive in the harsh environment (Ryan et al., 2009). Biotechnological potential of *S. maltophilia* was described in many papers (Alavi et al., 2013) and he was used as biocontrol agents up until the 1980s. However, in the early 1980s, *S. maltophilia* was reported as a new pathogen in the hospitals and now it is classified as an emerging, global, MDR opportunistic pathogen (Ryan et al., 2009; Brook, 2012). How this happen? Continuous development of the medical sciences with more aggressive why of diagnostics and therapy of many diseases, wider use of antibiotics (especially carbapenems)

led to increase in the isolation of *S. maltophilia* in hospitals. As an opportunistic pathogen, it does not infect healthy people. However, now we know that immunodeficiency (malignancy, chronic diseases, use of cytostatic and immunosuppressive therapy, neutropenia, burns, etc.), long hospitalization especially in the intensive care units, use of central venous and/or urinary catheter, intubation or tracheotomy, transplantation of artificial implants and prosthesis, in combination with overuse of broad spectra antibiotics are the main risk factors for infection with *S. maltophilia* and other hospital bacteria (Looney, 2005).

S. maltophilia usually causes pneumonia mostly in patients with chronicle respiratory diseases with long period-applied mechanical ventilation. Clinically important bacteremia is less frequent while infections of blood-stream infection, urinary and gastrointestinal tract, skin and soft tissues are rare (Looney, 2005). According to the clinical analysis, infections caused with *S. maltophilia* are not different from other hospital infections caused with clinical pathogens in immunodeficiency patients. Morbidity connected with these infections is hard to estimate since they are usually occurred in sever patients. Available data showed that morbidity as an effect of infection is found in 10-40% of patients (Sattler et al., 2000; Lai et al., 2004).

S. maltophilia is an environmental organism, which has also been isolated from human, animal feces, frozen fish, woodland ticks and milk. *S. maltophilia* has been found in the hospitals as a contaminant of medical devices, chlorhexidine-cetrimide disinfectant, edetic acid anticoagulant in vacuum-blood collection tubes, and sterile water (Spencer, 1995). High genomic diversity of the isolates obtained in previous studies (Valdezate et al., 2004) with rare exception (Garciá de Viedma et al., 1999) leads to the conclusion that patients were the route of introduction of the *S. maltophilia* to the hospital setting. This is suggesting that most patients acquire *S. maltophilia* from an independent source,

possibly before hospital entry, and the bacterium is then selected from the commensal flora (Caylan et al., 2004; Kagen et al., 2007).

2.2. Antibiotic resistance

Antibiotics are natural organic compounds produced by microorganisms that inhibited growth and kill other microorganisms. They are product of secondary metabolism, synthesized in the stationary phase of growth and selectively kill prokaryotic but not mammalian cells. Thanks to those characteristics, they are used for therapeutic purposes (Topisirović and Jovčić, 2013). According to the mechanism of action, antibiotics can be divided on inhibitors of: intracellular enzymes, bacterial wall synthesis, citoplasmatic membrane functions, nucleic acid synthesis and protein synthesis. The rising problem all over the world is the quick spreading of antibiotic resistance between different bacteria species. This problem becomes even greater since some strains develop multidrug resistance (MDR). The antibiotic resistance is acquired by the horizontal transfer of plasmids, transposons, integrons, integron-like elements, insertion elements common region (IECR) and biofilms (Topisirović and Jovčić, 2013; Looney, 2005). There is an urgent need for the new approach in the treatment of these bacteria. Synergy between antibiotics and different antimicrobial peptides, search for new antimicrobials or development of new antibiotics is some of the area of scientific research (Mataraci and Dosler, 2012).

2.2.1. Mechanism of resistance

S. maltophilia shows a high level of intrinsic and acquired resistance to various antibiotics such as β-lactam antibiotics (including imipenem), macrolides, quinolones, aminoglycosides, carbapenems, and tetracyclines, chloramphenicol, polymyxins, while trimethoprim–sulfamethoxazole (TMP/SMX) has been recognized as the antibiotic of choice in the treatment of these bacteria (Sánchez, 2015). Although, first it was thought that MDR in *S.*

maltophilia comes from hospital settings now we know that it was acquired in the natural nonhuman environment (Martínez, 2008; Sánchez, 2009). However, it can be broadened in the hospital settings through interactions with other hospital bacteria. In the last decades, intensive use in combination with misuse of antibiotics in the countries with poor regulation, not only for the human treatment but also in veterinary medicine led to contamination of the environment with antibiotics. This led to a higher incidence of antibiotic resistant bacteria or MDR bacteria and faster spread of resistance by other bacteria to pathogens (Cattoir et al., 2008). Numerous molecular mechanisms contribute to *S. maltophilia* antibiotic resistance, including, integrons, plasmids and transposons (Barbolla et al., 2004). Reduction in outer membrane permeability and β-lactamases contributes to resistance to β-lactams (Cullmann, 1991; Avison et al., 2001; Mercuri et al., 2002), presence of chromosomally encoded multidrug resistance efflux pumps (Gould et al., 2004), and antibiotic-modifying enzymes (Li et al., 2003) all contribute to the intrinsic antibiotic resistance of *S. maltophilia* clinical isolate (Sánchez et al., 2009; 2012; 2015).

2.2.1.1. Resistance to β-lactam antibiotics

β-lactam antibiotics disable peptidoglycan synthesis in the final step of bacterial cell wall synthesis. Their main targets are enzymes transpeptidase and carboxypeptidase that catalyze the reaction of peptidoglycan synthesis. These enzymes are also known as penicillin binding proteins-PBPs, since previously it was discovered that they bind penicillin. Binding of the antibiotic for the active site of enzyme led to formation of enzyme-antibiotic complex, which inhibits enzyme activity. This led to weakness of the peptidoglycan synthesis, inhibition of the bacterial growth and finally to the cell lyses (Wilke at al., 2005). Resistance to β-lactam antibiotics become usual for both Gram-negative and Gram-positive bacteria and are a consequence of antibiotic inactivation, target changes, lower membrane permeability or efflux pumps. Most frequent way is

synthesis of the β-lactamase, enzyme that degrades β-lactam antibiotics. Genes for β-lactamase could be located on plasmid, chromosomal DNA, transposons or integrons. The most relevant clinical β-lactamse is AmpC enzymes, Extended Spectrum Beta Lactamase – ESBL and carbapenemase.

Resistance of *S. maltophilia* on β-lactam antibiotics is due to synthesis of two types of β-lactamase, L1 and L2, whose genes are located on 200 kb plasmids. Interestingly, not all clinical isolates synthesized β-lactamase. The β-lactamase L1 is metallo-β-lactamase a homodimer of 118 kDa. It is a Zn^{2+}-dependent metalloenzyme that hydrolyzes almost all classes of β-lactams, including cephalosporins, penicillins, and carbapenems except monobactam, and it is not inhibited by clavulanic acid. The serine β-lactamase L2 is cephalosporinase that hydrolyzes aztreonam, it is a clavulanic acid-sensitive cephalosporinase (Walsh et al., 1997).

2.2.1.2. Resistance to aminoglycoside

Aminoglycosides are large and for chemotherapy important group of antibiotics, from streptomycin to highly potent amikacin and netilmicin. They are broad-spectrum antibiotics that inhibit protein synthesis in huge number of bacteria. However, their clinical importance is less and less since the number of resistance bacteria is increased dramatically. Many studies suggest that multiple mechanisms may contribute in aminoglycoside resistance by *S. maltophilia*, such as temperature-dependent resistance due to outer membrane changes, aminoglycoside-modifying enzymes, the efflux-mediated mechanism, and target modification which has been reported in some Gram-negative pathogens (Magnet and Blanchard., 2005). A family of enzymes that include O-phosphotransferases, O-nucleotidyltransferases, and N-acetyltransferase are responsible of aminoglycoside enzymatic modification. The changing in the lipopolysacharide (LPS) structure has been connected with changes in resistance to a variety of antimicrobial agents (Poole, 2002). It has been showed

the capability of *S. maltophilia* to modify the size of O-polysaccharide and the phosphate content of LPS at different temperatures, which increases the resistance to aminoglycosides at 30°C compared to 37°C (McKay et al., 2003). In addition, S. *maltophilia* also has several heavy-metal resistance mechanisms, and can tolerate silver-lined catheters.

2.2.1.3. Resistance to quinolones

Quinolones are synthetic broad-spectrum antibiotics that inhibited DNA replication by inhibiting enzyme DNA gyrase. There are three generations of these antibiotics, first are nalidicic acid, cinocoxain and pipemidic acid, second are norfloxacin, ciprofloxacin, lomefloxacin, ofloxacin, levofloxacin, etc. and third are gatifloxacin and moxifloxacin (Topisirović and Jovčić, 2013). The most frequent cause of *S. maltophilia* resistance on quinolones is specific mutation in the quinolone-resistance determining regions – QRDR inside the subunit for DNA gyrase enzyme (GyrA, GyrB) and (ParA, ParC). In addition, resistance on quinolones in *S. maltophilia* could be from reduction in outer membrane permeability and multidrug resistance efflux pumps (Valdezate et al., 2002).

2.2.1.4. Multidrug resistance efflux pumps

Multidrug resistance efflux pumps have been recognized as an important resistance mechanism in *S. maltophilia*. It is composed of membrane fusion proteins, an energy dependent transporter, Outer Membrane Proteins - OMPs. In general, they are responsible for secretion of antibiotics and toxins produced by bacterial cell and excretion of the compounds that came from surroundings to bacterial cell (antibiotics, disinfectant, colors, detergents, etc.) (Askoura et al., 2011). Genes that encodes efflux pump proteins are located on plasmids and chromosomal DNA. In *S. maltophilia* there are a few types of efflux pumps SmeABC, SmeDEF, SmeJKL and SmeYZ (Tanimoto, 2013). Genes from SmeDEF operon encodes genes responsible for resistance on β-lactams, tetracyclines, erythromycin, quinolones, aminoglycoside and choramphenicol

(Brooke, 2012; Nicodemo and Paez, 2007). *S. maltophilia* isolates rapidly develop resistance mechanisms against fluoroquinolones by making mutations in outer-membrane proteins. Recently study established the involvement of efflux mechanisms in acquired multidrug resistance in *S. maltophilia* (Zhang et al., 2000).

2.3. Selection of antimicrobial agents

The proper selection of antimicrobial agents for the treatment of *S. maltophilia* infection is a challenge in increased resistance prevalence and a high-level intrinsic resistance of this opportunistic pathogen. Recent treatment recommendations are based on historical evidence, case series and case reports, and in-vitro susceptibility studies. It is possibly wise to select a treatment program to which the clinical isolate is susceptible in *in vitro* tests, despite doubts about the clinical significance of such results. The drug of choice for treatment of *S. maltophilia* infections according to the World Health Organization is trimethoprim-sulfamethoxazole (TMP-SMX). However, there is increased number of reports from different hospitals about the resistance to TMP-SMX and susceptibility decreased from more than 98% to 30-40% (Toleman et al., 2007). Molecular mechanisms contributing in resistance of S. *maltophilia* to TMP-SMX are the class 1 integrons (Barbolla et al., 2004). Part of the class 1 integron are *sul1* gene found in TMP-SMX resistant *S. maltophilia* isolates recovered from Spain, Italy, Turkey, Germany, North and South America and *sul2* gene found on plasmid and chromosomal DNA in *S. maltophilia* TMP-SMX resistant isolates. Some *sul2* genes are connected to ISCR2 elements (Toleman et al., 2007). Vartivarian and coauthors (1994) reported an increase in the TMP-SMX susceptibility of isolates over 12-year period at the M. D. Anderson Cancer Center where there is discontinuation of its use as a common agent for antibacterial prophylaxis. Furthermore, they observed an increase in resistance to the quinolones the antibiotics that replaced TMP-SMX

in this treatment over the same period. Recent study suggests that a combination of TMP-SMX and either ticarcillin – clavulanate or cephalosporin may be superior to TMP-SMX alone (Muder et al 1996). Ticarcillin-clavulanate has been noted to display good activity against *S. maltophilia*, and it has been suggested that this agent should be used with individuals intolerant of TMP-SMX. Tigecycline and levofloxacin, alone or in combination, have shown promising efficacy in the treatment of *S. maltophilia* infections (Farrell et al., 2010; Wang et al., 2014). New treatment strategies point out the need for finding new and more effective antibiotics or selection of synergy antibiotics. In addition, search for new antimicrobial agents (lipopeptides, plant oils, bacteriocins and other antimicrobial peptides) and it use in combination with conventional antibiotic is also new area of scientific research. *In vitro* models suggest that antimicrobial combination therapy would be more effective than monotherapy particularly for treatment of difficult infections.

3. Molecular mechanisms involved in pathogenesis of *S. maltophilia*

Although molecular mechanisms involved in pathogenesis of *S. maltophilia* are not all discovered the main factors for the pathogenicity of *S. maltophilia* are adhesion capacity, biofilm formation, hydrophobicity, motility, and synthesis of extracellular enzymes (Pompilio, 2010; Looney, 2005).

3.1. Adhesion capacity

Adhesion capacity of bacterial cells is the first factor involved in the process of initial colonization and invasion to the tissue of the host or abiotic surfaces. Interaction between bacterial cell and epithelial cell is through flagella and/or pile on the bacterial surface (Looney, 2005). Positive charge of the bacterial surface is also one of the main factors which goes in favor to adhesion

and colonization of biotic (host tissue) and abiotic (plastic, glass, Teflon, medical devices, etc.) surfaces (Di Bonaventura et al., 2008). McKay and coauthors (2003) showed that *spgM* gene which encodes bifunctional enzyme that has both phosphoglucomutase and phospomannomutase activity is necessary for the tissue colonization with *S. maltophilia*. This enzyme is involved in synthesis of lipopolysaccharides that on the other hand have influence on the cell adhesion. Adhesion of *S. maltophilia* on abiotic surfaces enables direct or indirect contact of bacteria with the patient. For example, colonization of the endotracheal tubus with *S. maltophilia* enables direct entrance of bacteria into the patient's lungs and on that why influenced pathogenesis of pneumonia. In addition, bacteria could easier multiply inside of the tubus since that surface is not protected by the immune system cell and antibiotics do not have effect on them too (Looney, 2005). The adherence of bacterial cells to a surface is one of the early steps in biofilm formation process.

3.2. Biofilm

Biofilm formation is an important factor of bacterial virulence and pathogenesis, which usually contribute to the diseases progression. Biofilm is a highly organized, multicellular community of microorganisms encased in an extracellular polymeric matrix, made from polysaccharides and proteins that are affixed to a biotic or abiotic surface. Bacterial populations within a biofilm, as opposed to their planktonic counterparts, have a reduced growth rate and a distinct transcriptome. Moreover, they exchange genetic material at an increased frequency thereby augmenting their ability to acquire traits favorable to their persistence (Donlan and Costerton, 2012). Biofim protects microorganisms from the immunity system of host and from influence of some antimicrobial substances (Pompilio, 2010; de Oliveira-Garcia, 2002). Physical and molecular interactions that govern the adhesion of bacteria to these

surfaces require an understanding of specific and non-specific interactions (Oztuna et al., 2006).

Changing from planktonic growth to biofilm populations is due to response to the changes in the bacteria environment. It involves a huge regulatory network that translates signals which regulate genes expression causing cell reorganization (Kostakioti et al., 2017). This cellular reprogramming includes the expression of surface molecules, adapting the use of nutrients, expression of virulence factors, and equipping the bacteria with an arsenal of properties that allow them to survive under unfavorable conditions (Lenz et al., 2008).

Bacterial aggregation and biofilm maturation consists of reversible and irreversible levels involving numerous and specific factors. There are two basic processes in the formation of biofilm. The first phase involves binding of bacteria to the surface, followed by the second phase of aggregation of bacteria and the formation of multilayer structures. Bacteria in biofilm are associated with amorphous mucous material so called extracellular polysaccharide matrix (Gotz, 2004), which plays a special role as a cellular adhesive, which makes the layers sticky, tolerant to the environmental factors (Rode et al., 2007) and antimicrobial agents (Heiby et al., 2010). Within the biofilm there is a developed network of water and nutrient channels, thus providing cells with conditions for growth and diversity (Watnick and Kolter, 2000).

According to the information so far, more than 99% of the bacteria in the natural environment live in biofilm, and in 80% of cases of infection in humans, biofilm plays an important role (Stewart PS, 2001). In that sense, *S. maltophilia* is involved in approximately 65% of hospital-associated infections (Mah and O'Toole, 2001). Di Bonaventura et al. (2010) showed that *S. maltophilia* SM33 cells could adhere within 2h to polystyrene surfaces and form biofilms within 24 h of inoculation. Both the transmission and scanning electron microscopy

(TEM and SEM) recognized the *S. maltophilia* flagella on 46 clinical isolates, which suggest that the flagella and other thin pili arrangements are involved in adherence of bacterial cell on a plastic surface (de Oliveira-Garcia et al., 2002).

Previous study has determined that *S. maltophilia* can form biofilm on lung cells. The mucus that lining the surfaces of the respiratory, gastrointestinal, and reproductive systems provides a barrier against pathogens. It contains glycoproteins, which are composed of a peptide backbone that associated to carbohydrates, which act as a receptor for bacterial adhesions (Arora et al., 1998). The biofilms formed by cystic fibrosis (CF) isolate *S. maltophilia* OBGTC9 on CF sputum-derived bronchial epithelial IB3-1 cell monolayers showed that *S. maltophilia* formed microcolonies embedded in extracellular matrix (Pompilio et al., 2010). Interestingly, biofilm formed by *S. maltophilia* CF isolates on polystyrene surface did not resemble to the biofilm formation by the isolates on the cell monolayer. That evidence supports the thought that biofilm formation on abiotic surfaces may not reflect the biofilm formation on biotic surfaces. Furthermore, the environmental factors that can influence the biofilms formation by *S. maltophilia* include temperature, pH, phosphate, chloride concentrations, aerobic and anaerobic conditions, and the presence of silver and copper ions (Di Bonaventura et al., 2007; Critchley et al., 2003). *S. maltophilia* can form biofilms on wet surfaces that may contact direct or indirect with patients, including, dental suction tubing, water plumbing systems, respiratory tubing and unit waterlines, clinical sink drains, catheters, intravenous lines, dialysis equipment and domestic sink drains. It has been identified that the presence of sodium phosphate can alter the biofilms of clinical *S. maltophilia* isolates (Brooke et al., 2009). A study established that 9 of 11 clinical isolates have altered biofilm formation when cultured in Luria Bertani broth (LB) medium supplemented with sodium phosphate buffer (SPB). These findings have significance for applied situations where *S. maltophilia* may possibly form biofilms. Since for the phosphate has been demonstrated to alter the microbial communities in the

human water supply, the levels of sodium and phosphate in hospital water plumbing systems should be observed (Goss et al., 2004).

Clinical *S. maltophilia* isolates have been detected to form biofilms at 32°C more than at 18°C and 37°C (Di Bonaventura et al., 2007). The formation of biofilm was higher under aerobic conditions and in a 6% of CO_2 atmosphere than the biofilm formation under anaerobic conditions. The *S. maltophilia* isolates have reported to produce comparable biofilms at pH 8.5 and 7.5 and higher biofilm produced at pH 5.5 (Di Bonaventura et al., 2007).

3.2.1. Factors associated with biofilm formation in *S. maltophilia*

The environmental factors affecting *S. maltophilia* biofilm formation have been investigated, but the molecular basis of their regulatory mechanisms remains incompletely understood (Di Bonaventura et al., 2004; Di Bonaventura et al., 2007; Stoodley et al., 1999) and thus far only a few related *S. maltophilia* genes have been experimentally studied. For example, several structural genes associated with the cell envelope, including those encoding the proteins involved in lipopolysaccharide/exopolysaccharide-coupled biosynthesis (*rmlA*, *rmlC*, and *xanB*) and the pump-encoding genes *macABCsm* and *smeYZ* have been identified as necessary for biofilm formation (Huang et al., 2006; Lin et al., 2014; Lin et al., 2015). In addition, the genes encoding three transcription regulators (fleQ, fsnR, and bfmA) also control biofilm development. FleQ binds to the putative ATPase FleN to form a complex that directs flagellar gene expression (Yang et al., 2014). FsnR, designated as a response regulator with transcription-regulating activity, binds directly to the promoter regions of gene clusters involved in flagellar assembly to activate their transcriptional initiation (Kang et al., 2015; Zheng et al., 2016). Besides the aforementioned regulatory factors, recent studies have identified bis-3′, 5′-cyclic diguanosine monophosphate (c-di-GMP) as an important cellular second messenger broadly distributed among bacteria and critical to the control of bacterial physiology,

especially biofilm development and motility. c-di-GMP activates downstream cascades by binding to specific protein effectors or riboswitches embedded in the leader regions of mRNAs.

3.3. Synthesis of extracellular enzymes

Different extracellular enzymes including DNase, RNase, fibrinolysin, lipases, hyaluronidase, protease, hytinase, mucinase, elastase, may play a role in the pathogenesis of *S. maltophilia* associated infection. Bottone and coauthors (1986) reported a case of ecthyma gangrenous in a leukemic patient with *S. maltophilia* bacteremia. Since the production of protease and elastase by bacteria is considered an important in the pathogenesis of cases of ecthyma gangrenous associated with *P. aeruginosa* septicemia, they studied the *S. maltophilia* isolate for elaboration of these enzymes and established that it was an "avid protease and elastase producer" (Bottone et al., 1986). A study of 52 clinical and environmental strains tested for the production of nine extracellular enzymes showed that there is no production of lecithinase, hyaluronidase, or chondroitin sulfatase by any strains, however all of them produced protease and elastase. Production of elastase was variable, but there was no significant variation between clinical and environmental strains. Even though DNase, fibrinolysin and lipase were produced by all strains at 20°C, the clinical isolates showed higher production of these enzymes at 37°C. Moreover, the ability of the bacterium to grow in dialysis fluids and release their low molecular- weight pyrogens, might be important in the pathogenesis caused by the pyrogenic reactions during hemodialysis (Ganadu et al., 1996). A study reported that *S. maltophilia* clinical isolates were much more likely to exhibit this property than environmental isolates (Denton et al., 1998).

3.4. Hydrophobicity and motility

The interaction of microorganisms with different host organisms as well as with the environment is mediated through their surface; therefore, surface properties are important for these interactions. The behavior of microorganisms in different environment is largely determined by the composition and characteristics of their surface. This determined their adhesion capacity, colonization, biofilm formation and finally development of infections (Di Bonaventura et al., 2008). When microorganisms come to interaction with abiotic or biotic surface destiny of that interaction depends of nonspecific, especially hydrophobic interactions (Costa et al., 2006). A positive correlation was observed between cell surface hydrophobicity and adhesion and biofilm formation of *S. maltophilia* (Pompilio et al., 2008). Surface hydrophobicity is especially important in interactions with abiotic surfaces, in particular those found in hospital environments. However, isolates with different hydrophobicity characteristics could have similar biofilm formation ability what goes in favor that multiple factors affect biofilm formation.

Cell surface structures flagella, pili, fimbriae play an important role in adhesion capacity, colonization and biofilm formation in different microorganisms (Mandlik et al., 2008). *S. maltophila* is characterized with different structural appendages such as flagella and pili that both enable different types of motility to this bacterium (swimming, swarming and/or twitching) but also biofilm formation (De Oliveira-Garcia et al., 2002, 2003). Pompilio and coauthors (2008) did not found any correlation between motility and hydrophobicity, adhesion capacity and biofilm formation. However, they showed relationship between amount of biofilm formed and the extent of the initial adhesion. This finding suggests that first step in biofilm formation of *S. maltophila* is adhesion of bacteria to abiotic surfaces.

4. Cystic fibrosis

Cystic fibrosis (CF) is most frequent monoclonal inherited diseases (1:2500) of the white race people that is inherited in an autosomal recessive manner, and with high frequency (4-5%) of heterozygote carrier in human population (Govan and Deretic, 1996). A chronic, progressive, and multisystem disease is a consequence of one or more mutation in *cftr* gene. This gene encodes cystic fibrosis transmembrane conductance regulator, CFTR, which form chloride channel, which regulated transport of the ions through cell membrane. Mutations in this gene led to disturbance of Na^+ and Cl^- ions transport, dehydration of secret that normally cover airways forming viscose mucus layer which could not be easily excreted. This tick, viscose and high osmolarity secret "capture" bacteria and they increase their number, develop biofilm and cause chronic respiratory infections (Cantón and del Campo, 2010). Eradication of the respiratory tract due to chronic microbial colonization and infection is the main cause of morbidity and mortality of the CF patients. Interestingly, small number of microbes causes respiratory infections in CF patients, but they are usually polymicrobial (Rabin and Surette, 2012). In children, the most frequent cause of infections is *Staphylococcus aureus* and unencapsulated *Haemophilus influenzae*, while in adult's opportunistic pathogens such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* are isolated. In CF patients, not as a cause of respiratory infection *Burkholderia cepacia* complex (Bcc), *Burkholderia gladioli*, *Ralstonia* spp., *Cupriavidus* spp., *Pandoraea* spp., etc, could be found also (LiPuma, 2010). Isolation of some of these species is associated with bad prognosis of the disease but for another precise role is not determined (LiPuma, 2010). Simple explanation of this disease would be that it is the consequence of (impaired mucociliary transport) inefficient elimination of microorganism from

respiratory tract. However, it is probably more complex process. Lower activity of surfactants and natural peptides with local antimicrobial activity makes chronicle bacterial colonization and infection easier, and defense of natural immunity system is lower (Cantón and Del Campo, 2010). There is a constant interplay between infection and inflammation in airways, which is a key point of lung disease in CF patients (Hector et al., 2016).

4.1. *S. maltophilia* and cystic fibrosis

Prevalence of *S. maltophilia* in CF patients worldwide has increase in the last decade (de Vrankrijker et al., 2010). However, the exact role of this opportunistic pathogen in CF patients is still undiscovered. Bacterial population in CF patients is exposed to a harsh environment and intensive antibiotic treatment so changes in population content as well mutation in microorganisms occur intensively causing the constant changes in bacterial population (Tenaillon et al., 1999). The best-studied microorganism from CF patients is *P. aeruginosa* were it was documented that hypermutation is a key mechanism for increased antibiotic resistance (Oliver and Mena, 2010). Although, there is still some uncertainness in the precise role of *S. maltophilia* in the CF patients, nowadays it is considered as emerging opportunistic pathogen in the CF patients (Brooke, 2012). Diversity of *S. maltophilia* isolated from chronically colonized CF patients is high (Vidigal et al., 2014). Vidigal and coauthors (2014) showed that mutation in analyzed isolates confirmed fast adaptation of this bacterium population in order to survive in the CF lung airways, but they could not find significant correlation between mutations and increasing antibiotic resistance as it was shown for *Pseudomonas*. Aforementioned, polymicrobial infections in CF patients additionally impeded analysis of specific role of each species as well as their mutual interactions and role in the development and prognosis of the disease. However, Pompilio and coauthors (2011) suggested

existence of "CF phenotype" since their results for phenotypic characteristics of CF isolates significantly differ from those obtained for non-CF isolates.

4.2 Prevention of *S. maltophilia* infections

A number of prevention strategies to avoid *S. maltophilia* infection have been recommended. These include prevention of using the wrong antibiotics, prolonged embedding of foreign devices, and an appropriate sterilization of respiratory therapy equipment, cardiopulmonary bypass apparatus, hemodialyzers, and ice-making machines. In addition, it may be to avoid drinking of noncarbonated bottled water. To avoid hospital associated infections of *S. maltophilia,* colonization or infection, wearing of gloves and strengthening of hand hygiene practices when handling contaminated respiratory excretions or wound drainage were recommended. Transfer of *S. maltophilia* from tap water to patients is the problem, which could be prevented with continual education about the hygiene practice of the health care personal. The cost-effectiveness of screening programs for *S. maltophilia* colonization in high-risk patients needs further examination before they can be recommended (King et al., 2010). Scientific research, which will allow better understanding of this bacterium and its characteristics, would help in preventing infections as well as their adequate treatment. Biofilm-related infections are a therapeutic challenge of modern medicine and preventing of primary adhesion would prevent biofilm formation, consequently, this would lead to its faster eradication.

5. Future perspectives

Stenotrophomonas maltophilia is classified in the group of emerging, global, MDR pathogens. Environmental bacterium involved in hospital settings were due to its tremendous ability to adopt to these new surroundings from occasional isolations become usual in polymicrobial infections. Multiple

intrinsic antibiotic resistance makes its treatment difficult and development of new strategies are needed. It is necessary to consider its ecology, way of transfer in hospitals and their controlled use of antimicrobials in order not to encourage the spread of antibiotic tolerant *S. maltophilia* strains. Number of immunosuppressed individuals is increased and this points to the need to monitor the worldwide global emerging pathogens such as *S. maltophilia.* The most important is identification of molecular mechanisms, which lie in the basis of persistence of opportunistic pathogens in environment and clinical settings.

AIMS

2. AIMS

The prevalence of *Stenotrophomonas maltophilia* has increased in hospitals worldwide simultaneously with the appearance of a myriad of antibiotic resistant bacteria. One of the usually present virulence factors in pathogenic bacteria and opportunistic pathogens are ability to form biofilm. Therefore, biofilm-associated infections substantially affect human health, increasing antibiotic resistance of bacteria and making more challenging to combat with such infections.

The main aim of this study is genotyping, antibiotic resistance and biofilm formation of *S. maltophilia* clinical isolates.

On the basis of the above-mentioned specific aims of this research are:

1. Molecular identification and genotyping of *S. maltophilia* clinical isolates by sequencing of 16S rRNA gene and Pulse Field Gel Electrophoresis (PFGE).
2. Dendrogram construction based on the PFGE results and clustering collections of *S. maltophilia* clinical isolates
3. Multilocus sequencing typing (MLST) analysis of representative of each cluster
4. Analysis of antibiotic resistance of *S. maltophilia* clinical isolates by disc diffusion method for tetracycline, chloramphenicol, livofloxacin and ciprofloxacin
5. Analysis of antibiotic resistance of *S. maltophilia* clinical isolates by microdilution method for trimethoprim/sulfamethoxazole (TMP/SMX)

6. Analysis of virulence factors of *S. maltophilia* clinical isolates: hydrophobicity, motility, ability to adhere to mucin and biofilm formation

7. Determination of kinetics of biofilm formation for selected *S. maltophilia* strains

8. Analysis of influence of temperature, pH, CO_2 concentration and agitation on biofilm formation

9. Testing influence of TMP/SMX on formation and preformed biofilm of selected *S. maltophilia* strains

As the final aim of this study, we expect to determine differences between *S. maltophilia* strains isolated from patients with cystic fibrosis (CF) and non-CF patients, as well as potential determination of *S. maltophilia* CF-phenotype.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

1. Bacterial strains used in this study

Stenotrophomonas maltophilia isolates used in this study are clinical isolates from Institute for Mother and Child Health Care of Serbia "Dr Vukan Čupić", a 400-bed university-affiliated pediatric tertiary care hospital in Belgrade, Serbia. This institution is also referral hospital and is the national and regional reference CF specialist center for pediatric and adult patients with CF from Serbia, Montenegro and Bosnia and Hercegovina. All isolates were isolated in the Laboratory for clinical microbiology, during the period from April 2013 until April 2015. At least one isolate per patient was included, as well as subsequent isolations that were considered phenotypically different or were recovered with a time interval of more than 6 months. Isolates are from patient with (CF) and without (non-CF) cystic fibrosis (CF) but with different immunomodulatory, cancer, diabetes, neurological and other diseases. The collection comprises 88 clinical isolates, 42 for CF and 46 from non-CF patients (Table 1).

Table 1 – Clinical isolates of *S. maltophilia* used in this study

Strains	CF status	Date of isolation	Site of isolation	Strains	CF status	Date of isolation	Site of isolation
4065	non-CF	11.4.2013	Throat swab	252FA	non-CF	13.8.2013	Bronchial swab
4111	non-CF	12.4.2013	Throat swab	8770	non-CF	16.8.2013	Throat
4199	non-CF	15.4.2013	Throat swab	10021	non-CF	20.9.2013	Tube
4477	non-CF	22.4.2013	Throat swab	10030	non-CF	21.9.2013	Throat swab
4619	non-CF	25.4.2013	Abdominal wound	10137	non-CF	24.9.2013	Bronchial swab
4810	non-CF	30.4.2013	Throat swab	11124	non-CF	19.10.2013	Throat swab

4952	non-CF	7.5.2013	Throat swab	10668	non-CF	8.10.2013	Throat swab
5226	non-CF	13.5.2013	Throat swab	11370	non-CF	25.10.2013	Bronchial swab
5365	non-CF	16.5.2013	Urine	311FA	non-CF	28.10.2013	Bronchial swab
5389	non-CF	17.5.2013	Foreskin	320FA	non-CF	30.10.2013	Bronchial swab
5503	non-CF	20.5.2013	Throat swab	11863	non-CF	5.11.2013	throat swab
167FA	non-CF	28.5.2013	Bronchial swab	11774	non-CF	4.11.2013	Bronchial swab
6227	non-CF	7.6.2013	Throat swab	12049	non-CF	11.11.2013	Throat swab
6607	non-CF	17.6.2013	Throat swab	12144	non-CF	13.11.2013	Throat swab
7339	non-CF	8.7.2013	Throat swab	12572	non-CF	24.11.2013	Throat swab
7491a	non-CF	11.7.2013	Sputum	363F	non-CF	2.12.2013	Bronchial swab
7491b	non-CF	11.7.2013	Sputum	13029	non-CF	6.12.2013	Tube
223FA	non-CF	16.7.2013	Bronchial swab	374F	non-CF	13.12.2013	Bronchial swab
13589	non-CF	21.12.2013	Throat swab	3826	CF	9.4.2014	Sputum
13590	non-CF	21.12.2013	Throat swab	195a	CF	3.7.2014	Bronchial swab
13620	non-CF	22.12.2013	Throat swab	7316	CF	*16.7.2014*	Sputum
13761	non-CF	25.12.2013	Sputum	8757	CF	27.8.2014	Sputum
13839	non-CF	27.12.2013	Human milk	8935	CF	1.9.2014	Sputum
13889	non-CF	29.12.2013	Nose swab	9018	CF	3.9.2014	Throat swab
13879	non-CF	28.12.2013	Throat swab	10073	CF	30.9.2014	Throat swab
280H	non-CF	22.1.2014	Blood	10454	CF	9.10.2014	Throat swab
2275	non-CF	12.6.2014	Blood	11006	CF	22.10.2014	Sputum
1987	non-CF	19.2.2015	Throat swab	11279	CF	29.10.2014	Throat swab
4584	CF	23.4.2013	Throat swab	11304	CF	29.10.2014	Throat swab
5046	CF	8.5.2013	Throat swab	11382	CF	30.10.2014	Sputum
5310	CF	15.5.2013	Throat swab	11975	CF	12.11.2014	Sputum

6148	CF	5.6.2013	Throat swab	12606	CF	26.11.2014	Throat swab
6144	CF	5.6.2013	Sputum	12439	CF	23.11.2014	Throat swab
6603	CF	17.6.2013	Sputum	13373	CF	12.12.2014	Sputum
7711	CF	17.7.2013	Sputum	3F	CF	5.1.2015	FA
8339	CF	5.8.2013	Sputum	791/15	CF	22.1.2015	Sputum
8687	CF	14.8.2013	Throat swab	1394a	CF	4.11.2015	Throat swab
11600	CF	30.10.2013	Throat swab	1394b	CF	4.11.2015	Throat swab
12128	CF	13.11.2013	Throat swab	2234	CF	15.2.2015	Sputum
12682	CF	27.11.2013	Throat swab	2483a	CF	4.3.2015	Throat swab
13215	CF	12.12.2013	Throat swab	2483b	CF	4.3.2015	Throat swab
486/14	CF	15.1.2014	Throat swab	2484	CF	4.3.2015	Sputum
741	CF	22.1.2014	Sputum	3817	CF	8.4.2015	Throat swab
1874	CF	19.2.2014	Throat swab	3944a	CF	11.4.2015	Throat swab

2. Media used for bacterial cultivation

S. maltophilia isolate, as well as *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* DH5α were grown in Luria Bertani (LB) liquid medium (Sodium chloride (0.5%), tryptone (1%) and yeast extract (0.5%)). The solid media for growth was obtained by the addition of 1.5% agar into LB medium. For the purpose of testing the sensitivity of antibiotic disc diffusion test and determining the minimum inhibitory concentration (MIC) with microdilution test Mueller-Hinton (MH) medium (Oxoid, Hampshire, UK) was used. Motility assay for swimming, swarming and twitching characteristics was performed in medium with a low content of the agar. Swimming agar (10 g/l tryptone, 5 g/l NaCl, 0.3% agar), swarming agar (0.5% agar, 8 g/l nutrient broth, and 5 g/l glucose), and twitching agar (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 1% agar), respectively. Trypticase soy broth (TSB) medium (LabM, UK) was used in test for biofilm formation. All media were sterilized by autoclaving for 15 min at 121^{o}C, while bacterial growth was performed at 37°C with aeration.

3. DNA isolation

Isolation of total DNA from *S. maltophilia* was made according to the method described by Hopwood *et al.,* (1985) with appropriate modifications. The cell precipitate was obtained by centrifugation (2 min, 13000 rpm, centrifuge 5804, Eppendorf, Hamburg, Germany). Overnight culture, was washed in 500 µl of TEN buffer (50 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, pH 8). Lysis of cells was performed by adding 250 µl of 2% SDS, and gently rotating the sample, and then the multiple phenolic extraction was done, by adding 200 µl of the neutral phenol to the samples, with vigorous mixing, vortexing and centrifugation (10 min, 13000 rpm, centrifuge 5804, Eppendorf, Hamburg, Germany). The supernatant was carefully collected and transferred to a new microfuge tube. The precipitation of DNA was made by the addition of 1/10 volume of 3M Na-acetate and 0.7 volumes of isopropanol, followed by the centrifuging of samples (20 min, 13,000 rpm, centrifuge 5804, Eppendorf, Hamburg, Germany). The precipitate was washed by adding 500 µl of 70% ethanol and centrifugation (10 min, 13000 rpm centrifuge 5804, Eppendorf, Hamburg, Germany). The obtained precipitate was dried at 37°C, and then the remaining RNA is eliminated by resuspending the precipitate in 100 µl of RNase solution (10 mg/mL) in distilled water and allowed to incubate at 37°C for a period of 15 minutes.

4. Electrophoresis

Electrophoresis of total DNA as well as fragments obtained by the PCR was performed in a 1% agarose gel. The gels were made by dissolving the agarose in 1X TAE buffer (40 mM Tris-acetate, 2 mM EDTA and final pH 8.0) with the addition of ethidium bromide (0.5 mg/ml). Electrophoresis was run in 1X TAE buffer at a constant voltage of 1-10 V per cm of the gel. Sizes of

analyzed fragments were determined by comparing the length of obtained fragments with DNA fragments of standard, "GeneRulerTM 1 kb DNA Ladder Mix" (Thermo Scientific, Fermentas, Lithuania).

5. Molecular identification and genotyping of clinical isolates

5.1. Polymerase Chain Reaction (PCR) analysis of 16S rRNA gene

Laboratory identification of the isolates was carried out using standard biochemical testing and automated Vitek 2 system (BioMérieux, Marcy l'Etoile, France). Molecular identification of the strain was performed by PCR analysis of 16S rRNA gene with specific primers UNI16SF and UNI16SR (Table 2). PCR products were purified with GeneJET PCR Purification Kit (Thermo Scientific, Lithuania) and sequenced by the Macrogen DNA sequencing service (Macrogen Inc., Netherlands). Obtained sequences were aligned in the NCBI database by using BLAST program.

Table 2 – Specific primers used in this study.

Name of the primer	Sequence of the primer	Annealing Temperatures	References
UNI16SF	5-GAG AGT TTG ATC CTG GC-3	50°C	Jovcic *et al.*, 2009
UNI16SR	5-AGG AGG TGA TCC AGC CG-3	50°C	Jovcic *et al.*, 2009
atpD forw	5-ATGAGTCAGGGCAAGATCGTTC-3	62°C	MLST Data base
atpD rev	5-TCCTGCAGGACGCCCATTTC-3	62°C	MLST Data base
gapA forw	5-TGGCAATCAAGGTTGGTATCAAC-3	62°C	MLST Data base
gapA rev	5-TTCGCTCTGTGCCTTCACTTC-3	62°C	MLST Data base
gapA forw(2fwd)	5-AGGAGCTTGAGAAATGGCAA-3	48-58°C	MLST Data base
gapA rev(2r)	5-GAGTAGCCCCACTCGTTGTC-3	48-58°C	MLST Data base
guaA forw	5-AACGAAGAAAAGCGCTGGTA-3	62°C	MLST Data base
guaA rev	5-ACGGATGGCGGTAGACCAT-3	62°C	MLST Data base

mutM forw	5-AACTGCCCGAAGTCGAAAC-3	58,62°C	MLST Data base
mutM rev(2r)	5-GAGGATCTCCTTCACCGCATC-3	58,62°C	MLST Data base
mutM rev(4r)	5-TTACCGGCCTCGCGCAG-3	52,48°C	MLST Data base
nuoD forw	5-TTCGCAACTACACCATGAAC-3	48°C	MLST Data base
nuoD rev	5-CAGCGCGACTCCTTGTACTT-3	48°C	MLST Data base
nuoD forw(2f)	5-AGGAAATCCGCAACTACACC-3	48°C	MLST Data base
nuoD rev(2r)	5-AGCGCGACTCCTTGTACTTC-3	48°C	MLST Data base
ppsA forw	5-CAAGGCGATCCGCATGGTGTATTC-3	62°C	MLST Data base
ppsA rev	5-CCTTCGTAGATGAA(A/G)CCGGT (A/G)TC-3	62°C	MLST Data base
ppsA forw(2f)	5-TTCACCCTGGACACCGAGT-3	58°C	MLST Data base
ppsA rev(2r)	5-CGAAGTCGAAGGCACGTT-3	58°C	MLST Data base
recA forw	5-ATGGACGAGAACAAGAAGCGC-3	62°C	MLST Data base
recA REV	5-GGTGATGACCTGCTTGAACGG-3	62°C	MLST Data base
MutM_10f	5-CTGCCCGAAGTCGAAACAA-3	58°C	MLST Data base
mutM_803r	5-CAGTGGCTGCACCAGACG-3	58°C	MLST Data base
mutM_11f	5-TGCCCGAAGTCGAAACCAC-3	58°C	MLST Data base
mutM_804r	5-GCAGTGGCTGCACCAGAC-3	58°C	MLST Data base
mutM_13f	5-CCCGAAGTCGAAACCACCC-3	58°C	MLST Data base
mutM_802r	5-AGTGGCTGCACCAGACG-3	58°C	MLST Data base
mutM_14f	5-CCGAAGTCGAAACCACCCG-3	58°C	MLST Data base
mutM_800r	5-TGGCTGCACCAGACGC-3	58°C	MLST Data base
rmlA	5-GCAAGGTCATCGACCTGG-3	60°C	Pompilio et al. 2011
rmlA	5-TTGCCGTCGTAGAAGTACAGG-3	60°C	Pompilio et al. 2011
spgM	5-GCTTCATCGAGGGCTACTACC-3	60°C	Pompilio et al. 2011
spgM	5-ATGCACGATCTTGCCGC-3	60°C	Pompilio et al. 2011
rpfF	5-CTGGTCGACATCGTGGTG-3	60°C	Pompilio et al. 2011
rpfF	5-TGATCCGCATCATTTCATGC-3	60°C	Pompilio et al. 2011

PCR amplification was performed by mixing of total DNA (0.1-1 µg) with 1X reaction buffer (standard reaction buffer 10X with Mg^{2+}, Kapa biosystems, USA), a mixture of dNTPs (each dNTP in final concentration of 200 µM), primers (each 2.5 µM) and Taq DNA polymerase (1 U/µl, Kapa biosystems, USA). PCR reactions were performed using GeneAmp 2700 PCR Cycler "(Applied Biosystems, USA) according to the programme in Table 3.

Table 3 - PCR reaction for 16S rRNA gene amplification.

Reaction phase	Temperature	Time	Number of cycle
Initiation denaturation	94°C	5 min	
Denaturation	94°C	30 s	30 cycles
Hybridization (*annealing*)	50°C	30 s	
Elongation	72°C	30 s	
Final elongation	72°C	10 min	

5.2. Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed as previously described (Kojic *et al.,* 2005) and for this analysis *Xba*I enzyme (Thermo Scientific, Lithuania) was used, and obtained profiles were subject of statistical analysis. *In situ* preparation of samples for PFGE was performed as follows. Cells grown in LB broth at 37°C to early logarithmic phase and collected by centrifugation (13000 rpm for 5 min). The collected cells were washed twice with EET buffer (100 mmol/l EDTA, 10 mmol/l EGTA, 10 mmol/l Tris-HCI, pH 8.0) and resuspended in 50 µl of the same buffer to obtain 10^9 cells/ml. This cell suspension was warmed on water bath at 42°C and mixed with an equal volume of prewarmed gel (0.1% *mlv* agarose (InCert™ Agarose, Lonza, Rockland, MA, USA) in 5 ml of EET buffer), poured in 100 µl block modules, and allowed to solidify at 4°C. The agarose blocks with incorporated cells were then incubated overnight at water bath 50°C in 500µl of EET buffer with SDS (0.5 % *mlv*) and proteinase K (0.5 mg/ml)

with gentle shaking. Final treatment of agarose blocks constituted of washing two times in 100 volumes of TE buffer (10 mmol/l Tris-HCI, 1 mmol/l EDTA, pH 8.0) or water containing 0.1 mmol/l PMSF (phenylmethylsulfonyl fluoride) and two times in water, each time for 30 min at room temperature with slowly shaking. Prepared agarose blocks were sliced, and each slice was preincubated for 30 min in 500 µL of the *Xba*I restricting enzyme buffer at room temperature. After that, the buffer was removed and replaced with fresh buffer (100 µl) containing 20 units per blocks, of the *Xba*I restriction enzyme. Digestion was carried out for 3 h at 37°C. The reaction was stopped by adding a stop solution buffer (40% sucrose, 10 mmol/l EDTA, 0.01% bromophenol blue, pH 8.0) and samples were kept at 4°C before use.

PFGE was performed with a 2015 Pulsafor unit (LKB Instruments, Broma, Sweden) equipped with a hexagonal electrode array. Electrophoresis. Agarose gels (1.2% *m/v)* were run in 0.5x TBE running buffer (45 mmol/l Tris, 45 mmol/l boric acid, 1 mmol/l EDTA, pH 8.3) for 18 h at 300 V at 9°C. Pulse times were increased by step from 8s first 8 h to 18s additional 10 h, during electrophoresis. The gels were stained with 30µl ethidium bromide for 30 min with shaking at room temperature, then washed with 0.5x TBE buffer for 30 min with shaking at room temperature, and photographed under UV illumination. Lambda phage concatemers (Biolabs, England) were used as molecular size markers.

5.3. Multilocus sequencing typing (MLST) analysis

MLST was performed as was described in Kaiser *et al* (2009) and the primers and protocols were downloaded from the website of the *S. maltophilia* MLST database (http://pubmlst.ors/smaltophilia/). MLST was performed by PCR amplification and sequencing of seven housekeeping genes: *atpD* (H(+)-transporting two-sector ATPase), *gapA* (NAD-dependent glyceraldehyde-3-phosphate dehydrogenase), *guaA* (GMP synthase), *mutM* (DNA-

formamidopyrimidine glycosylase), *nuoD* (NADH dehydrogenase), ppsA (pyruvate, water dikinase), recA (RecA protein). Sequences of the primers are given in the Table 2. The PCR amplification was performed as described previously with addition of 1µl of Dimethyl sulfoxide (DMSO). PCR reactions were performed using GeneAmp 2700 PCR Cycler "(Applied Biosystems, USA) according to the programmes given in the Table 4. Allele profiles obtained after sequencing were used to determine specific sequence type (ST) for analyzed isolates using MLST Database at the University of Freiburg, Germany.

Table 4 - PCR reaction for MLST analysis.

Reaction phase	Temperature	Time	Number of cycle
Initiation denaturation	95°C	9 min	
Denaturation	94°C	20 s	30 cycles
Hybridization (*annealing*)	Recommended temperature	1 min	
Elongation	72°C	50 s	
Final elongation	72°C	5 min	
Exception for amplification of *mutM* gene			
Initial denaturation	95°C	9 min	
Denaturation	94°C	40 s	35 cycles
Hybridization (*annealing*)	58°C	40 s	
Elongation	72°C	1 min	
Final elongation	72°C	7 min	

6. Antimicrobial susceptibility testing

6.1. Disc diffusion methode

The sensitivity of the isolates to the following antibiotics was done using the disc diffusion method: tetracycline (30 µg), chloramphenicol (30 mg), livofloxacin (5 µg) and ciprofloxacin (5 µg). Commercial antibiotic discs were

used (Bio Rad, Marnes-la-Coquette, France) and interpretation of obtained results for inhibition zone according to CLSI, 2015. When specific critere for *S. maltophilia* were not present, relevant criteria for *Pseudomonas aeruginosa* or non-*Enterobacteriaceae* were used.

Table 5 – Determination of the results obtained with disc diffusion method.

Antibiotic disc	MIC (µg/ml)		
	S	I	R
Tetracycline 30µg/ml	≥15	12-14	≤11
Chloramphenicol 30µg/ml	≥18	13-17	≤12
Ciprofloxacin 5µg/ml	≥21	16-20	≤15
Levofloxacin 5µg/ml	≥17	14-16	≤13

For this purpose all tests were performed in Mueller-Hinton broth (Oxoid, UK) and suspension of eash isolate was adjusted to the density of 0.5 McFarland (Biosan, Latvia). Cell density was measured at OD_{600} in a microtitre plate reader (Tecan, Austria GmbH) after 24 h of incubation at 37°C. Prepared bacterial suspension was swabbed carfully on a thin layer of Muller Hinton agar to cover entire agar sarface in Petrie dishes. Antibiotic discs were placed on the surface of the agar, then the dishes were incubated overnight at 37°C. The diameter of the inhibition zone formed around antibiotic disc was measured.

6.2. Microdilution method

Antimicrobial susceptibility testing by using microdilution method was performed for trimethoprim–sulfamethoxazole (TMP/SMX) in order to determine Minimal inhibitory concentration (MIC). For this purpose all test were performed in Mueller-Hinton broth (Oxoid, UK) and TMP/SMX concentrations were 2, 4, 8, 16, 32 and 64 µl/ml, according to the criteria of the Clinical and Laboratory Standards Institute – CLSI (CLSI, 2015) guidelines.

When specific critere for *S. maltophilia* were not present, relevant criteria for *Pseudomonas aeruginosa* or non-*Enterobacteriaceae* were used. First step in this test was preparation of the strains: A single colony of bacteria grown on LB agar were added to 5ml of MH broth, and the suspension of each isolate was adjusted to the density of 0.5 McFarland (Biosan, Latvia). Second step was preparation of antibiotic stock solution trimthoprim was mixed with sulfamethoxazole ratio 1:19 respectivly. Different antibiotic concentration were mixed with bacterial cells suspension and added to the wells, the control wells were one for the antibiotic, one for strains suspension, and one for MH media. Cell density was measured at OD_{600} in a microtitre plate reader (Tecan, Austria GmbH) after 24 h of incubation at 37°C. All experiments were performed in triplicate. Microsoft Excel software was used for the calculation of the MIC_{50} and MIC_{90} (the concentration of antimicrobial agents which inhibited the growth rate by 50% and 90%, respectively) values. Obtained values for MIC_{50} and MIC_{90} are the results of three independent experiments.

7. Surface characteristics and motillity assay

Surface characteristics were determined as described previously (Begovic *et al.,* 2010). The cells from overnight culture in LB media were collected by centrifuge 1ml of overnight culture for 5min 13000 rpm, discard the supernatant and the cells were washed in 1ml of potassium phosphate buffer (0.1M, PH 7) centrifuged 5min, 13000 rpm, discard the supernatant and resuspended the cells with 500µl of the same 0.1M potassium phosphate buffer, vortex. on a glass tube we put 4ml of potassium phosphate buffer and the previously prepared suspension of bacterial cell with the buffer were add to this glass tube slowly, vortx and measure the OD_{600} of the cells on spectrophotometer (Ultrospec 3300 Pro, Amersham Biosciences) to get OD=1, then the organic solution Hexadecane 150µl was added to 3ml of prepared bacterial suspension, the mixture was

vortexed twice for 30s with 30s intermissions between vortexing, then 1ml of the lower phase was thaken and transferred to cuvitt after vortexing, the OD_{600} of the lower phase and the bacterial suspension before mixing with hexadecane were measured. Microbial adhesion to hexadecane (MATH) was analyzed for all strains. The optical density of the initial OD0 (OD of cell and buffer mixture) and extracted solution OD1 (OD of cells suspension and hexadecane) was measured at OD_{600} (Ultrospec 3300 Pro, Amersham Biosciences). According to this equation: θ = OD0 – OD1/OD0 the fraction of bacteria adhering to hexadecane/water interface was calculated. To determine strain hydrophobicity previously defined values were used as reference values: 0-35% low hydrophobicity, 36-70% medium hydrophobicity and 71-100% high hydrophobicity. Microbial adhesion to hexadecane (MATH) was analyzed for all 88 strains.

Motillity assay for swimming, swarming and twitching characteristics were performed as described previously (Pompilio *et al.,* 2011). A single colony from an overnight agar growth was inoculated onto swimming and swarming agar while for twitching agar, a single colony was inoculated at the bottom of a Petri dish containing twitching agar. After incubation at 37°C for 24 h, results were expressed as the diameter (mm) of the area observed at the agar surface.

8. Mucin binding assay

Ability of *S. maltophilia* strains to bind to mucin was tested as it was previously described (Muñoz-Provencio et al., 2009) with some modification. Briefly, flat-bottomed polystyrene 96-well plate (Sarsted, Newton, USA) were covered with the mucin (porcine stomach, Sigma, Germany), 30 mg/ml of mucin in 50 mM of carbonate buffer pH 9.6, while control plates were filled with the same volume of 50 mM carbonate buffer (200 µl). Plates were incubated overnight at 4°C. After immobilization, plates were

washed three times with 1X PBS and blocked at room temperature for 1 h with PBS plus 1% Tween 20 to saturate the uncoated binding places. After washing the plates again three times with 200µl of 1X PBS, 200 µl (20µl of overnight culture plus 180µl of 1X PBS) of bacterial suspension adjusted to the density of 0.5 McFarland were added and plates were incubated 2h at 37°C. Non-adhered cells were removed by washing three times with 1X PBS containing 0.05% Tween 20 and the plates were dried at 65°C. Adhered cells were stained with 0.1 mg/ml of crystal violet (200µl/well) incubated for 45 min at room temperature. Dye was discarded, plates were dried on 65^{o} C and the unbound stain were removed by washing three times with1X PBS. For dissolving the stain bounded to the bacterial cell 50 mM of citrate buffer pH 4.0 (200 µl/well) were added and incubated for 1h at room temperature and the absorbance was measured at 595 nm.

9. Biofilm formation assay

Biofilm formation assay was performed as described previously (Stepanović *et al.,* 2007). Over night cultures of *S. maltophilia* isolates in 3 ml of Trypticase soy broth (TSB) (oxoid) were washed and diluted in fresh TSB and standardized to contain 1 x 10^5 to 1 x 10^6 CFU/ml. Suspension of each isolate was adjusted to the density of 0.5 McFarland (Biosan, Latvia). The cultures were then diluted 1 : 100 in 200 µl tryptic soy broth (TSB) and then 200 µl of each strains were inoculated into the wells of a flat-bottomed polystyrene 96-well plate (Sarsted, Newton, USA). As positive control we used *Pseudomonas aeruginosa* PAO1, while negative control was Escherichia coli DH5α, and TSB without bacteria was the control of all test. Incubation was performed at 37°C for 24h, then plates were washed three times with 200µl of 1X sterile phosphate buffered saline PBS (pH 7.2). Adherent biofilms were fixed for 30 min at 65°C, the plate were stained for 30 min at room temperature with 160 µl of 0.01%

crystal violet (HI Media). Dye was discarded and the plates were dried at 65ºC and then washed three times with 1X PBS and dried again at 65°C. Biofilm samples were destained with 200 µl of solution containing 96% ethanol and aceton in ration 4:1 for 15 min and the optical density OD was read at 595 nm. The low cut-off (ODc) was calculated as the three standard deviations 3 x SD above the mean OD of control wells. Classification of strains were performed acording to the following criteria: no biofilm producer (OD ≤ ODc), weak biofilm producer (ODc < OD ≤ 2 x ODc), moderate biofilm producer (2 x ODc < OD ≤ 4 x ODc) and strong biofilm producer (4 x ODc < OD).

9.1. PCR based genotyping for *rmlA*, *spgM*, and *rpfF* genes

PCR with specific primers for *rmlA, spgM, rpfF* were performed as described previously by (Pompilio *et al.,* 2011). The PCR amplification was performed as described previously (section 5. Molecular identification of clinical isolates). Sequences of the primers are given in the Table 2. PCR reactions were performed using GeneAmp 2700 PCR Cycler "(Applied Biosystems, USA) according to the programme given in the Table 6.

Table 6 - PCR reaction for *rmlA, spgM, rpfF* genes amplification.

Reaction phase	Temperature	Time	Number of cycle
Initiation denaturation	94°C	5 min	
Denaturation	94°C	30 s	30 cycles
Hybridization (*annealing*)	60°C	30 s	
Elongation	72°C	30 s	
Final elongation	72°C	10 min	

PCR products were sequenced by the Macrogen DNA sequencing service (Macrogen Inc., Netherlands). Obtained sequences were aligned in the NCBI database by using BLAST program.

9.2. Kinetics of the biofilm formation

Kinetics of the biofilm formation was performed for seven strong biofilm producer strains. Overnight cultures of *S. maltophilia* in Trypticase soy broth (TSB) (Oxoid) were washed, diluted with fresh TSB, and standardized to contain 5 x 10^5 to 1 x 10^6 CFU/ml. Aliquots (200 µl) of standardized inoculum were added to the wells of sterile flat-bottom polystyrene 96 wells plates (Sarsted, Newton, USA), and incubated at 37°C. After incubation for 30 min, 1, 2, 4, 8, and 24 h plates were treated like it was described under subtitle Biofilm formation assay and biofilm formation was determined.

10. Infeluence of different factors on biofilm formation by *S. maltophilia.*

Influence of different factors on biofilm formation by *S. maltophilia* strais which, formed strong, moderate and week biofilm was determined. Biofilm formation assay were done as descibed above. Briefly, overnight cultures of *S. maltophilia*, prepared in TSB broth (Oxoid) were washed twice, and diluted with fresh TSB , and standardized to contain (1x 10^5 or 10^6 CFU/mL). Aliquots (200 µL) of standardized inoculum were added to the wells of sterile flat-bottom polystyrene 96 wells plates, and incubation was performed in different conditions: pH 5.5 and 8.5, temperature (cold shock 12°C, 30°C, 37°C and heat chock 45°C), 10% saturation with CO_2 (Heracell 150, Thermo Fischer Scientific Inc., Walthman, MA, USA), dynamic conditions (with agitation). The biofilm formation was evaluated as described above in Biofilm formation assay paragraph. All experiments were performed in three independent repetitions.

10.1. Effect of TMP/SMX on *S. maltophilia* formed biofilm

Biofilm formation by *S. maltophilia* was preformed in flat-bottomed polystyrene 96-well plates (Sarsted, Newton, USA) as described above (biofilm formation assay). After 24h incubation at 37°C, the supernatant from each well was gently aspirated by micropipette, each well was then washed three times with 200µl of 1X PBS, and 200µl of antimicrobial agents at two different concentrations (25 and 50µg/ml) was added to the wells. Controls were the formed biofilm by the strains without adding the antimicrobial agent. The plates were incubated at 37°C for 6h, after incubation the supernatant was discared and plates were washed three times with 200µl of 1X PBS, dried on 65°C for 30min, and dyed with 0.01% crystal violet for 30 min at room temperture. Biofilm formation assay was performed as described previously.

11. Statistical analysis

Statistical analyses was performed using the SPSS 20.0 statistical software package (IBM Corporation). Jaccard coefficient was used for calculation of similarity matrix to determine similarity coefficients. Two-tailed Mann-Whithey test was used to describe differences between the groups. Calculation of Spearmann's rho coefficient was used for correlations analysis.

In addition, some statistical analysis was performed with the Phoretix 1D Pro (TotalLab, free trial license) program with 1% tolerance, while for dendrogram creation Band difference and Complete Linkage algorithm (Defays, 1977) were used. Heatmaps and cluster analysis was performed using R packages gtools, hclust and gplots. To determine the statistical differences between the groups t test was used.

12. Ethics Statement

The authors assert that all procedures contributing to this work comply with the ethical standards of the Ethics Committee of The Institute for Mother and Child Health Care of Serbia "Dr Vukan Čupić" on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. Since the analysis was performed retrospectively on isolates collected through routine clinical work and patient identifiable information was anonymized, no written or verbal informed consent to participate in this study from patient was necessary. The authors had no contact or interaction with the patients. Patient demographics anonymization was performed in two steps. First, personal data was coded by the head of the clinical microbiology laboratory at the Institute for Mother and Child Health Care "Dr Vukan Čupić" where the isolates were obtained from, and secondly by assigning a different code by the principal investigator at the Institute of Molecular Genetics and Genetic Engineering where the molecular analysis was conducted. Ethics Committee of The Institute for Mother and Child Health Care of Serbia "Dr Vukan Čupić" specifically approved this study, approval No. 8/6a.

RESULTS

4. RESULTS

1. Clinical population and *Strenotrophomonas maltophilia* isolation

Sixty-eight patients from tertiary care pediatric hospital Institute for Mother and Child Health Care "Dr Vukan Čupić„ were included in the study, 32 males and 36 females. The median age of the patients was 0.7 years (range 3 days to 34 years). There were 27 CF patients that were treated as outpatients and inpatients. Out of 41 non-CF patients, 27 (65.9%) were hospitalized in three intensive care units (pediatric medical, surgical and cardiothoracic), while the remaining 14 were treated on different specialized clinical wards. A total of 88 clinical isolates from 68 patients were examined (Table 1). The 42 isolates from 27 CF patients were cultured from sputum samples (n=16), cough swabs (n=24) and bronchial washing fluid (n=2). The 46 isolates from non-CF patients were cultured from a number of sites, including blood (n=2), bronchial washing fluid (n=8), endotracheal aspirate (n=24), sputum (n=3), urine (n=1), abdominal cavity drainage fluid (n=1), cough swab (n=2), breast milk (n=1), nose/throat secretions (n=3) and wound (n=1). Single isolates were archived from each of 52 patients (16 CF and 36 non-CF) while from 16 additional patients (11 CF and 5 non-CF) more than one isolate were collected per patient.

2. Identification of *S. maltophilia* clinical isolates

Initial laboratory identification of the isolates was carried out using standard biochemical testing and automated Vitek 2 system (BioMérieux, Marcy l'Etoile, France). Molecular identification of the strain was performed by sequencing of PCR products of amplified 16S rRNA gene which, confirmed that all analyzed clinical isolates belong to *S. maltophilia* species with identity ranging from 95% to 99% with *S. maltophilia* strains from the NCBI database.

3. Genotyping of *S. maltophilia* clinical isolates

To determine genetic relatedness among the analyzed isolates PFGE analysis was performed. Obtained PFGE profiles are presented at the Figure 1. Immense diversity among PFGE profiles was observed and 11 different pulsotype was observed.

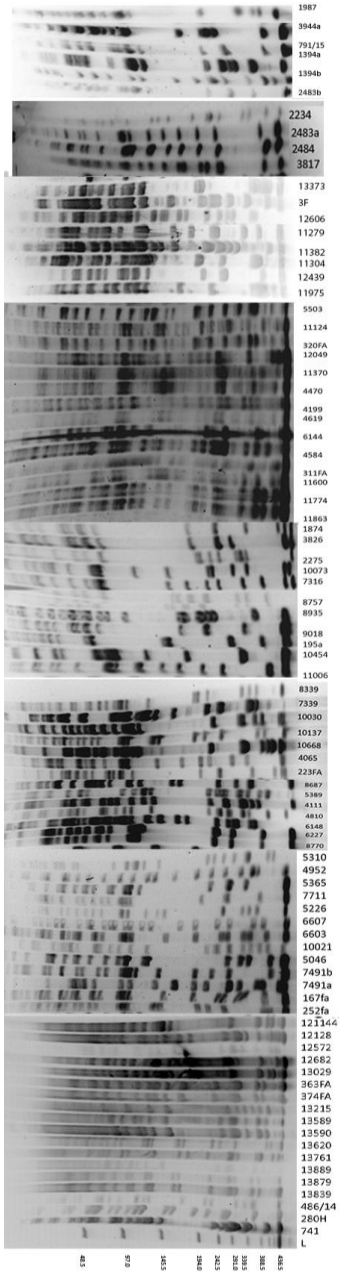

Figure 1. PFGE profiles for *S. maltophilia* clinical strains. L – λ concatemeres standard. Names of the strains are presented with numbers on the gels.

PFGE revealed that most of the strains do not show significant genetic relatedness among themselves. Based on the diversity of genetic profiles obtained, dendrogram that reconstruct phylogenetic relationships within collection was constructed using Complete linkage and is shown in Figure 2.

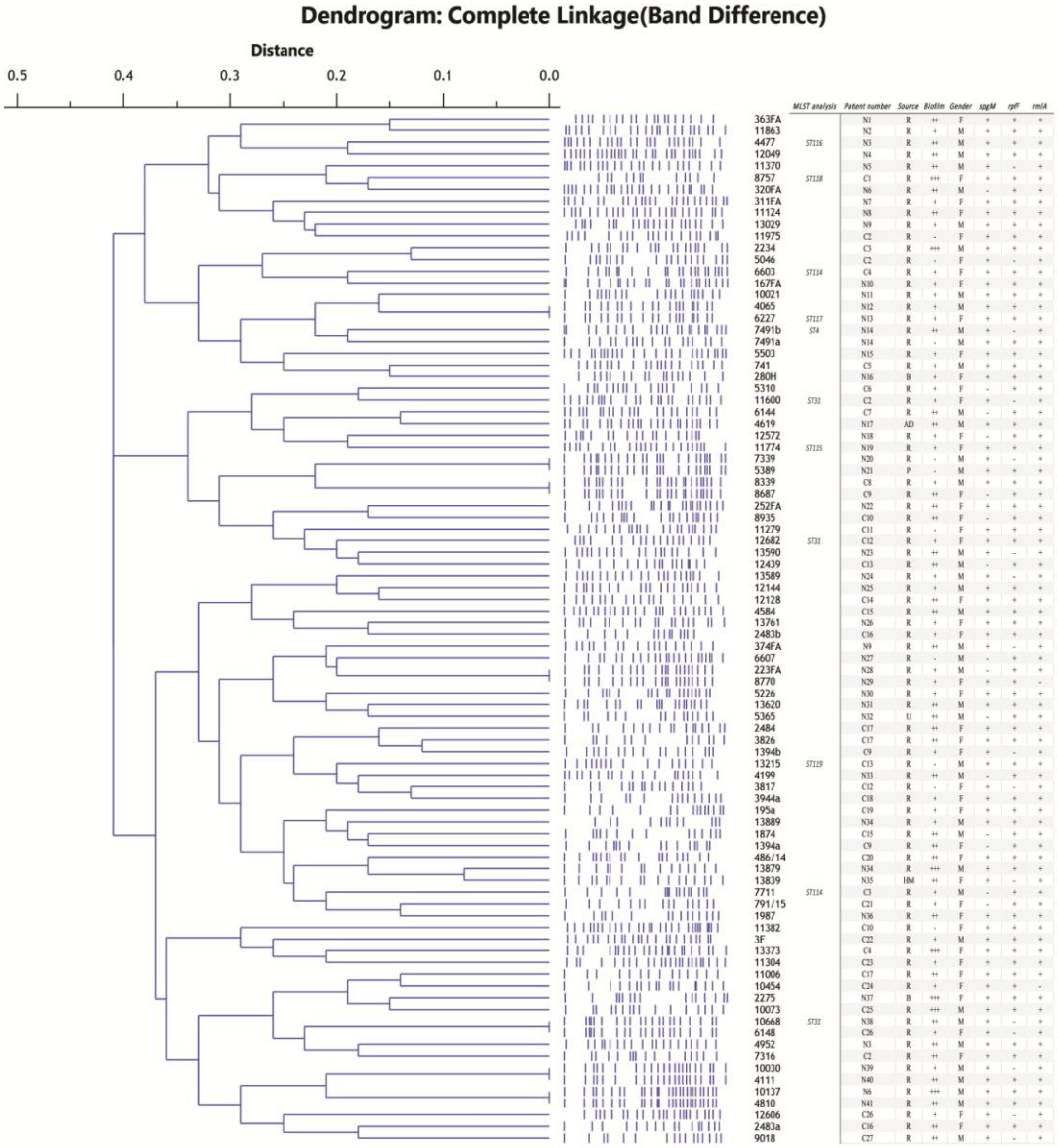

Figure 2. Phylogenetic analysis of obtained PFGE *Xba*I profiles. Genetic relatedness between the analyzed strains are shown above the dendrogram with the distance. Names of the strains are indicated with the numbers on the right side. Results of MLST analysis, CF origin – C, non-CF origin – N, source of isolation, biofilm formation, gender of the patients, *rmlA, spgM* and *rpfF* gene amplification for each strain are presented in the table on the right side.

Based on the PFGE results 11 pulsotypes were determined and selected for MLST analysis which, showed that six sequence types (ST) were novel (Table 7). Three isolates (11600, 10668 and 12682), belong to ST31, a sequence type isolated in Perth, Australia. One isolate, 7491b, belongs to group ST4, in which there are two other isolates from Europe, while other are novel. These previously identified STs are also clinical isolates of human origin. Sequences are deposited at the MLST Database at the University of Freiburg, Germany, http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_smaltophilia isolates&page=query.

Table 7. Sequence type (ST) of 11 *S. maltophilia* clinical isolates.

Strain	*atpD*	*gapA*	*guaA*	*mutM*	*nuoD*	*ppsA*	*recA*	ST
11600	allele 3	allele 4	allele 24	allele 7	allele 7	allele 22	allele 7	31
7491b	allele 1	allele 4	allele 7	allele 7	allele 28	allele 19	allele 6	4
10668	allele 3	allele 4	allele 24	allele 7	allele 7	allele 22	allele 7	31
12682	allele 3	allele 4	allele 24	allele 7	allele 7	allele 22	allele 7	31
6603	allele 3	allele 4	allele 18	allele 1	allele 7	allele 20	allele 1	114
7711	allele 3	allele 4	allele 18	allele 1	allele 7	allele 20	allele 1	114
11774	allele 3	allele 1	allele 84	allele 57	allele 25	allele 82	allele 6	115
4477	allele 3	allele 1	allele 84	allele 58	allele 25	allele 82	allele 6	116
6227	allele 1	allele 4	allele 43	allele 3	allele 70	allele 83	allele 7	117
8757	allele 2	allele 2	allele 93	allele 59	allele 63	allele 69	allele 5	118
13215	allele 4	allele 76	allele 92	allele 5	allele 70	allele 84	allele 9	119

4. Antibiotic susceptibility of *S. maltophilia* clinical isolates

Trimethoprim–sulfamethoxazole (TMP/SMX) demonstrated excellent inhibitory effect against all of the *S. maltophilia* tested, which confirmed its potential in clinical treatment. For all tested isolates, the MIC_{50} was ≤4 μg/ml, while the MIC_{90} was ≤32 μg/ml. Furthermore, the MIC_{90} was ≤10 μg/ml for 60 of the all 88 analyzed strains (68.18%). Additionally, we performed analyses by

disk diffusion methods with a few selected antibiotics. All of the tested strains were sensitive to all tested antibiotics, (Table 8).

Table 8. Antibiotic susceptibility of the *S. maltophilia* clinical isolates (n=88) obtained with two different methods.

	Disc diffusion method			
Antimicrobial agents	Zone diameter interpretative			Percentage of susceptibility strains
	S	I	R	
Ciprofloxacin**	≥ 21	16-20	≤ 15	100%
Chloramphenicol*	≥ 18	13-17	≤ 12	100%
Tetracycline**	≥ 15	12-14	≤ 11	100%
Levofloxacin	≥ 17	14-16	≤ 13	100%
	Microdilution method			
	S	I	R	
TMP/SMX	≤ 2/38	-	≥ 4/76	100%

* - breakpoints for *E. coli* ATCC25922, ** - breakpoints for *P. aeruginosa* ATCC27853
TMP/SMX – trimethoprim/sulfomethoxazole

5. Virulence factors analysis

5.1. Motility, surface characteristics and adhesion to mucin of *S. maltophilia* clinical isolates

Different types of motility swimming, swarming and twitching characteristics were analyzed in *S. maltophilia* clinical isolates. Interestingly, swimming motility was observed in all tested strains, while none of the tested strains showed swarming or twitching motility. Zones detected for swimming motility were in the range from 0.5 to 5 cm.

On the contrary, out of 88 analyzed strains, only one strain adhered to hexadecane (44% adherence) which classified it as a strain 13590 with medium hydrophobicity of the cell surface, while all other strains had low percentages of adherence to this non-polar solvent, indicating low hydrophobicity of their cell surfaces.

Ability of clinical isolates to adhere to mucin was calculated as the ratio of absorbance at 595 nm, measured in mucin-coated wells against absorbance in control non-coated wells. There were no differences in mucin-adhesion ability between CF and non-CF isolates. Aside from comparing mucin-binding ability of CF and non-CF isolates, the ability of each individual strain to adhere to mucin was compared to its affinity to adhere to a plastic surface (mucin-coated vs. non-coated wells) (Figure 3). Mann-Whitney test revealed significantly higher adhesion of the isolates to mucin-coated compared to adhesion to non-coated wells ($p<0.05$).

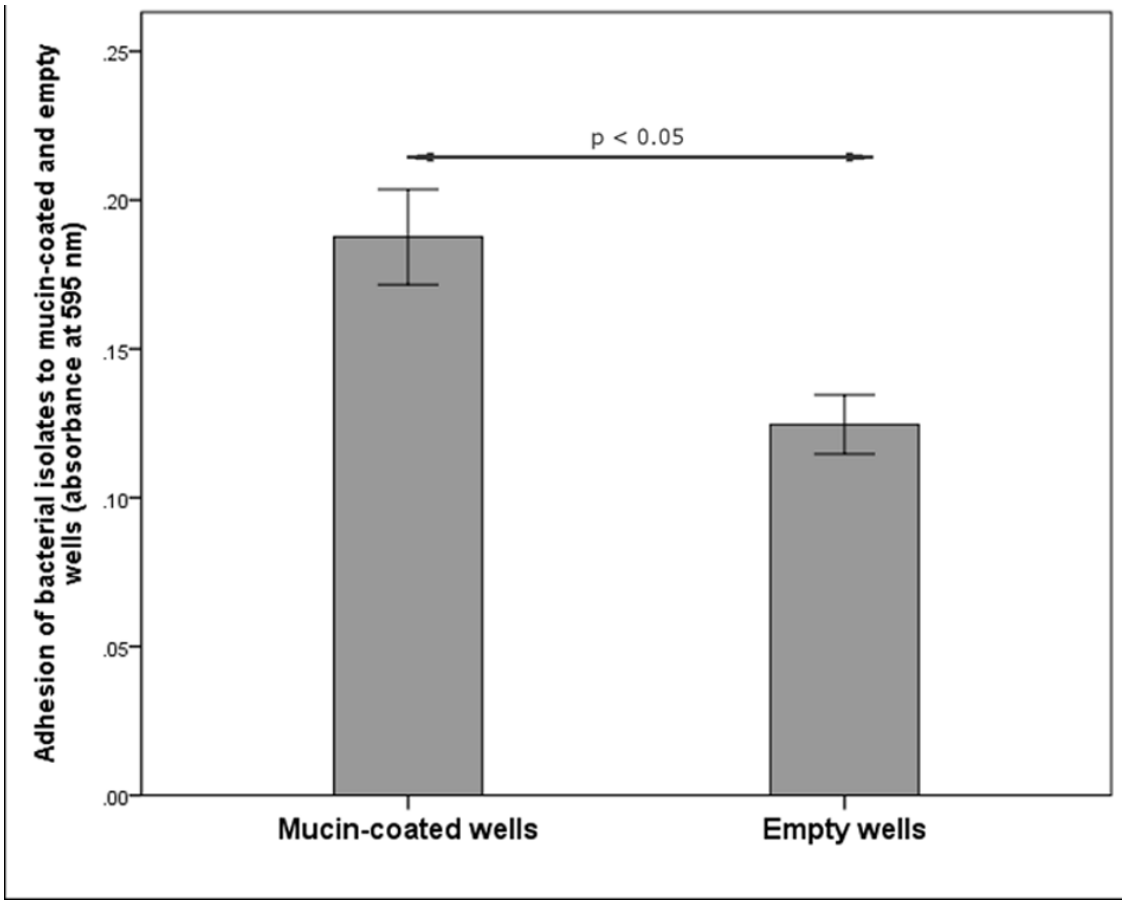

Figure 3. Adhesion of bacterial isolated to mucin-coated and non-coated wells of microtiter plate. Bars represent mean values ± standard errors.

5.2. Biofilm formation of *S. malt*ophilia clinical isolates

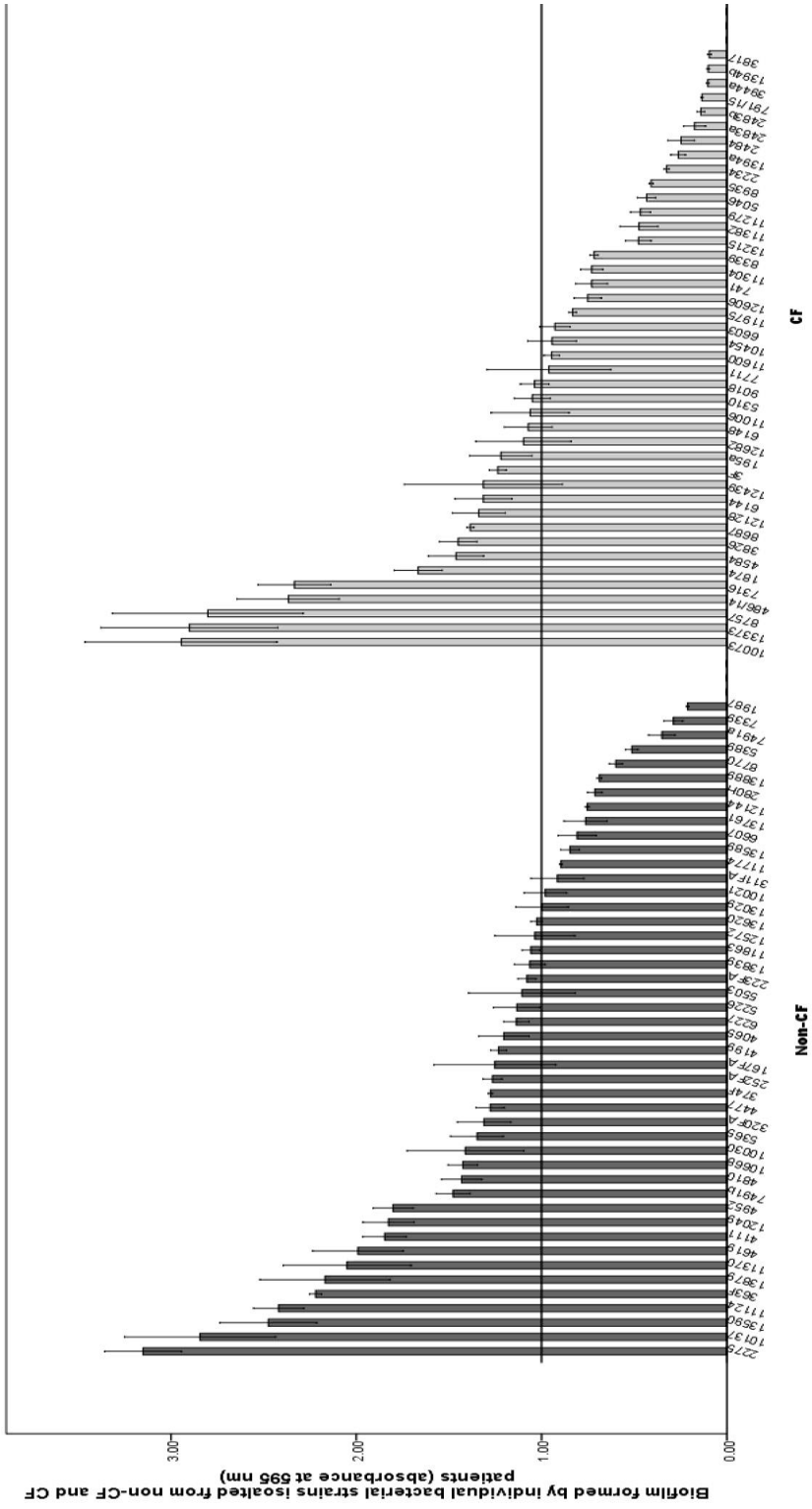

Figure 4. Biofilm formed by individual bacterial *S. maltophilia* strains isolated from CF and non-CF patients

Ability to form biofilm was detected in the most of the analyzed strains with almost equal representation in CF and non-CF strains (Figure 4). Biofilm formation assay was performed on polystyrene plates and results were strong biofilm was formed by seven strains (7.95%), only nine strains (10.2%) did not form biofilm, moderate biofilm was formed by 37 strains (42.05%), while weak biofilm was formed by 35 strains (39.8%) (Figure 5). From the seven strains forming strong biofilm four were from CF patients (9.5% vs. 6.5%), but among the 37 strains that formed moderate biofilm slight majority were from non-CF patients (45.7% vs. 38.1%).

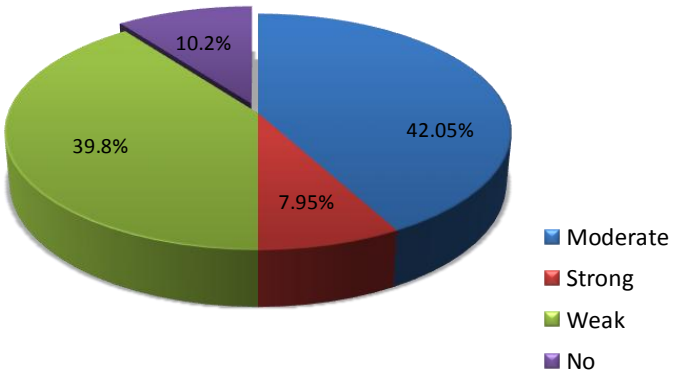

Figure 5. Percent of different biofilms formed by *S. maltophilia* strains

Source of strain isolation did not affect biofilm formation, except for strong biofilm producers, since all were respiratory isolates except one from blood culture. According to PCR – based analysis, *rmlA, rpfF* and *spgM* genes were present in 86 strains (97.7%), 62 strains (70.4%) and 63 strains (71.6%), respectively (Figure 2). There was no difference in the presence of the analyzed genes between CF and non-CF patients. Spearmann's rho coefficients (Table 9) showed that there was no statistical correlation between biofilm strength and the presence of *rmlA, rpfF* or *spgM* genes (presence of the signal was marked as 1 and absence as 0). Nevertheless, the presence of both *rpfF* and *spgM* genes in one strain was correlated with strong biofilm formation ($p<0.05$), while there

was no correlation in other gene combinations (*rmlA* + *rpfF* and *rmlA* + *spgM*). Moreover, a negative correlation was observed between *rpfF* and *spgM* presence ($p<0.05$).

Table 9. Correlations between presence of PCR signals for *spgM*, *rpfF* and *rmlA* genes and biofilm formation in bacterial isolates according to Spearmann's rho coefficients.

Correlations

			Presence of strong biofilm	Presence of spgM gene	Presence of rpfF gene	Presence of rmlA gene	Sum of presence of spgM and rpfF genes	Sum of presence of rpfF and rplA genes	Sum of presence of spgM and rplA genes
Spearman's rho	Presence of strong biofilm	Correlation Coefficient	1.000	.143	.149	.048	.233*	.160	.155
		Sig. (2-tailed)		.183	.166	.655	.029	.136	.150
		N	88	88	88	88	88	88	88
	Presence of spgM gene	Correlation Coefficient	.143	1.000	-.214*	-.069	.615**	-.230*	.926**
		Sig. (2-tailed)	.183		.046	.522	.000	.031	.000
		N	88	88	88	88	88	88	88
	Presence of rpfF gene	Correlation Coefficient	.149	-.214*	1.000	-.072	.639**	.930**	-.231*
		Sig. (2-tailed)	.166	.046		.506	.000	.000	.031
		N	88	88	88	88	88	88	88
	Presence of rmlA gene	Correlation Coefficient	.048	-.069	-.072	1.000	-.112	.301**	.312**
		Sig. (2-tailed)	.655	.522	.506		.297	.004	.003
		N	88	88	88	88	88	88	88
	Sum of presence of spgM and rpfF genes	Correlation Coefficient	.233*	.615**	.639**	-.112	1.000	.570**	.543**
		Sig. (2-tailed)	.029	.000	.000	.297		.000	.000
		N	88	88	88	88	88	88	88
	Sum of presence of rpfF and rplA genes	Correlation Coefficient	.160	-.230*	.930**	.301**	.570**	1.000	-.105
		Sig. (2-tailed)	.136	.031	.000	.004	.000		.328
		N	88	88	88	88	88	88	88
	Sum of presence of spgM and rplA genes	Correlation Coefficient	.155	.926**	-.231*	.312**	.543**	-.105	1.000
		Sig. (2-tailed)	.150	.000	.031	.003	.000	.328	
		N	88	88	88	88	88	88	88

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

However, according to Mann-Whitney test, non-CF isolates showed higher biofilm forming potential and motility than CF isolates ($p = 0.021$ and $p = 0.0001$) (Fig 6A and 6B).

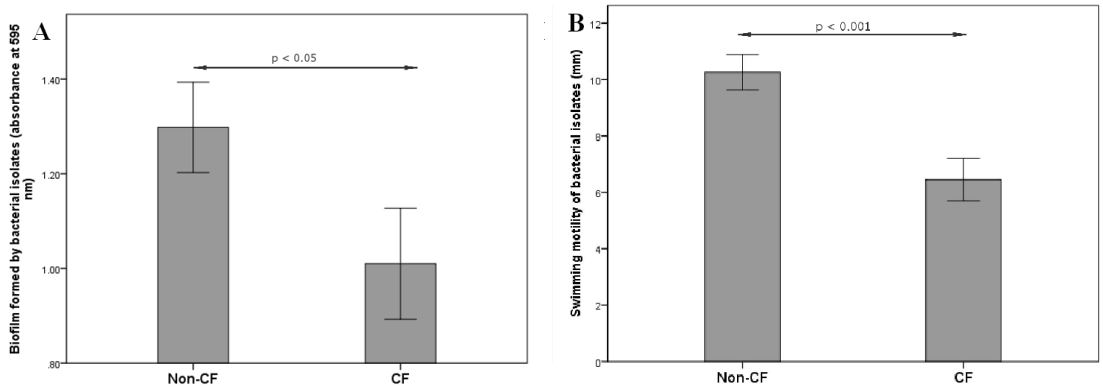

Figure 6. Biofilm forming potential (A) and motility (B) of non-CF and CF bacterial isolates. Bars represent mean values ± standard errors.

In addition, the same trend in changing motility and strength of biofilm formation was observed (Figure 7). Strains forming stronger biofilm show high motility with no statistically important differences in motility between CF and non-CF isolates forming strong biofilm (p = 0.78).

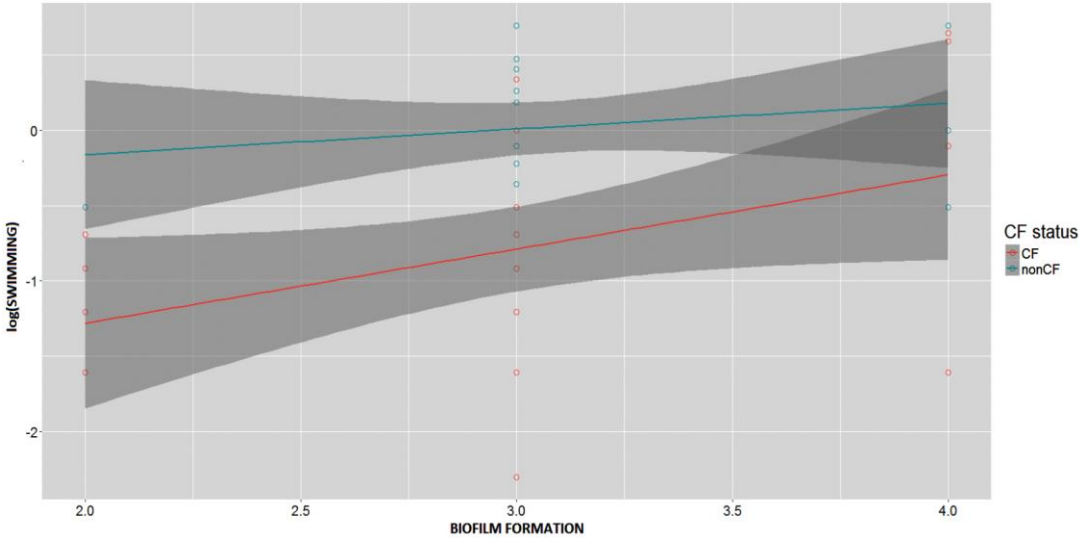

Figure 7. Correlation between the strength of formed biofilm and motility of *S. maltophilia* strains isolated from CF and non-CF patients.

Spearmann's rho coefficients were calculated to check for correlations between three tested parameters (mucin adhesion, motility and biofilm formation) and results were that motility has shown positive correlations ($p < 0.01$) with both biofilm formation and the mucin-adhesion ability of the strains (Table 10).

Table 10. Correlations between tested physiological parameters of bacterial isolates according to Spearmann's rho coefficients.

Correlations

			Biofilm formed by bacterial isolates (absorbance at 595 nm)	Motility of bacterial isolates (mm)	Mucin binding ability of bacterial isolates (adhesion in mucin-coated wells relative to adhesion in control wells)	Adhesion of bacterial isolates to mucin-coated wells	Adhesion of bacterial isolates to empty wells
Spearman's rho	Biofilm formed by bacterial isolates (absorbance at 595 nm)	Correlation Coefficient	1.000	.398**	.170	.033	.013
		Sig. (2-tailed)		.000	.113	.762	.908
		N	88	88	88	88	88
	Motility of bacterial isolates (mm)	Correlation Coefficient	.398**	1.000	.307**	.270*	.244*
		Sig. (2-tailed)	.000		.004	.011	.022
		N	88	88	88	88	88
	Mucin binding ability of bacterial isolates (adhesion in mucin-coated wells relative to adhesion in control wells)	Correlation Coefficient	.170	.307**	1.000	.669**	.461**
		Sig. (2-tailed)	.113	.004		.000	.000
		N	88	88	175	88	88
	Adhesion of bacterial isolates to mucin-coated wells	Correlation Coefficient	.033	.270*	.669**	1.000	.944**
		Sig. (2-tailed)	.762	.011	.000		.000
		N	88	88	88	88	88
	Adhesion of bacterial isolates to empty wells	Correlation Coefficient	.013	.244*	.461**	.944**	1.000
		Sig. (2-tailed)	.908	.022	.000	.000	
		N	88	88	88	88	88

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

5.2.1. Kinetics of strong biofilm formation

Kinetics of biofilm formation was determined for selected strains (Figure 8) which showed that non-CF isolates formed biofilm faster than CF isolates.

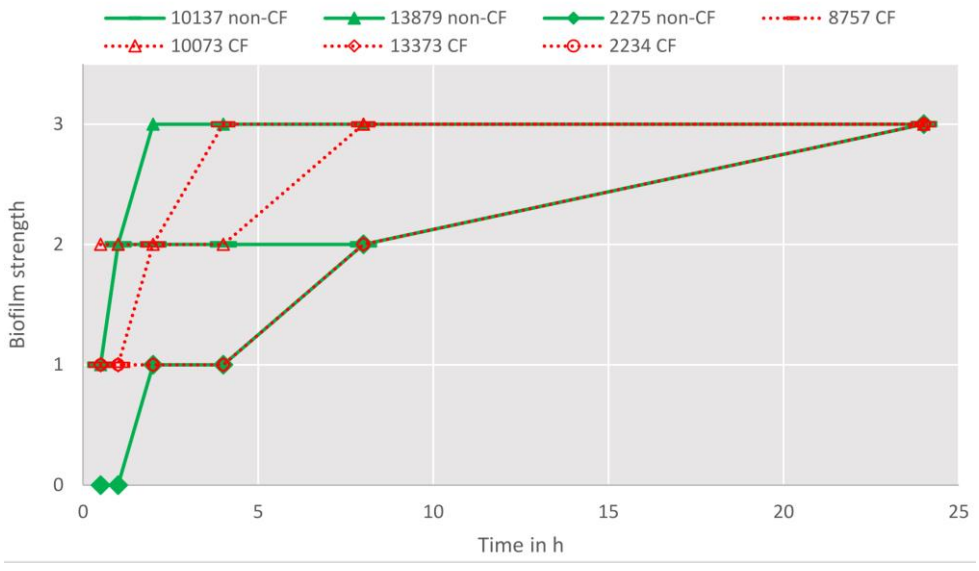

Figure 8. Kinetics of the biofilm formation by selected *S. maltophilia* strains isolated from CF and non-CF patients. Biofilm strength was designated from 0 to 3 where 3 is strong, 2 is moderate, 1 is weak, and 0 is no biofilm.

Although, they were all strong biofilm producer biofilm formation dynamic was significantly different between them correlating with the CF vs. non-CF phenotype. In addition, shaking conditions did not affect biofilm formation both in CF and non-CF isolates strong biofilm producers (data not shown).

6. Influence of different factors on biofilm formation of *S. maltophilia* clinical isolates

Biofilm-associated infections substantially affect human health, increasing antibiotic resistance of bacteria and making more challenging to combat with such infections (Balcázar et al., 2015). However, biofilm formation is influenced by different factors (Pompilio et al., 2008; Di Bonaventura et al., 2007). According to the obtained results among 88 *S. maltophilia* clinical isolates strong biofilm producer represented 7.95% and only nine strains (10.2%) did not form biofilm. All strong and moderate biofilm producers as well as five selected weak biofilm producers were subject of further analysis regarding influence of different factors on biofilm production. Obtained results were present as a heatmap (Figure 9).

In order to access the differences among the isolates abilities to form the biofilm, hierarchical clustering was performed. All isolates are divided in four differentiated clusters in agreement with hierarchical clustering analysis, though isolate clusters slightly overlapped. Clusters represent groups of isolates for which similar results in testing different factors on biofilm formation are obtained. Interestingly, both groups are present in the CF (black) and non-CF (gray) isolates suggesting that origin of strain did not influence the obtained results. Decrease or increase of temperature (12°C and 45°C) and changing pH of media on 8.5 were the factors which had the highest effect on biofilm formation. In addition, importance of the optimal temperature for biofilm

formation was shown for the weak biofilm producer also (2483b, 791/15 and 280H), which formed moderate biofilm on 30°C (Figure 9). Difference was demonstrated between CF and non-CF isolates where it was shown that CF isolates were more sensitive on changing of temperature, pH and CO_2 concentration.

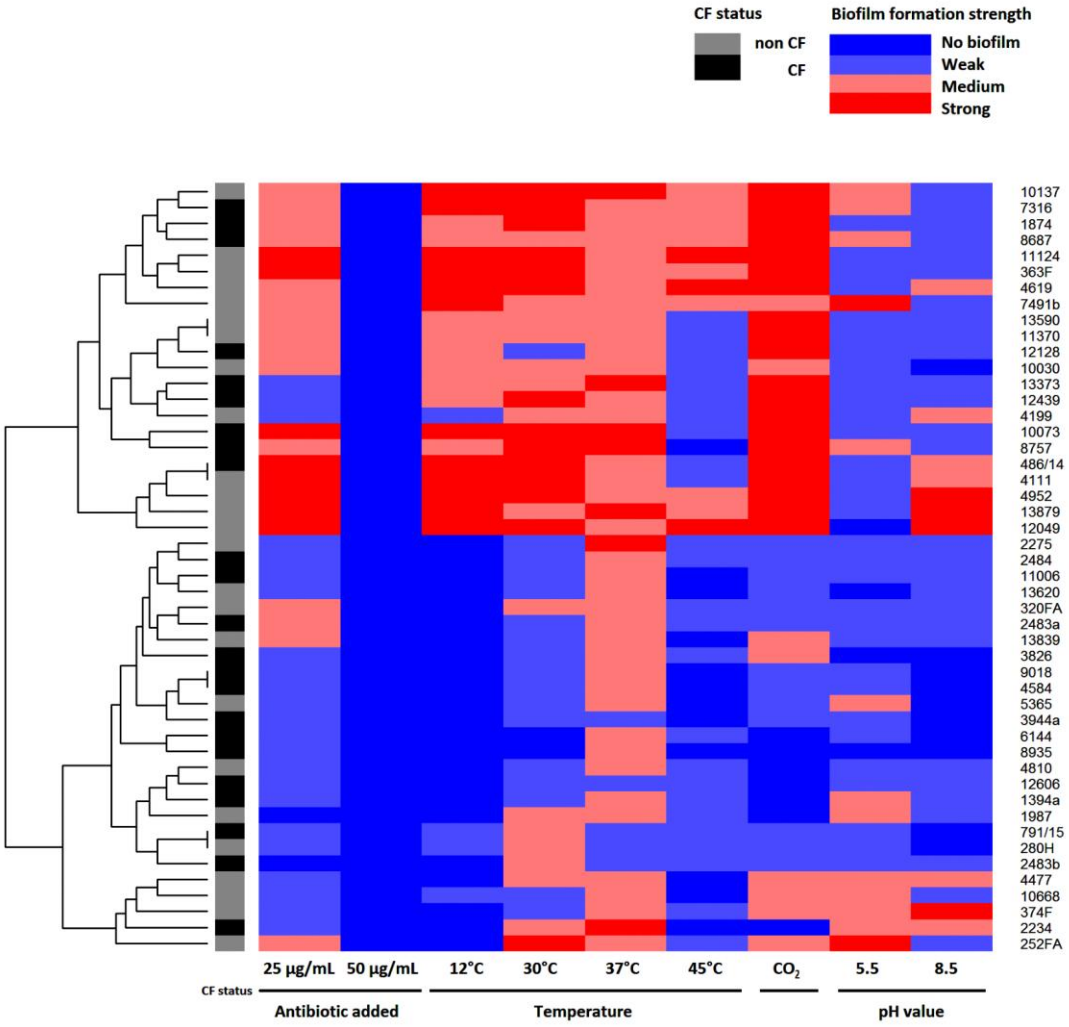

Figure 9. Heatmap for the biofilm formation under different growth conditions of *S. maltophilia* clinical isolates. Conditions were grouped by antibiotic addition (first two), various temperature growth conditions, increased CO_2 and various environmental pH values.

6.1. Effect of trimethoprim-sulfamethoxazole on *S. maltophilia* formed biofilm

Biofilm as a specific community of bacteria enable them among other advantages higher resistance on different antimicrobial agents. In that context, the effects of TMP/SMX in two concentrations (25 μg/ml and 50 μg/ml) on *S. maltophilia* formed biofilms was investigated. Both TMP/SMX concentrations were found to significantly contribute to the eradication of 24 h old biofilms (Figure 9), while 50 μg/ml of TMP/SMX completely eradicated formed biofilm in all tested strains, 25 μg/ml of TMP/SMX affects biofilm in a strain-dependent manner, from complete eradication to no, effect. Interestingly, strains more sensitive to other tested factors showed the higher sensitivity on 25 μg/ml of TMP/SMX.

DISCUSSION

5. DISCUSSION

Stenotrophomonas maltophilia is an environmental, multidrug resistant global opportunistic pathogen. First reports about this bacterium were associated with plant growth promoting (PGP) potential. It was isolated from plant rhizosphere, which was suggested to be a source of different bacteria possessing antibiotic resistance genes (Berg et al., 2005). *S. maltophilia* can acquire and transfer the antibiotic resistance genes to other bacteria species through horizontal gene transfer (HGT) (Berg et al, 2017). In addition, this bacterium can acquire the genes from Gram-positive bacteria (Alonso et al., 2000). Ability of *S. maltophilia* to adapt to the local environment, to interact with different bacteria species to receive genetic material from and to transfer genetic material to other bacteria makes this opportunistic pathogen one of the major challenges in the clinical/medical settings.

The prevalence of *S. maltophilia* has increased in hospitals worldwide simultaneously with the appearance of a numerous other antibiotic resistant bacteria (Brooke JS, 2012; 2014). Serbia was, for decades, among the countries where the misuse of antibiotics was high (http://www.who.int/drugresistance/documents/situationanalysis/en/) due to poor and unenforced regulation. The years of lax restriction and enforcement could and probably did lead to a higher incidence of antibiotic resistant strains. For example, in the recent past Serbia, among other Balkan countries, was pointed out as a potential endemic region and the second common putative country of origin of isolates carrying the New Delhi Metallo-beta-lactamase-1(NDM-1) gene, $bla_{\text{NDM-1}}$ (Berrazeg et al, 2014; Novovic et al, 2016). Although research of prevalence of *S. maltophilia* in the hospital setting is in the focus of scientific research in the last decade, there is no published data about the incidence of *S. maltophilia* from Serbian hospitals. The aim of this study was genotyping, antibiotic resistance and determination of virulence factors in the collection of 88 *S. maltophilia*

clinical isolates. In addition, phenotypic characterization of strains isolated from patient with cystic fibrosis (CF) and from patients with other diseases (non-CF) in order to determine possible existence of CF phenotype.

For genotyping of *S. maltophilia* PFGE has proven to be a powerful and discriminatory method (Berg et al., 1999) and the same was observed in this study. Genomic variability among the 88 analyzed strains was high and indicated that there was no cross-transmission among patients. Similar results were obtained in previous studies (Valdezate et al., 2004) with rare exception (Garciá del Videama et al., 1999). This is not a surprise, since *S. maltophilia* is an environmental bacterium. High genomic diversity of the isolates leads to the conclusion that patients were the route of introduction of the analyzed *S. maltophilia* to the hospital setting. This suggestion is also supported by the fact that 16 patients from our research had more than one isolate and they were all genetically different. Additionally, to the PFGE analysis for identification and genotypization of *S. maltophilia* strains Multilocus sequencing typing (MLST) was performed in order to put our results in broader pictures. The results of MLST analysis of 11 chosen isolates determined two previously described STs, one from Europe and the other from Australia, and six novel STs described for the first time in this study (ST114, ST115, ST116, ST117, ST118 and ST119). Interestingly, the most dominant STs in Europe (Kaiser et al., 2009) were not present among patients from Serbia. The determination of new STs is keeping with the high plasticity and capacity of bacterial organisms to adapt to specific niches and develop new characteristics. On the other hand, selective pressure on these bacteria in the hospital environment could be a reason for the selection of certain STs, which have an adaptive advantage in this environment, and this could lead to their clonal spread. Accordingly, these results indicate good situation in Serbian hospitals since we were not able to detect the most clinically prevalent European clinical isolates (Kaiser et al., 2009) and we determined the lack of clonal spread.

Due to the multiple applications of antibiotics, presence of other multidrug-resistant strains, such as *P. aeruginosa*, *Acinetobacter* spp. and *Burkholderia cepacia,* in the same ecological niche (hospital settings and CF patients) with *S. maltophilia* is usual. *S. maltophilia* is intrinsically resistant to many antibiotics and easily acquires new resistance phenotypes as well as spread it to other bacteria. An increased number of reports from different hospitals about *S. maltophilia* resistant to timethoprim/sulfamethoxazole (TMP/SMX) (Toleman et al., 2007; Hu et al., 2016) rose serious concerns, especially since TMP/SMX was considered as the main antibiotic for the treatment of *S. maltophilia*. What's more, for *S. maltophilia* EUCAST set a breakpoint for TMP/SMX, even while the Clinical and Laboratory Standards Institute (CLSI) approved standards for levofloxacin, minocycline, ticarciline/calvulanate and ceftazidime. On the other hand, results obtained with TMP/SMX are the most reproducible, with no relation to the methods in susceptibility testing used (Masgala et al., 2010). Our results suggest that all of the *S. maltophilia* analyzed had high susceptibility not only to TMP/SMX but also to chloramphenicol, ciprofloxacin, levofloxacin and tetracycline. Then again, there is an increasing number of scientific papers on the high rate of *S. maltophilia* resistant to TMP/SMX that point out the need for finding new and more effective antibiotics. So far tigecycline and levofloxacin, alone or in combination, have shown promising efficancy in the treatment of *S. maltophilia* infections (Farell et al., 2010; Wang et al., 2014).

The results obtained in our study are even more important since the Institute for Mother and Child Health Care "Dr Vukan Čupić" is a paediatric tertiary care referral hospital and is the national and regional reference CF specialist center for pediatric and adult patients with CF from Serbia, Montenegro and Bosnia and Hercegovina. One of the main problems in the treatment of CF patients is the emergence of multidrug-resistant strains, so it is necessary to establish a pattern of sensitivity to antibiotics in order to apply the

appropriate treatment for these patients. Although, results from different hospitals, not only in Europe but also worldwide, suggest that *S. maltophilia* is not transmitted from patient to patient and is still susceptible to TMP/SMX and/or fluoroquinolones, higher incidence of isolation as well as increased percentage of resistant strains pointed the importace of this type of research/analysis. Moreover, it is important to get a complete (global-regional) epidemiological picture, since from Southeast Europe only data from Greece and Hungary are available (Samonis et al., 2012; Juhász et al., 2014).

Formation of biofilms is a significant problem both in medicine and in the food industry. Many antibiotics that are effective against planktonic bacterial cells are less effective or completely ineffective against bacteria in biofilm (Shanks et al., 2012). Numerous studies have been carried out in recent years with the aim of detecting a compound that would inhibit the formation of biofilms or lead to the degradation of the already formed biofilm (Saising et al., 2012, Balaji et al., 2013). Although *S. maltophilia* is not a highly virulent pathogen, its virulence factors, such as adhesion capacity, biofilm formation, hydrophobicity, motility and synthesis of extracellular enzymes, contribute to the inflammatory process (Di Bonaventura et al., 2010) together with intrisic resistance on different antibiotics and aviability for HGT. Dispite its clinical relevance, very little is knowen about the pathogenic mechanism of infections. A positive correlation between motility, biofilm formation and adhesion to mucin was shown in our study. These results are different from those previously published (Pompilio et al., 2008), where authors did not find a correlation between these three characteristics. They also showed an influence of hydrophobicity on adhesion and biofilm formation which was not detected in our study. However, another study on CF and non-CF clinical isolates pointed to motility as crucial for biofilm development in CF isolates (Pompilio et al., 2011). Strains analyzed in this study forming stronger biofilm show high motility with no statistically important differences in motility between CF and

non-CF isolates. Although, one study suggested that motility was important for biofilm formation in CF isolates (Pompilio et al., 2015) in our study lower motility in CF pathogens was described. Thus, opposite results point to the complexity of the process of biofilm formation especially in the specific environment such as lung of CF patients. We also have to consider that most of these characteristics are at the bottom line strain specific. Adhesion of *S. maltophilia* to mucin and factors involved in this ability has not been elucidate yet. According to our results, clinical isolates of *S. maltophilia* exhibited the ability to adhere to mucin. So far only in one study it was shown that *S. maltophilia* could adhere to mouse tracheal mucus with the help of flagella (Zgairt and Chhibber, 2011).

Surface characteristics, motility of strains, genes involved in biofilm formation, and other factors are responsible for abitlty of some strains to form biofilm and can be correlated with a higher level of resistance to antibiotics (Balcázar et al., 2015). Different factors influenced biofilm formation in *S. maltophilia*, SmeYZ efflux pump, which is not only responsible for antibiotic resistance (Lin et al., 2015), level of iron in the media (García et al., 2015) or histidin kinase and BfmAK system (Zheng et al., 2016). However, not only biofilm formation but also other physiological functions are also regulated with above meshed factors such as swimming motility, oxidative stress regulation, etc. The molecular basis of biofilm formation in *S. maltophila* has not been characterized yet. In our study we analyzed influence of different factors on biofilm fomration. Positive corelation between the simultaneous presence of genes *spgM* and *rpfF* in one bacterial strain and strong biofilm production in the same strain was determined in our study. Interestingly, this correlation was not affected by the presence or absence of an *rmlA* signal. However, the negative correlation observed between *spgM* and *rpfF* signals could mean that the presence of one of these genes, either *spgM* or *rpfF*, is required for biofilm formation, but the presence of both genes could lead to stronger biofilm

production. Still, further examination at the level of *spgM* and *rpfF* expression is required to support this assumption.

An interesting observation of this study is the higher motility and biofilm-forming potential of non-CF versus CF isolates. Although the loss of motility of CF pathogens has already been described as part of their adaptation process to the CF environment, the decrease in biofilm formation could not be easily explained. *Pseudomonas aeruginosa*, which is another relevant CF-related pathogen, increases biofilm formation in CF lungs, which strengthens its resistance to the host's antimicrobial factors. Pompilio et al. (2015) reported the prevalence of *P. aeruginosa* in mutual biofilm communities formed by *P. aeruginosa* and *S. maltophilia* in CF lungs. Actually, *S. maltophilia* stimulates biofilm formation by *P. aeruginosa*. This altruistic behavior of *S. maltophilia* facilitates its survival in these mutual biofilms. Considering this, we can assume that only those strains of *S. maltophila* that are poor biofilm producers survive in CF lungs. Otherwise, they would be outcompeted by more prevalent *P. aeruginosa* strains. This might be the reason for the higher incidence of poor biofilm-producing strains among CF *S. maltophilia* isolates in our study.

Ability of *S. maltophilia* to survive and adhere within intravenous infuses catheters and in dialysis, fluids may contribute to the pathogenesis of hemodialysis, intravenous line-related infections, and catheter related bacteremia and urinary tract infections. Adhesion is usually followed with the biofilm formation. Kinetics of the biofilm formation contributed to level of spread of bacteria and its resistance. For selected strains, we determined the kinetics of biofilm formation, which showed that non-CF, isolates formed biofilm faster than CF isolates. Although, they were all strong biofilm producer biofilm formation dynamic was significantly different between them correlating with the CF vs. non-CF phenotype. This might be, at least to some extent, attributed to the higher motility of non-CF isolates, which was shown in our

study also. Kinetics for *S. maltophilia* attaches and colonizes the polystyrene surface suggests that the bacteria could quickly adhere and form biofilm on medical instrument and devices that is why the only changing an old medical device with a new one could be unproductive, as it may result in adhering the planktonic bacteria to the new devices leading to persistent infection. Thus, an understanding of biofilm dynamics is important in order to improve better control strategies to combat with bacteria in hospital setting. Treatment of an infection after biofilm formation is less effective because the biofilm protects microorganisms from antimicrobial agents, particularly in immunocompromised patients. Once a biofilm has been formed, the bacterial cells become extremely robust against different antimicrobial agents. In this study, we further investigated the effects of TMP/SMX in two concentrations (25 μg/ml and 50 μg/ml) on *S. maltophilia* formed biofilms. Mechanisms providing resistance in biofilm to antimicrobial agents are important to understand and determine since they are responsible for inability of the antimicrobial agent to penetrate into bacterial biofilm.

Taking in mind importance of biofilm formation of pathogenic bacteria strains in different environments in the present study, we tested the effect of environmental factors on biofilm formation of *S. maltophilia* clinical isolates. For the majority of strains optimal temperature for biofilm formation was 37°C. Biofilm formation was the most affected with decrease or increase of temperature (12°C and 45°C) and changing pH of media on 8.5. The importance of the optimal temperature for biofilm formation was shown not only for strong and moderate biofilm producers but for the weak biofilm producer also, which formed moderate biofilm on 30°C. The temperature was showed to be the most relevant factor in biofilm formation of different strains not only in *S. maltophilia* but also in other bacteria species (Di Bonaventura et al., 2007; Di Bonaventura et al., 2008; The et al., 2016). In addition, CF isolates were more sensitive on changing of temperature, pH and CO_2 concentration. Overproduction of thick

and sticky mucus in patients with cystic fibrosis forms a specific environment that certainly indirectly influences the characteristics of the bacteria that colonize it (Cantón and del Campo, 2010). This could be the reason for the slight difference in characteristics of CF vs. non-CF isolates, because of adaptation to specific environmental conditions.

Contrary to the aerobic conditions used for *in vitro* biofilm studies, usual situation in the human body in which concentration of CO_2 and oxygen fluctuates. We found that the maximum levels of *S. maltophilia* biofilm formation were accomplished under aerobic conditions and in CO_2 atmosphere. These outcomes have significant consequences regarding the pathogenicity of individual strains of *S. maltophilia* in certain infection sites, such as the lung of cystic fibrosis patients, which are characterized by either decreased oxygen concentration or anaerobic conditions (Worlitzsch *et al*. 2002; Donaldson and Boucher 2003). Such environment could be favorable for the increase of the growth of bacterial biofilms and persistent infection.

In our study, we found that *S. maltophilia* strains were able to form biofilms under both static and dynamic conditions, although in the human host biofilms commonly developed under dynamic conditions (*i.e*. fluid flow through catheters, movements of artificial joints, *etc*.). Regardless of the conditions (dynamic *vs*. static) *S. maltophilia* strains analyzed in this study were biofilm producer. In contrast, in *Staphylococcus* spp. and *Salmonella* spp. strains the formation of biofilms was remarkably influenced by dynamic conditions (Stepanovic et al., 2001; 2004). We revealed that the regulation of biofilm production by *S. maltophilia* is complex and influenced in a strain-specific manner by several abiotic factors such as temperature, CO_2 concentration, and pH. The divergent biofilm responses suggest that *S. maltophilia* clinical strains have the potential to form biofilm but that the capability of individual strains to cause disease is also influenced by host factors and environmental conditions at the site of infection.

In summary, this work represents the first study of clonal relatedness and antibiotic resistance of *S. maltophilia* clinical isolates in Serbia. Clonal diversity detected in this study indicates low cross-transmission of *S. maltophilia* in the hospital settings. The susceptibility testing gained unremarkable results, as strains were universally susceptible to the tested antibiotics. Interestingly, six novel *S. maltophilia* STs were revealed while none of the STs prevalent in Europe were identified. Biofilm formation was the prevalent treat in the most of the analyzed strains. Complexity of this important virulence factor involves mutual influence of strains characteristics and environmental conditions. However, we could conclude that for factors tested in this study temperature and pH had the strongest effect on biofilm formation. Correlation between motility and biofilm formation was confirmed, more motile strain formed stronger biofilm. Nevertheless, additional experiments are needed to completely evaluate mechanism of action of each factor on biofilm formation of this important opportunistic pathogen. A comparison of phenotypic characteristics of CF and non-CF isolates suggested that there was a difference between the two populations but we could not speak about CF phenotype. This study accentuates the need for continuous surveillance for *S. maltophilia* in hospital settings in Serbia and monitoring their evolution towards antibiotic resistance.

CONCLUSIONS

6. CONCLUSIONS

Based on the results presented in this paper, the following conclusions can be drawn:

1. With the application of the PFGE method and the Phoretix 1D Pro program, phylogenetic relationships in a collection of 88 *Stenotrophomonas maltophilia* clinical isolates were determined, most of the strains do not show significant genetic relatedness among themselves and the total diversity was grouped into 11 groups.
2. Multilocus sequencing typing (MLST) analysis of 11 representative of each cluster determined six novel ST (ST114, ST115, ST116, ST117, ST118, ST119) while most common ST in Europe were not detected.
3. All of the *S. maltophilia* analyzed were susceptible not only to trimethoprim/sulfamethoxazole (TMP/SMX) but also to chloramphenicol, ciprofloxacin, levofloxacin and tetracycline.
4. Only one strain had moderate hydrophobicity, 13590, while all other strains had low hydrophobicity.
5. Swimming motility was observed in all tested strains, while none of the tested strains showed swarming or twitching motility.
6. Clinical isolates of *S. maltophilia* exhibited the ability to adhere to mucin. There were no differences in mucin-adhesion ability between CF and non-CF isolates.
7. Strong biofilm was formed by seven strains (7.95%), nine strains (10.2%) did not form biofilm, moderate biofilm was formed by 37 strains (42.05%), and weak biofilm was formed by 35

strains (39.8%) with almost equal representation in CF and non-CF strains.

8. PCR – based analysis revealed that *rmlA, rpfF* and *spgM* genes were present in 86 strains (97.7%), 62 strains (70.4%) and 63 strains (71.6%), respectively. There was no difference in the presence of the analyzed genes between CF and non-CF patients. The presence of both *rpfF* and *spgM* genes in one strain was correlated with strong biofilm formation.

9. Non-CF isolates showed higher biofilm forming potential and motility than CF isolates.

10. Strains forming stronger biofilm show high motility. Motility has shown positive correlations with both biofilm formation and the mucin-adhesion ability of the strains.

11. Kinetics of biofilm formation of strong biofilm producers showed that non-CF isolates formed biofilm faster than CF isolates.

12. Decrease or increase of temperature (12°C and 45°C) and changing pH of media on 8.5 were the factors which had the highest effect on biofilm formation.

13. TMP/SMX in 50 μg/ml concentration completely eradicated 24 h old formed biofilm in all tested strains, while 25 μg/ml of TMP/SMX affects biofilm in a strain-dependent manner.

14. A comparison of phenotypic characteristics of CF and non-CF isolates suggested that there was a difference between the two populations but we could not speak about CF phenotype.

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7. REFERENCES

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BIOGRAPHY

Haowa Mohamed Agila Madi was born in Bani walid, Libya, on November 19th 1986. She started her Bachelor studies in 2005 at The Department of Medical laboratories, Faculty of Medical technology, University of 7th of October and got her Bachelor degree in 2008 with grade point average (92.53%). From 2009, she works as teaching assistant in Faculty of Medical technology, University of 7th of October. In 2010, she got a scholarship to do her Master study at University of Belgrade, Faculty of Biology. She defended her Master thesis entitled "Correlation between bacteriocin production and plasmid content in BGIS29 and BGJAV1 strains of *Lactococcus lactis* subsp. *lactis*" in October 2012 and got her Master degree, with average mark 9.50 of 10 scales. In 2012, she started PhD study at University of Belgrade, Faculty of Biology, at study programme Molecular Biology, module Molecular biology of prokaryotes.

In her bibliography, she has two papers published in international scientific journals and two poster presentations at international and one at national congresses.

Образац 5.

1 Изјава о ауторству

Име и презиме аутора Haowa Madi

Број индекса M3013/2012

Изјављујем

да је докторска дисертација под насловом

Генотипизација, резистенција на антибиотике и формирање биофилма клиничких изолата *Stenotrophomonas maltophilia* из Србије

- резултат сопственог истраживачког рада;
- да дисертација у целини ни у деловима није била предложена за стицање друге дипломе према студијским програмима других високошколских установа;
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио/ла интелектуалну својину других лица.

Потпис аутора

У Београду, 20. 07. 2017.

Образац 6.

2 Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Haowa Madi

Број индекса M3013/2012

Студијски програм Молекуларна биологија

Наслов рада Генотипизација, резистенција на антибиотике и формирање биофилма клиничких изолата *Stenotrophomonas maltophilia* из Србије

Ментор Проф. др Јелена Лозо, ванредни професор, Биолошки факултет, Универзитет у Београду

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањена у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, 20. 07. 2017.

Образац 7.

3 Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Генотипизација, резистенција на антибиотике и формирање биофилма клиничких изолата *Stenotrophomonas maltophilia* из Србије

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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(Молимо да заокружите само једну од шест понуђених лиценци.

Кратак опис лиценци је саставни део ове изјаве).

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6. Ауторство – делити под истим условима. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца дозвољава комерцијалну употребу дела и прерада. Слична је софтверским лиценцама, односно лиценцама отвореног кода.