Clinical microbiologic examination of *Stenotrophomonas maltophilia* strains

Ph.D. thesis

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Budapest
2018

1. Introduction

Stenotrophomonas maltophilia has emerged as an important opportunistic and nosocomial pathogen in the past two decades worldwide. Behind *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, *S. maltophilia* is the third most common non-fermenting Gram-negative bacillus responsible for healthcare-associated infection. The World Health Organisation listed *S. maltophilia* as one of the leading antibiotic-resistant pathogens in hospitals. Community acquired infections have also been reported. Pneumonia and bacteraemia are the most frequent infections. Due to enzyme production (L1- and L2-β-lactamases, different aminoglycoside transferases, etc), efflux pumps (SmeABC – SmeYZ, MacABC, etc) and low outer membrane permeability, it has inherent extended antibiotic resistance. Therapeutic options are strongly limited. Currently only trimethoprim-sulfamethoxazole (SXT) is recommended for therapy, but some circumstances (hypersensitivity of the patient, resistance of the bacterium) can limit the use of this drug. In such cases, susceptibility of *S. maltophilia* isolates for other antimicrobials, for example fluoroquinolones or tetracycline derivates must be tested, even if clinical evidences for their efficacy are lacking yet. Furthermore, the recommendation by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) includes species specific clinical breakpoint only for SXT. Antibiotic combination therapy can be required in severely immunocompromised patient, in cystic fibrosis patients, in patients with SXT intolerance or in case of SXT-resistant (defined as MDR) *S. maltophilia* infection. Growing number of patients at risk (immunocompromised patients, cancer patients, patients under long-term intensive care, etc) and the necessity of the usage of broad-spectrum antibiotics such as carbapenems (which cause selective pressure for the inherently carbapenem-resistant *S. maltophilia*) have the consequence of increasing number of *S. maltophilia* infections. This fact together with the natural features of this bacterium (biofilm forming ability, ubiquiter, colonizing moist hospital environments) and its inherent and acquired antibiotic resistance mean that *S. maltophilia* is a continuous threat we have to face. The high attributable mortality rates (14-69%) in *S. maltophilia* infection highlight the clinical significance of this bacterium. The aim of this work is to describe the clinical microbiology of this well-known, but still news

hiding, globally challenging bacterium, especially focusing on antibiotic treatment options.

2. Objectives

The aim of our studies was to investigate *S. maltophilia* isolates collected in Diagnostic Laboratory of Clinical Microbiology, Institute of Laboratory Medicine, Semmelweis University in order to find answers to the following questions:

1. How susceptible were clinical *S. maltophilia* isolates to the first-line antibiotic agent SXT and to the second-line drugs?
2. What was the clinical background of *S. maltophilia* strains?
   a. Were they infective or just colonized patients?
   b. What was the outcome of *S. maltophilia* infections?
   c. What kind of risk factors influenced the outcome of infections?
3. How frequent was the *S. maltophilia* co-colonization or co-infection?
4. How susceptible were SXT-resistant *S. maltophilia* isolates to other antibiotic agents and what was the genetic background of their SXT resistance?
5. Was there any *in vitro* effective antibiotic combination against extremely drug resistant *S. maltophilia* isolates?
6. Was colistin+SXT combination *in vitro* effective against *S. maltophilia*, MDR *P. aeruginosa* and MDR *A baumannii* strains isolated together from lower respiratory tract co-infections?

3. Methods

3.1. Identifications and collection of strains

A total of 160 consecutive non-duplicate *S. maltophilia* isolates from a three-year collection period (2009-2011) were investigated in the first part of the study. If more strains were collected from the same patient, the first clinically relevant one (presumed on the type of the sample) was included in the study. Thirty consecutive non-duplicate *S. maltophilia* isolates collected in a five-year period (2010 – 2014) in Diagnostic Laboratory of Clinical Microbiology, Institute of Laboratory

Medicine were resistant to SXT. These ones were tested in the second part of the study. The identification of the isolates was done initially by the classic methods (OF probe, oxidase test, catalase test) and VITEK 2 Gram-negative identification cards (bioMérieux), later by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics). Strains were stored at -20°C in brain-heart infusion broth containing 30% glycerine until investigations. Strains identified by biochemical methods were re-tested by MALDI-TOF MS technique. The MALDI-TOF MS identification was performed by direct overlay method (isolates were smeared on target plate and overlayed by 1 µl matrix solution). Bruker Biotyper 2.0 softver was used for analysis. Matrix solution contained 5 mg α-cyano-4-OH-cinnamic acid dissolved in one milliliter of water, acetonitril and trifluoroacetic acid 4,75:5:0,25 mixture. Parameters of mass spectrometer device (ion source 1 20kV, ion source 2 18,5 kV, lenses 8,5 kV, detector 2,65 V, without gating, positive linear mode, spectra detected in 2000-20000 Da range, maximal laser frequency) were not changed. Biotyper scores ≥2.0 were accepted as valid species level identification. Identification results as „*Stenotrophomonas maltophilia*", „*Stenotrophomonas maltophilia* (*Pseudomonas beteli*)", „*Stenotrophomonas maltophilia* (*Pseudomonas hibiscicola*)" and „*Stenotrophomonas maltophilia* (*Pseudomonas geniculata*)" were all accepted, as these species were belonging to *Stenotrophomonas* rRNA lineage.

In our centre 58% of *S. maltophilia* isolated from LRT specimens was cultured as co-pathogen or co-colonizer in 2013-2014. *Pseudomonas aeruginosa* was found to be the most frequent co-pathogen, but *A. baumannii* was also a significant co-habitant. Those lower repiratory tract samples (tracheal aspirate or bronchoalveolar lavage sample) were included in our study, from which MDR *P. aeruginosa*, MDR *A.*

Clinical data and laboratory findings (white blood cell number (WBC), C-reactive protein (CRP), procalcitonin (PCT)) of patients at the time of *S. maltophilia* infections were collected retrospectively from clinical final reports. Based on clinical data, isolates were divided into two groups: isolates of patients who were infected by *S. maltophilia* and of those who were colonized only. Infection or colonization was distinguished according to clinical diagnoses given in final reports. The definitive diagnosis of infection was clinically established. Colonization was defined as the presence of on skin, mucous membranes, in wounds, or in excretions or secretions without causing adverse clinical signs or symptoms.

baumannii and *S. maltophilia* were isolated at the same time. In the study period 20 bacterial triplets were collected from different patients.

3.2. Antibiotic susceptibility testing

The minimal inhibitory concentrations (MIC) of antibiotics were determined by the broth microdilution method in cation-adjusted Mueller-Hinton broth (BectonDickinson) on 96-well plates (TOMTEC), according to CLSI testing conditions. The antibiotics tested included SXT (Sigma-Aldrich) in 0,25-128 mg/l, ciprofloxacin (Fresenius Kabi) in 0,5-256 mg/l, moxifloxacin (Bayer Pharma) in 0,064-32 mg/l, levofloxacin (TEVA) in 0,064-32 mg/l, colistin (colistin-sulphate, Sigma-Aldrich) in 0,25-256 mg/l, doxycycline (Pfizer) in 0,064-32 mg/l and tigecycline (Wyeth) in 0,064-32 mg/l concentrations. The MIC values of invasive isolates were tested by agar dilution method in case of SXT and fluoroquinolones and by gradient diffusion tests (bioMérieux Etest® and Liofilchem) in case of SXT, fluoroquinolones, tigecycline and colistin, too.

Isolates resistant to SXT were tested for ceftazidime (Fresenius Kabi) in range 1-512 mg/l and chloramphenicol (Sigma-Aldrich) in range 0,5-256 mg/l, too. The SXT and colistin ranges were modified to 0,5-256 mg/l and 1-512 mg/l, respectively. Colistin was tested at 0,06-32 mg/l and SXT at 2-1024 mg/l in case of MDR *P. aeruginosa* strains isolated from co-infections. Colistin was tested at 0,06-32 mg/l and SXT at 0,5-256 mg/l in case of MDR *A. baumannii* strains isolated from co-infections.

To interpret MIC results, EUCAST breakpoints were applied. The *S. maltophilia* specific breakpoint for SXT, and the non-species related breakpoints for fluoroquinolons and tigecycline were applied. For doxycyline – lacking either specific or non-species related breakpoints – the epidemiological cut-off (ECOFF) value of *S. maltophilia* (8 mg/l) was applied. For colistin – lacking non-species related breakpoints or determined ECOFF - *Pseudomonas* sp. specific breakpoint (4 mg/l) was used (according to EUCAST clinical breakpoints version 4.1.). For chloramphenicol the *S. maltophilia* specific, Clinical Laboratory Standards Institute (CLSI) declared breakpoints were used. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used as

quality control strains, which correspond with the quality control recommendation of CLSI.

3.3. Testing of antibiotic combinations

The extremely resistant *S. maltophilia* strains (n=4) were tested for antibiotic combinations. The twenty different combinations tested were followings: a) ceftazidime plus ciprofloxacin, moxifloxacin, levofloxacin, doxycyline, tigecycline, colistin, SXT; b) colistin plus ciprofloxacin, moxifloxacin, levofloxacin, doxycyline, SXT; c) tigecycline plus ciprofloxacin, moxifloxacin, levofloxacin, colsitin, SXT; d) SXT plus ciprofloxacin, moxifloxacin, levofloxacin. Antibiotic combinations were analysed by checkerboard (CB) technique on 96-well plates. Microbroth plates were inoculated with bacteria to yield $5x10^5$ CFU/mL in 100 µL final volume of Mueller-Hinton broth completed with antibiotic agents. Plates were incubated at 35°C for 18-22 h. When synergy was detected, results were confirmed by E-test combination method. Double disk agar diffusion method was tried to use for screening, placing disks in 1 cm distance from each other.

Fractional inhibitory concentration indices (ΣFICI) were calculated following the formula: FIC(A)+FIC(B)= ΣFICI, where FIC(A)= MIC of antibiotic agent A in combination with antibiotic agent B/MIC of antibiotic agent A alone and FIC(B)= MIC of antibiotic agent B in combination with antibiotic agent A/MIC of antibiotic agent B alone. The ΣFICI of two antibiotics tested defines the effects of antimicrobial combinations as antagonistic (ΣFICI > 4), indifferent (0,5 = ΣFICI ≤ 4) or synergic (ΣFICI < 0,5). The 0,5 = ΣFICI ≤ 1 values can be defined as additive or partial synergic.

Susceptible breakpoint indices (SBPI) were also used to interpret the results. SBPI was calculated as follows: SBPI = (susceptible breakpoint of antibiotic agent A / MIC of antibiotic agent A in combination with antibiotic agent B) + (susceptible breakpoint of antibiotic agent B / MIC of antibiotic agent B in combination with antibiotic agent A). An SBPI of 2 indicates that the MIC values of the two antibiotics tested in combination are either equivalent to their respective susceptible breakpoints or the MIC of one of the antimicrobials in a combination is less than its susceptible breakpoint. Greater SBPI values indicate more effective combinations

Antibiotic combination of colistin-plus-SXT on *S. maltophilia*, *P. aeruginosa* and *A. baumannii* co-isolates was analysed initially by CB technique. *Stenotrophomonas maltophilia* isolates were tested in 7 doubling dilutions of colistin and 11 doubling dilutions of SXT, whereas *P. aeruginosa* and *A. baumannii* strains were tested in 7 doubling dilutions of SXT and 11 doubling dilutions of colistin. When synergy was detected by CB, a time-kill assay (TKA) was performed at 1xMIC. When MIC values were above the therapeutic level, SXT was used at 8 mg/l and colistin at 4 mg/l, which fits the peak serum levels of these agents. Twenty ml of SXT, colistin and SXT-plus-colistin containing Mueller-Hinton broth were inoculated with bacteria to yield a density of 10^6 CFU/ml in the final volume. Tubes were incubated at 37°C with constant agitation. After 1, 2, 4, 6 and 24 h incubation aliquots were removed, serially diluted ($10^{-1} – 10^{-6}$) in 0,9% sodium chloride solution and plated on sheep blood agar plates (bioMérieux). Colony-forming units were counted on agar plates after 24 h incubation at 37°C. The lower limit of detection by this method was 20 CFU/ml. Synergy was defined as a $\geq 2 \log_{10}$ decrease in CFU/ml at 24 h for the antibiotic combination compared with CFU/ml decrease of *S. maltophilia* in presence of SXT, and CFU/ml decrease of *P. aeruginosa* and *A. baumannii* in presence of colistin.

3.4. Genetic testing

Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) was used for molecular typing of isolates. Bacteria were suspended in 100 µl of PCR-grade water and heated at 100 °C for 15 minutes. After centrifugation at 12000 rpm for 2 minutes, supernatant was removed. One µl of the supernatant was used as DNA for PCR. Primers of ERIC1 5'-ATGTAAGCTCCTGGGGATTCAC–3' and ERIC2 5'-AAGTAAGTGACTGGGGTGAGCG-3' (Bio Basic Inc.) and REDTaq Ready Mix PCR reaction mix (Sigma-Aldrich) were used for DNA amplification, in 50 µl final PCR reaction volume. PCR conditions were the following: initial denaturation at 95°C for 2 minutes, 30 cycles at 90°C for 30 seconds, 52°C for 1 minute, 65°C for 8 minutes. Electrophoresis was performed in 1.5% agarose gel (Sigma Aldrich), in Tris-boronicacid-EDTA buffer (Sigma Aldrich), stained with 0.01% GelRed (Biotium). Isolates from the same wards were tested in the same PCR and gelelectrophoreses run.

Band patterns were visually evaluated in the absence of appropriate software. Isolates with two or more different bands were interpreted as unrelated.

The presence of *sul*1 gene was tested by F 5'-ATGGTGACGGTGTTCGGCATTCTGA-3' and R 5'-CTAGGCATGATCTAAC CCTCGGTCT-3' primers, the *sul*2 gene by F 5'-GAATAAATCGCTCATCATT TTCGG-3' and R 5'-CGAATTCTTGCGGTTTCTTTCAGC-3' primers. The parameters of PCR were the followings: 1 x 95°C 10 minutes, 30 x 94°C 45 seconds, 64°C 45 seconds, 72°C 2 minutes, 1 x 72°C 10 minutes. For detection of class 1 integron, primers F 5'-CAACACATGCGTGTAAAT-3' and R 5'-CTTGCTGC TTGGATGCC-3' were used. The annealing temperature was 55°C, the other parameters of PCR were the same as described above.

3.5. Statistical analysis

Patients' characteristics were tested for their association with overall mortality. First, univariate analysis using χ^2 test or Fisher test was performed. A p value of <0,05 was considered as significant. Variables with significant association with mortality in the univariate analysis were entered in a multivariate forward stepwise logistic regression model to identify independent risk factors for death. The odds ratio (OR) with the corresponding 95% confidence interval (95% CI) for each variables were calculated. A p value of <0,05 was considered as indicative of statistical significance. Stata 12 software (StataCop LP) was used for the analysis.

4. Results

Biotyper 2.0 software of Bruker MALDI-TOF MS classified 74% of the isolates as *S. maltophilia*, 16% as *S. maltophilia* (*P. hibiscicola*), 7% as *S. maltophilia* (*P. beteli*), 3% as *S. maltophilia* (*P. geniculata)*. As *P. hibiscicola*, *P. beteli* and *P. geniculata* belong to *Stenotrophomonas* rRNS lineage, they were reclassified to *Stenotrophomonas* genus. Now they are synonims of *S. maltophilia*.

Isolates of infected patients (n=100) were cultured from blood (n=25), bronchoalveolar aspirate (n=30), tracheal aspirate (n=31), sputum (n=7), central venous catheter (n=4), peritoneal fluid (n=3). Isolates of colonized patients (n=60) were cultured from rectal swab (n=11), urine (n=8), ear swab (n=6), throat - (n=3),

nose - (n=4), eye sample (n=7), catheter (n=1), sputum (n=7), tracheal aspirate (n=7) and wound sample (n=6).

Isolates resistant to SXT were cultured from blood (n=2), bronchoalveolar lavage sample (n=8), tracheal aspirate (n=3), sputum (n=3), endotracheal tube (n=3), rectal swab (n=7), urine (n=1), ear swab (n=1) and throat swab (n=2). The most of these isolates (n=23) were considered clinically to be colonizer or contaminant bacteria. Only 7 isolates were infective; they caused lower respiratory tract infections or bacteriaemia. Thirteen strains were isolated from neonates cared in neonatal intensive care unit (ICU), six strains from adult patients treated in ICUs.

4.1. Results of ERIC-PCR

ERIC-PCR resulted in highly diverse patterns, however seven identical patterns among the colonizer (4 timer 2 isolates and 3 timers 3 isolates with the same band patterns) and twelve among the infective isolates (6 timers 2 isolates, 4 times 3 isolates, once 5 isolates and one 6 isolates with tha same band patterns) were found. In order to prevent the distortion of values by clonal isolates, 10 colonizer and 23 infective isolates were excluded from the final evaluation of antibiotic susceptibility testing results.

Four identical patterns were found among SXT resistant isolates (2 times 2 isolates, once 3 isolates and once 9 isolates with the same band patterns). Despite the detected clonality, isolates with the same band pattern showed different MIC values. All isolates were included in the final evaluation of antibiotic susceptibility testing results.

4.2. Results of antibiotic susceptibility testing

The results obtained by microdilution, gradient diffusion and agar dilution methods were in concordance. The MIC50 and MIC90 values, MIC ranges and susceptibility in % of selected isolates with different ERIC-PCR patterns (77 infective and 50 colonizer) were summarized in Table 1. However, MIC values did not differ significantly if the total of 100 infective and 60 colonizer isolates were evaluated.

Susceptibility for doxycycline was investigated instead of the often tested minocycline, since the latter is not distributed in Hungary. Applying *S. maltophilia* specific ECOFF, all the isolates seemed to be sensitive to doxycyline. The

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susceptibility rates of of infective and colonizer isolates were compared. Both groups high rate of susceptibility to SXT. Apart from ciprofloxacin, the infective isolates had higher rates of non-susceptibility than the colonizers. Non-susceptibility rates for colistin and tigecycline were 1,2-1,3 times higher in the infective groups.

Table 1. Summary of MIC values and interpretations of 127 *S. maltophilia* strains (77 infective, 50 colonizer) with different ERIC-PCR patterns.

Antimicrobial agent breakpoints (mg/l)	Isolates	MIC (mg/l)			% of isolates		
		MIC range	MIC50	MIC90	Resistant	Intermediate	Susceptible
SXT	infective	<0,25->32	0,25	1	1	0	99
S≤4 , R>4	colonizer	<0,25->32	0,5	1	2	0	98
ciprofloxacin	infective	<0,5-64	2	8	54	22	24
S≤0,5 , R>1	colonizer	0,5-128	2	8	76	12	12
levofloxacin	infective	0,125-16	1	2	7	18	75
S≤1 , R>2	colonizer	<0,064-4	0,5	2	4	12	84
moxifloxacin	infective	<0,064-8	0,25	1	7	6	87
S≤0,5 , R>1	colonizer	<0,064-4	0,125	0,5	4	6	90
doxycycline	infective	0,125-4	1	2	0	0	100
ECOFF 8 mg/l	colonizer	0,125-4	1	2	0	0	100
tigecycline	infective	0,125-16	0,5	2	50	38	12
S≤0,25 , R>0,5	colonizer	0,125-16	0,5	2	14	51	35
colistin	infective	1->256	64	>256	91	0	9
S≤4 , R>4	colonizer	0,25->128	16	>128	77	0	23

The antibiotic susceptibility of SXT resistant isolates is summarized in Table 2. The MIC values of SXT ranged 8 - 128 mg/l. None of the isolates was susceptible to ciprofloxacin, tigecycline, ceftazidime or colistin. Only 37% of them were susceptible to levofloxacin or moxifloxacin. Interpreting isolates with MIC value under ECOFF as susceptible, 50% of the strains were susceptible to doxycycline. Thirteen % of the isolates were susceptible to chloramphenicol. Two isolates resistant to all tested antibiotic agents and two others susceptible only to doxycycline were detected. These four isolates were further investigated; susceptibilities for combinations of 20 different antibiotics were tested on them.

Table 2. Summary of MIC values and interpretations of 30 SXT-resistant *S. maltophilia* strains (n=30).

Antimicrobial agent	MIC (mg/l)			% of isolates		
breakpoints (mg/l)	MIC range	MIC50	MIC90	Resistant	Intermediate	Susceptible
SXT : S≤4 , R>4	8-128	32	64	100	0	0
ciprofloxacin : S≤0,5 , R>1	1-32	4	16	83	17	0
levofloxacin : S≤1 , R>2	0,5-16	2	8	20	43	37
moxifloxacin : S≤0,5 , R>1	0,125-8	1	4	23	40	37
doxycycline : ECOFF 8 mg/l	0,5-64	4	16	33	17	50
tigecycline : S≤0,25 , R>0,5	0,5-8	2	4	97	3	0
ceftazidime : S≤4, R>8	16-512	128	512	100	0	0
colistin : S≤4 , R>4	8->512	128	>512	100	0	0
chloramphenicol : S≤8 , R≥32	8-64	16	32	44	43	13

The rate of SXT-resistant *S. maltophilia* isolates didn't change significantly in 2009-2017. Annually 4-12 patients were detected with MDR *S. maltophilia* positive samples. In the whole nine-year period 81 consecutive, non-duplicate SXT-resistant isolates were cultured. The overall resistance rate was 1,9%. The stagnating app. 2% resistance rate indicates that neither an MDR clone nor a mobile genetic element spread effectively. Furthermore, there isn't any effective pressure selecting SXT resistance.

All *P. aeruginosa* and *A. baumannii* strains were susceptible to colistin, with MIC50 1 mg/l and MIC90 2 mg/l. Fifteen percent of *A. baumannii* strains were susceptible to SXT, MIC50 32 mg/l and MIC90 128 mg/l was found. *Pseudomonas aeruginosa* strains showed a high level of intrinsic resistance to SXT, with MIC50 256 mg/l and MIC90 512 mg/l. All *S. maltophilia* strains were sensitive to SXT, with MIC50 0,25 mg/l and MIC90 1 mg/l, and resistant to colistin, with MIC50 256 mg/l and MIC90 >512 mg/l.

4.3. Results of antibiotic combination testing

Most of the combinations, tested by CB, had indifferent effect on extremely resistant *S. maltophilia* strains (n=4), based on the calculated ΣFICI values from the lowest MIC values. Antagonistic combination was not found. Synergic effect at least in one isolate was found in combinations of ceftazidime+ciprofloxacin, ceftazidime+moxifloxacin, ceftazidime+levofloxacin, ceftazidime+colistin,

colistin+ciprofloxacin, colistin+moxifloxacin, colistin+levofloxacin, colistin+doxycycline, tigecycline+colistin, SXT+moxifloxacin, SXT+levofloxacin. Ceftazidime+moxifloxacin and ceftazidime+colistin were the only combinations that showed synergic or at least partial synergic effect on each tested isolate. Taking into consideration the peak serum levels during antibiotic treatment (ceftazidime: 60 mg/l, ciprofloxacin: 1,8-4,6 mg/l, levofloxacin: 5,7-8,6 mg/l, moxifloxacin: 4,5 mg/l, colistin: 5-7,5 mg/l, SXT: 1-2/40-60 – 9/105 mg/l, tigecycline: 0,63 mg/l, doxycyline: 1,5-2,1 mg/l, chloramphenicol: 11-18 mg/l), only the combinations of ceftazidime+fluoroquinolons and colistin+fluoroquinolons can be accepted as synergic, in certain cases. Furthermore, if EUCAST susceptibility breakpoints are also taken into consideration, just the MIC values of fluoroquinolons (in combination with ceftazidime: $SBPI_{CAZ+MXF} = 2.125$, $SBPI_{CAZ+LEV} = 4.125$) and colistin (in combination with fluoroquinolons: $SBPI_{COL+MXF} = 2.125$) decreased into the susceptible range, and only in one-one isolate out of the tested four ones.

Synergic and indifferent effects of colistin+SXT were found by CB in cases of co-isolated *S. maltophilia*, MDR *P. aeruginosa* and MDR *A. baumannii* strains. Synergy was detected in 50%, 35% and 45% of *S. maltophilia*, *P. aeruginosa* and *A. baumannii* strains, respectively. Antagonistic effect was not found.

Results gained by TKA are summarized in Table 3. The tested *S. maltophilia*, *P. aeruginosa* and *A. baumannii* strains showed synergy in 70% (7 strains out of 10), 57% (4 strains out of 7) and 56% (5 strains out of 9) of cases, respectively. Considering that for most combinations with colistin against Gram-negative species initial killing is usually dramatic, but is followed by significant regrowth, the rates of $\log_{10}$ decrease after 6 and 24 h are summarized in Table 3. Synergic activity of the combination was already detected after 6 h incubation in 86% of *S. maltophilia* isolates and 50% of *P. aeruginosa* strains. In the case of *A. baumannii*, synergy was detected just after 24 h incubation. The results gained by CB and TKA methods correlated in 61% of cases, but the ΣFIC values did not correlate with the rate of $\log_{10}$ decrease in TKA. Colistin-plus-SXT combination had synergic effect on seven *S. maltophilia* (35%), four *P. aeruginosa* (20%) and five *A. baumannii* (25%) strains by both methods. The combination had synergic effect on all co-isolates from two patients.

Table 3. Summary of the synergic results gained by TKA. Colistin+SXT combination was tested on strains which were previously tested by CB method and ΣFICI values were <0,5. Decrease of colony count was compared with that in presence of SXT in case of *S. maltophilia,* and in presence of colistin in case of *P. aeruginosa* and *A.baumanni* strains.

colistin+SXT	Synergy by TKA n (%)	Decrease in log10 after 6 hours	Decrease in log10 after 24 hours	Previously determined ∑ FICI value by CB
S. maltophilia	7 (70)	5,9	2,4	Sm#1 ΣFICI=0,375
		7,7	3,8	Sm#3 ΣFICI=0,265
		7,5	3,9	Sm#6 ΣFICI=0,281
		2,4	4,7	Sm#7 ΣFICI=0,375
		2,7	2,9	Sm#9 ΣFICI=0,312
		6,3	3,1	Sm#10 ΣFICI=0,187
		6	6	Sm#20 ΣFICI=0,125
P. aeruginosa	4 (57)	7	4,2	Pa#1 ΣFICI=0,375
		3,4	2,3	Pa#5 ΣFICI=0,156
		6,7	4,1	Pa#14 ΣFICI=0,375
		7,2	3,7	Pa#18 ΣFICI=0,266
A. baumannii	5 (56)	3,1	7,3	Ab#2 ΣFICI=0,25
		3,3	5,3	Ab#4 ΣFICI=0,094
		3,1	7,3	Ab#6 ΣFICI=0,375
		2,7	4,8	Ab#11 ΣFICI=0,375
		3,6	5,5	Ab#12 ΣFICI=0,375

4.4. Results of resistance genes testing

Genetic testing could be performed only from 27 strains out of the 30 (three strains were unculturable after storage). Except of two isolates carrying *sul2*, all the others were positive for *sul1*. None of the strains contained both genes. Our study found that 7 strains (23%) carried a class 1 integron when integrase-specific primers were used.

4.5. Results of clinical data analysis

Analysis of clinical background of 100 infective isolates revealed that 70 % of the isolates were cultured from patients admitted to intensive care units. Sixty-two isolates were obtained from patients with pneumonia, 12 of them developing respiratory failure. Fourty-six patients had sepsis (23 of them developing severe sepsis, septic shock or multiorgan failure), in 19 cases *S. maltophilia* was considered

as the etiological agent. The co-morbidity was chronic obstructive pulmonary disease (COPD) in 19 cases and malignancy in 19 cases, respectively. Nine patients were immunsuppressed (3 of them had lung transplantation).

The all-cause mortality was 45%. In 25% of fatal cases *S. maltophilia* was regarded to have direct role in death. Clinical data and their correlation with mortality are shown in Table 4. Twenty patients did not receive specific therapy for *S. maltophilia* (13 of them died); however, patients received antimicrobial agents against the co-infective bacteria. In 11 cases colistin was applied, 9 of them were fatal. Twenty-nine patients were treated with SXT, 7 of them died. Six patients were treated with ciprofloxacin (5 died), 17 with moxifloxacin (3 died), 16 with levofloxacin (7 died), one with tigecycline (died).

The mortality was significantly associated with the following variables: ICU admission, need for mechanical ventilation, vasopressor therapy, presence of multiorgan failure and central venous catheter. The association with mortality remained significant after their adjustment for age and gender. Multivariate analysis with forward stepwise logistic regression identified central venous catheter (OR: 0,15, 95% CI: 0,03-0,59, p=0,007) and central venous catheter with vasopressor therapy (OR: 0,23, 95% CI: 0,08-0,65, p=0,006) as independent determinants of mortality. The count of WBC of infected patients ranged 0,05-37,7 G/l, median value was 11,2 G/l. Values of CRP and PCT ranged 0,4-423 mg/l and 0,15-100 µg/l, median values were 86 mg/l and 1,6 µg/l, respectively. Laboratory results were determined from blood samples collected at the same time when specimens for microbiologic testing were collected. When WBC, CRP and PCT values were not tested on the day of specimen collection for microbiologic culturing, those determined at the nearest date (within two days) were accepted.

Table 4. Univariate analysis of overall mortality of 100 patients infected by *S. maltophilia*, performed by χ^2 test or Fisher test. p <0,05 values are highlighted.

	Died (n=45)	Survived (n=55)	p vaue
	n (%)	n (%)	
Age in years, median (range)	67 (0-88)	62 (0-88)	-
Gender, male	23 (51,1)	35 (63,6)	0,29
Hematological malignancy	3 (6,6)	3 (5,4)	0,31
Advanced cancer	10 (22,2)	8 (14,5)	0,54
Diabetes mellitus	16 (35,5)	16 (29,1)	0,22
Corticosteroid use	4 (8,8)	8 (14,5)	0,31
Chemotherapy	4 (8,8)	11 (20)	0,2
Neutropenia (<0,5 G/l)	5 (11,1)	2 (3,6)	0,24
Post-transplantation stage	0	5 (9,1)	-
Chronic heart disease	15 (33,3)	18 (32,7)	0,88
Chronic kidney disease, hemodialysis	10 (22,2)	5 (9,1)	0,12
Chronic lung disease	13 (28,8)	14 (25,4)	0,87
Chronic liver disease	5 (11,1)	7 (12,7)	0,95
Hypertension	30 (66,6)	25 (45,4)	0,05
Admission to intensive care unit	43 (95,5)	32 (58,2)	**0,00005**
Need for vasopressors	26 (57,7)	8 (14,5)	**0,00001**
Central venous catheter	42 (93,3)	29 (52,7)	**0,00001**
Need for mechanical ventilation	41 (91,1)	28 (50,9)	**0,00004**
Severe sepsis, septic shock, multiorgan failure	23 (51,1)	5 (9,1)	**0,00001**
Non-*S. maltophilia* bloodstream infection	15 (33,3)	9 (16,3)	0,08
S. maltophilia bloodstream infection	12 (26,6)	13 (23,6)	0,9
Recent surgery	18 (40)	12 (21,8)	0,08
Polymicrobial infection	35 (77,7)	33 (60)	0,09

Other microorganisms were isolated together with *S. maltophilia* from 68% of specimens. Numbers of these isolates are shown in Table 5.

Table 5. Other microorganisms isolated together with 160 *S. maltophilia* isolates.

* *Alcaligenes faecalis, Achromobacter xylosoxidans, Pseudomonas fluorescens.*
** *Proteus mirabilis, Serratia marcescens, Escherichia coli, Klebsiella pneumoniae,*
Klebsiella oxytoca, Enterobacter aerogenes, Enterobacter cloacae

Isolates	Infective (n=100)	Colonizer (n=60)
	n	n
Gram-negative bacteria		
Acinetobacter baumannii	7	1
Pseudomonas aeruginosa	24	7
other non-fermenting*	0	3
*Enterobacterales**	17	13
Gram-positive bacteria		
coagulase-negative *Staphylococcus spp.*	4	9
Staphylococcus aureus	8	7
Enterococcus spp.	10	8
Candida spp.	36	6

5. Conclusions

1. Our results show that SXT is still the most potent antibiotic drug against *S. maltophilia*; the susceptibility rate was 98-99%. Due to the low frequency of moxifloxacin and levofloxacin resistance, these drugs can be used either in high dose monotherapy or rather in combination with other antibiotics. Doxycycline was proved to have excellent *in vitro* activity against *S. maltophilia*. Based on this results and previous reports, minocycline would have similar effect. Considering SXT resistant isolates, severe infections of patients with chronic respiratory illnesses and the guideline given by CLSI, the distribution of minocycline in Hungary would be suggested. Colistin was found to have weak *in vitro* activity against *S. maltophilia*, therefore, it cannot be used in monotherapy in *S. maltophilia* infections. The establishment of *S. maltophilia* specific clinical breakpoints for agents other than SXT by EUCAST is strongly required in the near future. Furthermore, the most reliable antibiotic susceptibility testing method for alternative antibiotics should be declared.

2. Most infections caused by *S. maltophilia* were associated with severe co-morbidity and long-term, extensive ICU treatment. The high all-cause in hospital

mortality rate (45%) we observed can be connected to the serious underlying illnesses rather to *S. maltophilia* itself. However, the strict attributable mortality rate (11%) was also high. Especially patients admitted to ICU have the risk of *S. maltophilia* infections, therefore screening them for *S. maltophilia* colonization should be considered. Patients with central venous catheter must be taken special attention, because central venous catheter is associated with mortality in *S. maltophilia* infections.

3. Physicians have to consider that *S. maltophilia* as a co-pathogen or co-colonizer in polymicrobial infections can have negative impact on the success rate of antibiotic treatment and clinical outcome. The carbapenem, cephalosporine and aminoglycoside treatment of *P. aeruginosa,* which was found to be the most frequent co-pathogen, can be negatively effected by co-colonizer *S. maltophilia.* As *S. maltophilia* can influence the virulence of surrounding bacteria, its eradication can be reasonable even if it just colonize a patient.

4. The occurance of SXT-resistant, MDR *S. maltophilia* is rare yet. While *sul* genes are associated with mobile genetic elements, ongoing resistance surveillance is required. The higher antibiotic resistance rates of MDR strains have significant clinical consequences: levofloxacin and moxifloxacin mean alternative therapeutic options yet, but in case of resistance to them just few antibiotic combinations or tetracycline derivates can be used. However, the clinical evidence of their efficacy is lacking yet.

5. Combinations including fluoroquinolons – especially moxifloxacin – can be effective, even if the isolate was resistant to them. Ceftazidime improves in vitro effect of other antibiotic agents in combination, despite the high level resistance of the strains to itself. An antibiotic agent in aerosolised form in combination with an intravenous one can be effective, even if the ΣFICI and SBPI do not show synergism. It has to be kept in mind that results of synergy testing are isolate specific.

6. According to our *in vitro* results colistin-plus-SXT combined therapy can be used efficiently in clinical practice as no antagonistic effect was detected. Moreover, in 20-35% it was found to have synergic effect against co-isolated MDR *P. aeruginosa*, *A. baumannii* and *S. maltophilia* strains. Regrowth of MDR *A. baumannii* after 24 hour in the presence of colistin can be prevented by colistin-plus-SXT combination.

List of publications

Publications related to present thesis:

Juhász E, Krizsán G, Lengyel G, Grósz G, Pongrácz J, Kristóf K. (2014) Infection and colonization by Stenotrophomonas maltophilia: antimicrobial susceptibility and clinical background of strains isolated at a tertiary care centre in Hungary. Ann Clin Microbiol Antimicrob, 13: 333.

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