ANTIBIOTIC SUSCEPTIBILITY OF SULFAMETHOXAZOLE-TRIMETHOPRIM RESISTANT *STENOTROPHOMONAS MALTOPHILIA* STRAINS ISOLATED AT A TERTIARY CARE CENTRE IN HUNGARY

EMESE JUHÁSZ*, JÚLIA PONGRÁCZ,
MIKLÓS IVÁN and KATALIN KRISTÓF

Diagnostic Laboratory of Clinical Microbiology, Institute of Laboratory Medicine,
Semmelweis University, Budapest, Hungary

Sulfamethoxazole-trimethoprim (SXT) is the drug-of-choice in *Stenotrophomonas maltophilia* caused infections. There has been an increase in resistance to SXT of *S. maltophilia* over recent years. In this study 30 *S. maltophilia* clinical isolates resistant to SXT were investigated. Antibiotic susceptibilities for ciprofloxacin, moxifloxacin, levofloxacin, doxycycline, tigecycline, ceftazidime, colistin and chloramphenicol were determined by broth microdilution method. None of the strains were susceptible to ciprofloxacin, tigecycline, ceftazidime or colistin. Only 37% of the isolates were susceptible to levofloxacin or moxifloxacin. Two isolates resistant to all tested antibiotic agents and two others susceptible only to doxycycline were further investigated: susceptibility for combinations of antibiotics was analyzed by checkerboard technique. According to the fractional inhibitory concentration indices calculated, moxifloxacin plus ceftazidime combination was found to be synergistic in each case. Genetic testing revealed the predominance of *sul1* gene. Our study concluded that the range of effective antibiotic agents is even more limited in infections caused by SXT-resistant *S. maltophilia*. In these cases, *in vitro* synergistic antibiotic combinations could be potential therapeutic options.

Keywords: *Stenotrophomonas maltophilia*, sulfamethoxazole-trimethoprim resistance, antibiotic resistance, synergism

*Corresponding author; E-mail: juhasz.emese@med.semmelweis-univ.hu

Introduction

Stenotrophomonas maltophilia is an emerging, multidrug-resistant, Gram-negative pathogen. After *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, it is the third most frequent non-fermenting Gram-negative bacteria causing nosocomial infections [1]. Pneumonia and bacteraemia are the most common clinical presentations of *S. maltophilia* infection, related to high mortality rate (14–69%) [2]. It causes infections predominantly in hospitalized and immunocompromised patients, but community-acquired infections have also been reported. Differentiation of colonization or infection by *S. maltophilia* can be difficult, especially if bacteria are isolated from respiratory tract samples. Moreover, *S. maltophilia* is often isolated from polymicrobial infections, in which its role is difficult to evaluate. Due to its extended inherent antibiotic resistance (characterized by production of L1-, L2-β-lactamases, extended spectrum β-lactamases, multidrug-efflux systems, low permeability of outer membrane, modifing enzymes and target site modifications), therapeutic options are strongly limited. The recommended first-line agent is sulfamethoxazole-trimethoprim (SXT). According to European Committee on Antimicrobial Susceptibility Testing (EUCAST), SXT is the only antibiotic agent currently that can be interpreted in microbiology results, based on established *S. maltophilia* specific clinical breakpoints [3].

Resistance to SXT has been reported mainly due to modified target genes *sul1* (carried by class 1 integrons) and *sul2* (carried by insertion sequence common region elements) [4, 5]. Resistance rates of SXT ranges from 2 to 10% globally; however studies from Turkey (15%), Spain (27%), Taiwan (25%) and China (30–48%) reported higher resistance rates [4, 6–8]. In patients suffering from cystic fibrosis (CF), the frequency of SXT-resistant *S. maltophilia* is higher (24–84%) [2, 9–11]. Resistance level of isolates from patients with cancer and those treated in intensive care unit (ICU) might be also higher [4]. In case of SXT resistance – as well as in cases when SXT cannot be used due to hypersensitivity of the patient or adverse effects in high dose therapy – alternative agents have to be taken into consideration. Fluoroquinolones, tetracycline derivates, colistin, ceftazidime and chloramphenicol can have activity against *S. maltophilia,* among few other antibiotic agents [1].

Aims of this study were to test the antibiotic susceptibility of *S. maltophilia* isolates resistant to SXT, to examine the *in vitro* effect of few antibiotic combinations and to reveal the presence of the most frequent SXT-resistance coding genes.

Materials and Methods

Our study included 30 consecutive non-duplicate *S. maltophilia* isolates collected in a five-year period (2010–2014) in the Diagnostic Laboratory of Clinical Microbiology, Institute of Laboratory Medicine, Semmelweis University (Budapest, Hungary). Isolates were identified by conventional methods and MALDI-TOF mass spectrometry (Bruker Daltonics, Bremen) technique. Isolates 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 the isolates (n = 23) were considered clinically to be colonizer or contaminant bacteria. Only seven isolates were infective as they caused lower respiratory tract infections and bacteraemia. Thirteen strains were isolated from neonates cared in neonatal intensive care unit (ICU), six strains from adult patients treated in ICUs.

The minimal inhibitory concentrations (MICs) of nine antibiotics were determined by broth microdilution method in cation-adjusted Mueller–Hinton broth, according to CLSI testing conditions [12]. The antibiotics tested included 0.5–256 mg/L SXT (Ratiopharm), 0.5–256 mg/L ciprofloxacin (Fresenius Kabi), 0.064–32 mg/L moxifloxacin (Bayer Pharma), 0.064–32 mg/L levofloxacin (TEVA), 1–512 mg/L colistin (Sigma-Aldrich), 0.064–32 mg/L doxycycline (Pfizer), 0.064–32 mg/L tigecycline (Wyeth), 1–512 mg/L ceftazidime (Fresenius Kabi) and 0.5–256 mg/L chloramphenicol (Sigma-Aldrich). While EUCAST has *S. maltophilia* specific breakpoint only for SXT, the non-species related breakpoints were applied for fluoroquinolones, ceftazidime and tigecycline. For doxycycline – due to absence of non-species related breakpoints – the epidemiological cut-off (ECOFF) value of *S. maltophilia* was applied. For colistin and chloramphenicol – lacking non-species related breakpoints and approved ECOFF – the *Pseudomonas* sp. specific colistin breakpoint and the *S. maltophilia* specific Clinical Laboratory Standards Institute (CLSI) chloramphenicol breakpoints were used, respectively. *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.

The 20 different antibiotic combinations tested for extremely resistant *S. maltophilia* strains were the following: a) ceftazidime plus ciprofloxacin, moxifloxacin, levofloxacin, doxycycline, tigecycline, colistin, SXT; b) colistin plus ciprofloxacin, moxifloxacin, levofloxacin, doxycycline, SXT; c) tigecycline plus ciprofloxacin, moxifloxacin, levofloxacin, colistin, SXT; d) SXT plus ciprofloxacin, moxifloxacin, levofloxacin. Antibiotic combinations were analysed by checkerboard technique and in selected cases (when synergy was detected) by E-test

combination method and disc agar diffusion method, too [13, 14]. Fractional inhibitory concentration indices (FICI) were calculated following the formula described in microbiology procedure guidelines [15]. The summation of FICIs of two antibiotics tested define the effects of antimicrobial agent combinations as antagonistic (Σ FICI > 4), indifferent (0.5 < Σ FICI ≤ 4) or synergistic (Σ FICI ≤ 0.5). 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) + (susceptible breakpoint of antibiotic agent B / MIC of antibiotic agent B in combination). 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 [11].

Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) was used for molecular typing of isolates, as described by Silbert et al. [16]. Isolates from the same ward were tested within the same PCR reaction and in the same gel electrophoreses run. Band patterns generated by ERIC-PCR were visually evaluated in the absence of appropriate software. Isolates that differed by two or more bands were interpreted as unrelated.

The occurrence of two frequent genes coding sulfamethoxazole resistance: *sul1* and *sul2,* and the presence of class I integron were tested by PCR as described previously [17].

Results

The MIC values, MIC ranges, breakpoints and interpretations are summarized in Table I. All isolates tested were resistant to SXT, however, MIC values ranged between 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 antibiotics were tested. The calculated FICIs from the lowest MIC values are shown in Table II. Most of the combinations had indifferent effect. Antagonistic combination was not found. Synergistic effect at least in one isolate was found in combinations of a) ceftazidime plus ciprofloxacin, moxifloxacin, levofloxacin or colistin, b) colistin plus ciprofloxa-

Table I. Summary of MIC values and interpretations

Antimicrobial agent : breakpoints (mg/L)*	MIC (mg/L)			% of isolates		
	MIC range	MIC 50	MIC 90	Resistant	Intermediate	Susceptible
TMP-SMX : 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 : **	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

*Breakpoints according to EUCAST. SXT breakpoints are specific for *S. maltophilia*.
Fluoroquinolone, tigecycline and ceftazidime breakpoints are non-species related. Colistin breakpoint is *Pseudomonas* sp. specific.
**ECOFF of doxycycline is 8 mg/L.
***Breakpoints according to CLSI.

cin, moxifloxacin, levofloxacin or doxycycline, c) tigecycline plus colistin, and d) SXT plus moxifloxacin or levofloxacin. Ceftazidime plus moxifloxacin and ceftazidime plus colistin were the only combinations that showed synergistic 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/mL, 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, doxycycline: 1.5–2.1 mg/L, chloramphenicol: 11–18 mg/L), only the combinations of ceftazidime plus quinolons and colistin plus quinolones can be accepted as synergistic, in certain cases. Furthermore, if EUCAST susceptibility breakpoints are also taken into consideration, just the MIC values of quinolones (in combination with ceftazidime: $SBPI_{CAZ+MXF} = 2.125$, $SBPI_{CAZ+LEV} = 4.125$) and colistin (in combination with quinolons: $SBPI_{COL+MXF} = 2.125$) decreased into the susceptible range, and only in one isolate out of the tested four ones.

Table II. Results of antibiotic synergy testing of 4 multidrug-resistant *S. maltophilia* strains

Combination	S. maltophilia 1					S. maltophilia 2					S. maltophilia 3					S. maltophilia 4				
	AB 1	AB 2	AB 1 + AB 2	AB 2 + AB 1	∑FICI	AB 1	AB 2	AB 1 + AB 2	AB 2 + AB 1	∑FICI	AB 1	AB 2	AB 1 + AB 2	AB 2 + AB 1	∑FICI	AB 1	AB 2	AB 1 + AB 2	AB 2 + AB 1	∑FICI
CAZ+CIP	128	16	16	4	**0.375**	128	4	64	0.25	0.562	128	32	32	2	**0.312**	128	16	32	2	**0.375**
CAZ+LEV	128	8	32	2	**0.5**	128	4	64	0.25	0.562	128	16	32	4	**0.5**	128	8	32	0.5	**0.312**
CAZ+MXF	128	4	32	0.5	**0.375**	128	2	32	0.125	**0.312**	128	8	32	1	**0.375**	128	4	32	1	**0.5**
CAZ+DOX	128	16	64	8	1	128	4	64	0.25	0.562	128	32	64	1	0.531	128	32	64	2	0.562
CAZ+TIG	128	16	64	8	1	128	4	64	0.25	0.562	128	8	64	0.5	0.562	128	8	64	0.25	0.531
CAZ+COL	128	64	16	16	**0.375**	128	8	32	2	**0.5**	128	128	32	16	**0.375**	128	512	16	64	**0.25**
CAZ+SXT	128	64	32	32	0.75	128	64	64	4	0.562	128	128	128	4	1.031	128	128	64	4	0.531
TIG+CIP	16	16	8	1	0.562	4	4	2	2	1	8	32	4	2	0.562	8	16	4	1	0.562
TIG+LEV	16	8	8	0.5	0.562	4	4	2	0.25	0.562	8	16	4	0.5	0.531	8	8	4	0.5	0.562
TIG+MXF	16	4	8	0.25	0.562	4	2	2	0.125	0.562	8	8	2	2	**0.5**	8	4	4	2	1
TIG+COL	16	64	8	2	0.531	4	8	1	2	**0.5**	8	128	4	16	0.625	8	512	4	32	0.562
TIG+SXT	16	64	8	32	1	4	64	2	4	0.562	8	128	4	4	0.531	8	128	4	64	1
COL+CIP	64	16	32	1	0.562	8	4	2	1	**0.5**	128	32	32	4	**0.375**	512	16	8	8	0.515
COL+LEV	64	8	16	2	**0.5**	8	4	2	2	0.75	128	16	32	2	**0.5**	512	8	64	1	**0.25**
COL+MXF	64	4	32	0.25	0.562	8	2	2	1	0.75	128	8	32	2	**0.5**	512	4	64	0.5	**0.25**
COL+DOX	64	16	32	8	1	8	4	4	2	1	128	32	64	1	0.531	512	32	128	8	**0.5**
COL+SXT	64	64	32	32	1	8	64	4	32	1	128	128	64	4	0.531	512	128	256	4	0.531
SXT+CIP	64	16	32	1	0.562	64	4	32	0.25	0.562	128	32	64	2	0.562	128	16	32	8	0.75
SXT+LEV	64	8	32	0.5	0.562	64	4	32	0.25	0.562	128	16	32	4	**0.5**	128	8	64	0.5	0.562
SXT+MXF	64	4	32	0.25	0.562	64	2	32	0.125	0.562	128	8	32	2	**0.5**	128	4	32	2	0.75

Abbrevations: CAZ: ceftazidime, CIP: ciprofloxacin, LEV: levofloxacin, MXF: moxifloxacin, DOX: doxycycline, TIG: tigecycline, COL: colistin, SXT: sulfamethoxazole-trimethoprim, AB1: MIC value of the first antibiotic agent, AB2: MIC value of the second antibiotic agent, AB1: MIC value of the first antibiotic agent in combination with the second one, AB2 + AB1: MIC value of the second antibiotic agent in combination with the first one AB1 + AB2: MIC value of the first antibiotic agent in combination with the second one, AB2 + AB1: MIC value of the second antibiotic agent in combination with the first one

Genetic testing was performed only in 27 strains out of the 30. 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. ERIC-PCR resulted in diverse patterns, but 4 identical patterns among the isolates (2 times 2 isolates, once 3 isolates and once 9 isolates with the same band pattern) were found. 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.

Discussion

The frequency of SXT-resistant *S. maltophilia* isolates was found to be 2% in our centre [18]. Despite this low frequency and the dominantly colonizing presence, emergence of resistance to SXT in *S. maltophilia* means a real challenge. Comparing the detected MIC values to our previous results, frequency of antibiotic resistance was found to be higher in SXT-resistant *S. maltophilia* strains than in SXT-susceptible ones [18]. Even if the resistance rate was 20–23% to levofloxacin and moxifloxacin, these newer fluoroquinolones mean real therapeutic options. Both can disrupt *S. maltophilia* biofilms [1, 2]. It was demonstrated that moxifloxacin possesses considerable time-kill effect on SXT-resistant *S. maltophilia* [19]. The high achievable concentration of quinolones in respiratory tract by applying them as inhalation therapy makes these antibiotic agents more valuable. As it was revealed from the final clinical reports retrospectively, 4 out of the 7 infected patients were successfully treated with levofloxacin or moxifloxacin. Susceptibility for doxycycline was investigated instead of the often tested minocyclin, since the latter has not been distributed in Hungary. Doxycycline looked to be the most effective antibiotic agent in this study, but it has to be emphasized that this result was based on *S. maltophilia* specific ECOFF. It is known that ECOFF and well-established clinical breakpoints can differ significantly; so it has to be assessed if this agent is a real therapeutic option. However, our result is in concordance with a previous study, in which doxycycline was found to be the most active agent against SXT-resistant *S. maltophilia* strains isolated from CF patients [10]. For the interpretation of tigecycline, the lowest breakpoints were applied. With other breakpoints (*Enterobacteriaceae* specific ones) the interpreted results of tigecycline susceptibility testing would have been more favourable. Even if chloramphenicol is a recommended drug according to CLSI and 13% of SXT-resistant *S. maltophilia* strains were susceptible in this study, the severe adverse effects of this drug must be born in mind.

Combination therapy is recommended for severe invasive infections, for immunocompromised patients and for empirical therapy in areas with high frequency of local resistance against SXT [4]. Combinations can be more active than monotherapy and can reduce the risk of developing antibiotic resistance during treatment, but superiority of combination therapy is not proved [1]. Clinical trails are missing yet. *Stenotrophomonas maltophilia* infections are often polymicrobial, where the use of a combination therapy may be also advantageous [14]. Against biofilm-growth *S. maltophilia* isolates combinations were *in vitro* effective, too [20]. In case of extremely drug-resistant *S. maltophilia* infections, combination therapy can be useful (maybe the only) therapeutic alternative. Several studies tested *in vitro* antibiotic combinations on SXT-susceptible *S. maltophilia* isolates, but only few ones focused on SXT-resistant ones [21]. By different methods, a lot of synergistic combinations were described; including those we tested [8, 11, 22–25]. According to our results, synergy is strain dependent. Ceftazidime and colistin can be synergistic with quinolones. The level of colistin or quinolones in aerosolised form can achieve 4–5 times higher concentration in airways than in serum during respiratory tract infections. Therefore, an antibiotic agent in aerosolised form in combination with an intravenous one can be effective, even if the FIC and SBPI do not show synergism [26].

Limitation of our study is that results gained by checkerboard method were confirmed only by combined E-test method (BioMérieux) [27]. Multiple-combination bactericidal testing and time-kill assay were not performed. However, there is no true gold standard for synergy testing. The literature suggests that no two synergy methods produce comparable results. Moreover, the true clinical relevance of synergy testing is unknown because of missing outcome-based studies [27]. A further limitation is the number of combinations tested. Many other combinations, including ticarcillin-clavulanate, aztreonam, azithromycin, rifampin, etc. could have been tested. We focused just on the most commonly used and available antibiotic agents in Hungary.

Methodically, synergic combinations detected by checkerboard method could be detected also by combined E-test method in our study. Maybe the E-test method is more practical in the daily routine in a busy diagnostic laboratory, but it requires very precise work. Moreover, the filmy growth of *S. maltophilia* microcolonies can make the evaluation of this test difficult. For antibiotic susceptibility testing of *S. maltophilia*, disc diffusion cannot be applied (except SXT), but synergic effects might be detected by double disc diffusion assay [14]. Ceftazidime plus levofloxacin or moxifloxacin synergy was detected also by this method in our study, but only tight inhibition zones were seen, not the typical keyhole shape.

Twenty-five isolates carried *sul*1, but only 7 were positive for class 1 integron with *int*I primers. It is possible that class I integrons may not have the same 5' and 3' ends, as described by Hu et al. [7]. Therefore the absence of a class 1 integron could not be confirmed. In combination with *dfr*A and *sul*2 genes, *sul*1 gene can lead to high resistance to SXT as well as multidrug resistance. While *sul1* genes were associated with class 1 integrons and *sul2* genes were found on plasmids, the mobilisation of *sul* genes is probable and likely to increase with SXT consumption. Ongoing resistance surveillance is needed [28].

In summary, SXT-resistant *S. maltophilia* strains are rarely isolated in our laboratory and the most of the isolates are colonizers. We concluded that levofloxacin and moxifloxacin are the most effective antibiotic agents against multidrug-resistant *S. maltophilia*. Combinations including fluoroquinolones – especially moxifloxacin – can be effective, even if the isolate was resistant to them. It has to be kept in mind that results of synergy testing are isolate specific.

Authors' Contributions

JE and KK conceived and designed the experiments. JE performed the experiments, analysed the data, wrote and corrected the manuscript. PJ and IM contributed in collecting the isolates and in correction of the manuscript. KK contributed reagents/materials.

Conflict of Interest

The authors declare that they have no competing interests.

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