Original Article

EMERGENCE OF CLINICAL STRAINS OF *Stenotrophomonas maltophilia* RESISTANT TO TRIMETHOPRIM/SULFAMETHOXAZOLE IN A BULGARIAN UNIVERSITY HOSPITAL

Sashka A. Mihaylova, Mariya P. Sredkova, Liselott A. Svensson[1], Edward R. B. Moore[1]

Sector of Microbiology and Virology, Department of Microbiology, Virology and Medical Genetics, Medical University, Pleven, Bulgaria
[1]Culture Collection University of Göteborg (CCUG), Department of Clinical Bacteriology, Sahlgrenska University Hospital, University of Göteborg, Göteborg, Sweden

Corresponding Author:
Sashka A. Mihaylova
Sector of Microbiology and Virology
Department of Microbiology, Virology and Medical Genetics
Medical University
1, St. Kliment Ohridski str.
Pleven 5800,Bulgaria
e-mail: sashkam@yahoo.com

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Summary

Clinical strains of *Stenotrophomonas maltophilia*, identified by a commercial system as resistant to trimethoprim/sulfamethoxazole (TMP/SMX), were analysed in detail, to confirm their taxonomic positions, to determine their susceptibilities to various classes of antibiotics and to assess this information with respect to the epidemiological relevance. The majority of strains were isolated from respiratory and wound specimens from patients admitted to intensive care units. Multi-locus sequence analyses (MLSA) of 16S ribosomal RNA (rRNA) and gyrase subunit B (*gyr*B) genes were applied for genotypic-based characterisation. The minimal inhibitory concentrations (MICs) of ten antimicrobial agents were determined, using the E-test method. The MIC values of TMP/SMX for the clinical isolates of *S. maltophilia* were greater than 32 mg/L, which confirmed their preliminary assessment as resistant. Minocycline, levofloxacin and ciprofloxacin exhibited the lowest MICs. All strains were observed to be susceptible to minocycline and levofloxacin. The emergence of clinical strains of *S. maltophilia* resistant to TMP/SMX is increasingly problematic as this antimicrobial agent is accepted as the "drug of choice" for treating infections caused by this bacterium. However, minocycline and levofloxacin demonstrated excellent *in vitro* activities and could be considered as alternative options to counter TMP/SMX-resistant strains of *S. maltophilia*.

Key words: *Stenotrophomonas maltophilia*, trimethoprim/sulphamethoxazole, resistance, 16S rRNA, *gyr*B

Introduction

Stenotrophomonas maltophilia, formerly classified as *Xanthomonas maltophilia* [1] and *Pseudomonas maltophilia* [2], is a Gram-negative, non-fermenting, obligately-aerobic bacterium. It is found ubiquitously in nature, in water, sediment and soil; it may be found in plant rhizosphere as a growth-promoting agent [3, 4, 5]. The genomic and physiological flexibility of the bacterium enables it to adapt readily to different environments, including those with relatively little available nutrient sources [6]. Importantly and due to its physiological plasticity, *S. maltophilia* is increasingly prevalent in hospitals as an opportunistic human pathogen,

causing infections in immuno-compromised individuals [7, 8]. Clinical manifestations of *S. maltophilia* infection include bacteraemia, endocarditis, pneumonia, meningitis, ophthalmologic syndromes, urinary tract infection, skin and soft tissue infection [9].

S. maltophilia may be identified in the clinical microbiology laboratory by various phenotypic tests. Several comparative studies have evaluated manual, semi-automated and automated systems and significant differences have not been observed in their abilities to identify this species [10, 11]. However, some reports indicate that commercial systems might identify other taxa, for example, species or genomovars of the *Burkholderia cepacia* complex, as *S. maltophilia* [12]. Misidentifications are significant for assessing the choice of antimicrobial treatment, particularly for individuals with cystic fibrosis and infections of immuno-compromised patients. In such cases, accurate identification of *S. maltophilia* is essential. The development and application of genotypic methods for the characterisation and identification of bacteria are well documented [13, 14, 15]. The combination of 16S rRNA and gyrase subunit B (*gyr*B) gene sequencing enables a reliable and highly differential multi-locus sequence analysis (MLSA) approach for identification of strains of closely related *Stenotrophomonas* species.

S. maltophilia is considered to be a multidrug-resistant bacterium [16], with trimethoprim/sulphamethoxazole (TMP/SMX) accepted as the "drug of choice" for treating infections. During the last decade, reports have appeared, concerning the recognition of TMP/SMX-resistant clinical isolates of *S. maltophilia* and their emergence as a serious threat in the treatment of infections [16]. Commercial systems used for antibiotic susceptibility testing evaluate only the *in vitro* effect of TMP/SMX on *S. maltophilia* and include a comment that other antimicrobials are not recommended. Thus, in the case of *S. maltophilia* strains resistant to TMP/SMX, no *in vitro* data regarding their susceptibilities to alternative antibiotics are available.

Materials and Methods

Bacterial strains

Fifteen strains were chosen from the Culture Collection of the University of Medicine - Pleven (CCUMP), Bulgaria, which holds clinically-relevant microorganisms. In order to be included in the study, the strains met the following criteria: they were identified by the VITEK® 2 GN card (bioMérieux) as *Stenotrophomonas maltophilia*; and they were defined by the VITEK® 2 AST-NO22 card (bioMérieux) as resistant to TMP/SMX.

Bacterial DNA preparation

S. maltophilia strains were inoculated in Tryptic Soy Broth medium and incubated at 35°C, with shaking, for 48 hours. Broth cultures were centrifuged (14 000 x *g*), to harvest the cell biomass. Genomic DNA was prepared, using the BIO101 Systems FastDNA Kit, according to the guidelines of the manufacturer (Qbiogene, Inc.). The concentrations of extracted DNA were measured by spectrophotometry (Bio-Photometer, Eppendorf) at 260 nm.

Primary DNA sequence analysis of 16S rRNA genes

The genes for the nearly complete 16S rRNA were amplified by PCR, using the primers M16F28 (5' - AGAGTTTGATCKTGGCTCAG-3') and M23R458 (5' - CCCCTTTCCCTCACGGTAC - 3'), hybridising at 16S rRNA gene sequence nucleotide positions 9-28 (*Escherichia coli* 16S rRNA gene sequence numbering) and at 23S rRNA gene sequence nucleotide positions 458-473 (*E. coli* 23S rRNA gene sequence numbering), respectively. PCRs were carried out in duplicate 50 µl reaction volumes containing 25 µl *Taq* PCR Master Mix Kit (Qiagen), 1 µM forward and reverse primers and 100 ng template DNA. PCR products were visualised by agarose gel electrophoresis and staining with ethidium bromide. PCR-DNAs from the duplicate reactions were combined, purified, using the QIAquick PCR purification Kit, according to the recommendations of the manufacturer (Qiagen), and sequenced directly, using the BigDye Terminator v3.1 Cycle Sequencing Kit and oligonucleotide primer 16R518 [17]. Sequencing was carried out with an ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems, Inc.). Sequences were analysed with the Kodon version 3.0 software package (Applied Maths, BVBA), edited and a uniform sequence length was used for sequence similarity and cluster analyses. Reference sequence data used for comparative analyses were obtained from the EMBL Nucleotide Sequence Database [18].

Secondary DNA sequence analysis of gyrB genes

The genes for *gyr*B were amplified by PCR, using the primers UP-1 and UP-2r [19] and the reaction conditions described above. Sequencing of PCR-products was performed as described above, but using the *gyr*B amplification primers. Sequence data were edited and analysed as described above.

Susceptibility testing

The minimal inhibitory concentrations (MICs) of TMP/SMX, ceftazidime, cefepime, ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin/clavulanic acid, amikacin, minocycline, ciprofloxacin and levofloxacin were determined, using the E-test method, according to the guidelines of the manufacturer (AB Biodisk, Solna, Sweden).

Epidemiological assessment

Patient records were reviewed retrospectively for epidemiological information. The following points were registered: age; gender; clinical diagnosis; and hospital ward at the time of isolation of *S. maltophilia*.

Results

The strains of *Stenotrophomonas maltophilia* included in this study were isolated from samples of patients admitted to Pleven University Hospital between 1999 and 2007 (Table 1). The bacteria were isolated mainly from respiratory tract and wound specimens. Other types of samples from which *S. maltophilia* were isolated included blood and urine specimens. The majority of the patients were being treated in ICUs. They were adults with ages varying from 18 to 71 years. Eight patients were female and seven patients were male. No temporal clustering of infections was observed.

Table 1. *Stenotrophomonas maltophilia* strain information, demographic and clinical data of patients with *S. maltophilia* infection

CCUMP number	Collection Date	Specimen type	Patient gender	Patient age	Clinical diagnosis	Hospital ward
151	11.01.1999	Tracheal aspirate	F	Adult	Polyradiculoneuritis	Neuro-surgical ICU
131	15.01.1999	Tracheal aspirate	F	28 y	General trauma	Neuro-surgical ICU
152	22.02.1999	Tracheal aspirate	F	60 y	Haemorrhagic insult	Neuro-surgical ICU
135	20.08.1999	Blood	M	25 y	General trauma	Medical-surgical ICU
3	30.01.2000	Drainage	M	66 y	Status after nephrectomy	Medical-surgical ICU
64	03.03.2000	Wound	M	66 y	General trauma	Medical-surgical ICU
115	02.05.2000	Tracheal aspirate	F	71 y	Ischaemic insult	Neuro-surgical ICU
116	08.05.2000	Tracheal aspirate	F	18 y	General trauma	Medical-surgical ICU
139	13.06.2000	Urine	F	68 y	Disseminated intravascular coagulation syndrome	Nephrology
160	21.03.2001	Drainage	F	66 y	Necrotic pancreatitis	Medical-surgical ICU
273	05.12.2002	Bronchoalveolar lavage	F	35 y	Haemorrhagic insult	Medical-surgical ICU
275	10.01.2004	Blood	M	36 y	Duodenal ulcer	Surgery
337	17.02.2004	Drainage	M	Adult	Status after thoracocenthesis	Medical-surgical ICU
300	06.04.2006	Tracheal aspirate	M	54 y	Aortic rupture	Operating room
451	19.01.2007	Drainage	M	Adult	Pneumothorax	Surgery

ICU, intensive care unit

Sequencing of PCR-amplified 16S rRNA genes, using a single primer reaction, allowed the determination and analysis of a stretch of 435 nucleotide positions, after editing. These partial gene sequences corresponded to approximately 30% of the complete 16S rRNA gene primary sequence, based upon the length of the gene sequence of *S. maltophilia*. The 16S rRNA gene sequences of the clinical strains were compared with the gene sequences of the type strains of the genus *Stenotrophomonas*. The gene sequences of the clinical strains were observed to be most similar to those of the type strain for *S. maltophilia* (CCUG 5866-T), with similarity levels ranging from 99.4 % to 100 %, or *S.* "*africana*" (*S.* "*africana*" is a later synonym of *S. maltophilia*), with similarity levels ranging from 98.5 % to 99.4 % (Figure 1).

The *gyr*B gene sequences used in this study were generated by two sequence reactions, comprising 916 nucleotide positions, after editing. Sequence similarities for the *gyr*B genes

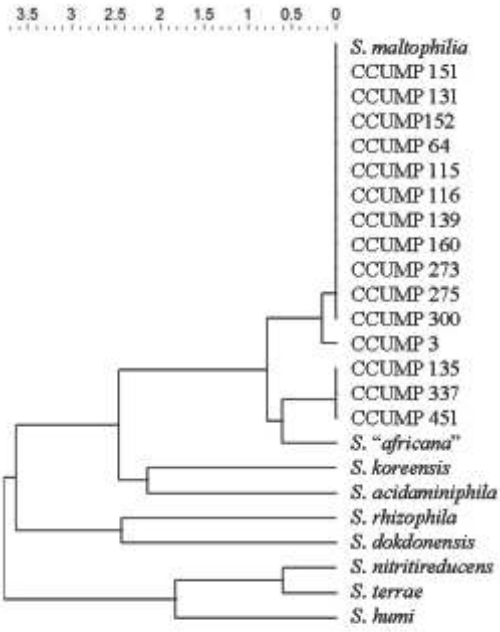

Figure 1. UPGMA cluster analysis of 16S rRNA gene partial sequences from 15 clinical strains of *S. maltophilia* and related species of the genus *Stenotrophomonas*

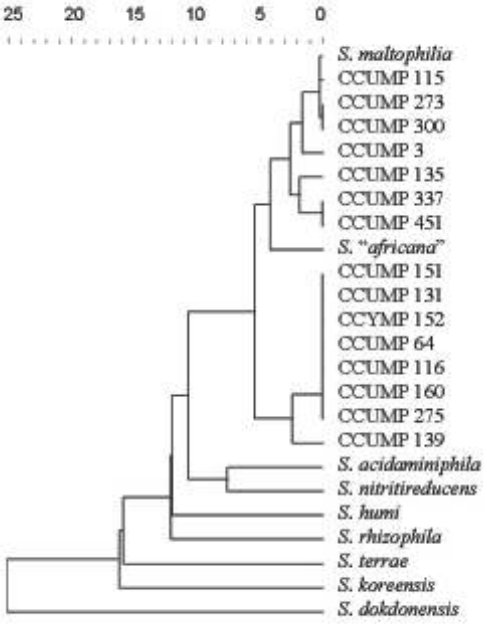

Figure 2. UPGMA cluster analysis of *gyr*B partial sequences from 15 clinical strains of *S. maltophilia* and related species of the genus *Stenotrophomonas*

of the clinical strains with that of *S. maltophilia* varied from 99.8 % to as low as 93.9 % (Figure 2).

The MIC values of the ten antimicrobials that were tested and the susceptibilities of clinical strains, determined using the E-test method, are presented in Table 2 and Figure 3. The MICs of TMP/SMX were observed to be greater than 32 mg/L for all clinical strains, which confirmed the preliminary assessment by the VITEK® 2 system (bioMérieux). The $MICs_{90}$ of ceftazidime, ampicillin/sulbactam, piperacillin/tazobactam were greater than 256 mg/L and all strains were resistant or intermediately susceptible to them. Only one strain was susceptible to ticarcillin/clavulanic acid, three strains were susceptible to cefepime and eight strains were susceptible to amikacin. Minocycline and the tested fluoroquinolones exhibited the lowest MIC values. All clinical strains of *S. maltophilia* tested were observed to be susceptible to minocycline and levofloxacin.

Table 2. Results from susceptibility testing of 15 *S. maltophilia* strains to 10 antimicrobial agents, using the E-test method

Antimicrobial agent	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC_{Range} (mg/L)
TMP/SMX	>32	>32	>32
Ceftazidime	24	>256	12 – >256
Cefepime	16	128	2 – >256
Ampicillin/sulbactam	>256	>256	16 – >256
Piperacillin/tazobactam	>256	>256	32 – >256
Ticarcillin/clavulanic acid	48	>256	12 – >256
Amikacin	16	>256	4 – >256
Minocycline	0.25	0.38	0.023 – 0.5
Ciprofloxacin	0.75	1.5	0.047 – 2
Levofloxacin	0.38	0.5	0.125 – 0.75

$MIC_{50,}$ Minimal inhibitory concentration required to inhibit the growth of 50% of organisms
$MIC_{90,}$ Minimal inhibitory concentration required to inhibit the growth of 90% of organisms

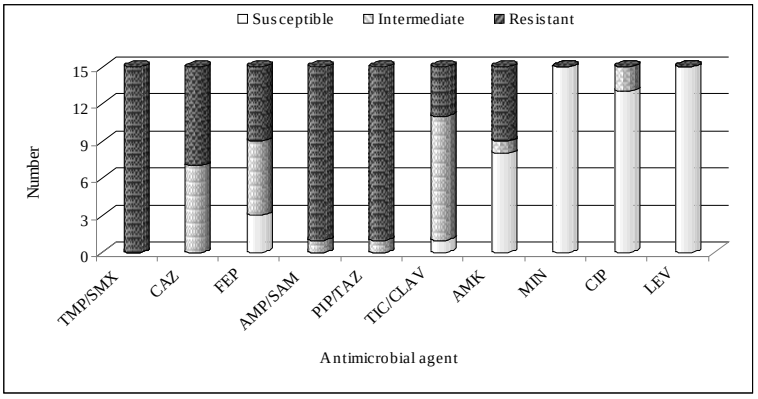

Figure 3. Comparative data on the susceptibilities of 15 *S. maltophilia* strains to 10 antimicrobial agents (CAZ, ceftazidime; FEP, cefepime; AMP/SAM, ampicillin/sulbactam; PIP/TAZ, piperacillin/tazobactam; TIC/CLAV, ticarcillin/clavulanic acid; AMK, amikacin; MIN, minocycline; CIP, ciprofloxacin; LEV, levofloxacin)

Discussion

Stenotrophomonas maltophilia was isolated initially in 1943 from pleural fluid by J. L. Edwards, and classified as *Pseudomonas maltophilia* by Hugh and Ryschenkow [20] At that time, the bacterium was recognised as a significant isolate of clinical specimens, causing infections in compromised human hosts [2]. *P. maltophilia* was transferred to the genus *Xanthomonas*, including plant pathogenic species, primarily on the basis of genotypic characteristics [1]. Subsequently, *X. maltophilia* was transferred into a separate and new genus due to noted differences between *X. maltophilia* and the other species of the genus *Xanthomonas* [21]. Since the reclassification of *S. maltophilia*, eight other species have been added to the genus.

More than sixty years later, *S. maltophilia* continues to pose problems in clinical microbiology. Accurate and reliable identification of *S. maltophilia* in certain patient populations is vital since misidentification has important consequences [12]. False positive identifications, for example, as *B. cenocepacia*, would lead to unnecessary segregation of patients, while false-negative identifications (for example, if a strain of *B. cenocepacia* is misidentified as *S. maltophilia*) would result in risks of "cepacia syndrome", with potential spread to other patients.

In this study, a comparative MLSA strategy, targeting 16S rRNA and *gyr*B genes, provided definitive identifications of clinical strains of *S. maltophilia.*

The role of 16S rRNA gene sequence analysis for identifications of clinically-relevant bacteria has been described [22, 23]. The primary genotypic marker sequence of the MLSA of this study employed partial 16S rRNA gene sequences, using 435 nucleotide positions of the 5'-region of the gene derived from a single primer reaction. Comparisons of complete 16S rRNA gene sequences of the type strains of the nine species of the genus *Stenotrophomonas*, defined a total of 112 nucleotide positions observed to vary. Thus, only 7.5% of the gene sequence is susceptible to variation between the sequences of the different species of the genus *Stenotrophomonas*. The region of 435 nucleotide positions determined in this study included 36 variable positions, or approximately 30% of the potential number of variable positions contained in the complete gene sequence, and enabled putative identifications to the genus and sub-genus levels. However, the 16S rRNA gene sequences of the species of *Stenotrophomonas* are so conserved that reliable differentiation of species can be problematic.

Because of the high similarities between the 16S rRNA gene sequences of the species of *Stenotrophomonas* and the resulting limited levels of resolution, the sequences for the *gyr*B housekeeping gene were used as a secondary genotypic marker for species level resolution and identification. The similarities of *gyr*B gene sequences between the different species of the genus *Stenotrophomonas* ranged from 96.0% (between *S. maltophilia* and *S.* "*africana*") to as low as 74.1% (between *S. koreensis* and *S. dokdonensis*). Although *S.* "*africana*", isolated from cerebrospinal fluid, was described initially as a new species of *Stenotrophomonas* [24], it was reanalysed and described subsequently as a

later synonym of *S. maltophilia* [25]. Thus, the *gyr*B gene sequence similarity of *S.* "*africana*" with *S. maltophilia* can be seen as typical for strains of *S. maltophilia*. Similarities of *gyr*B gene sequences between species of *Stenotrophomonas*, excluding *S.* "*africana*", extended to as high as 92.8%, which can be applied as a presumptive "cut-off" for delineating the different species of the genus. The *gyr*B gene sequence similarities of the clinical isolates, ranging from 99.8% to 93.9% with *S. maltophilia*, thus, fall within the range expected for strains of a species. These MLSA data further confirm the initial identifications of the isolates, supporting the performance of the commercial VITEK® 2 system (bioMérieux) for identification of the species *S. maltophilia*.

S. maltophilia is considered to be a multidrug-resistant bacterium [16]. In an extensive study of clinical and environmental isolates of *S. maltophilia*, the vast majority of strains exhibited marked resistance to 17 of 19 tested antibiotics [26]. Interestingly, the clinical isolates exhibited higher resistances to most of the antibiotics tested than the isolates obtained from environmental samples. Several studies have compared the different methods for antibiotic susceptibility testing of *S. maltophilia*. The commercial VITEK® system (bioMerieux, Hazlewood, MO) has correlated poorly with microbroth and agar diffusion methods [27], whereas, the E-test method has shown excellent agreement with the agar dilution method, which is considered to be the reference method [28]. In this study, antibiotic susceptibility was assayed with E-test strips on Mueller-Hinton agar. All strains were observed to be resistant to TMP/SMX, with MICs greater than 32 mg/L. Confirmation of TMP/SMX resistance in clinical isolates of *S. maltophilia* required establishing new options for treating infections. Ticarcillin/clavulanic acid is another agent of potential therapeutic value, although most of the strains tested in this study exhibited resistance or intermediate susceptibility to it. A review of the literature reveals that the newer fluoroquinolones demonstrate enhanced activities against *S. maltophilia*, compared with ciprofloxacin [16, 29, 30, 31]. In this study, levofloxacin displayed excellent *in vitro* activities against the tested strains. In fact, no strain of *S. maltophilia* fully resistant to ciprofloxacin was detected. These results indicate that the fluoroquinolones could be an effective therapeutic option for the treatment of infections. The potential benefit of minocycline against *S. maltophilia* has recently been stressed and has led to increased frequency in use [32]. The high *in vitro* activity of minocycline detected in this study demonstrates that this drug could be considered as an additional agent in the treatment of infections caused by *S. maltophilia*. In general, treatment strategies for *S. maltophilia* are similar to those employed for other multidrug-resistant microorganisms, whereby high doses of two or more parenteral agents, with different mechanisms of action, are used to manage an infection. Gabriel et al. [33] performed the largest *in vitro* study of antibiotic combinations against clinical strains of *S. maltophilia*. Synergistic or additive activities were demonstrated by TMP/SMX paired with ticarcillin/clavulanic acid (65% of strains), ciprofloxacin paired with ticarcillin/clavulanic acid (64% of strains), ciproofloxacin paired with piperacillin/tazobactam (59% of strains), and TMP/SMX paired with piperacillin/tazobactam (55% of strains).

This study did not focus on identifying the risk factors for the emergence of TMP/SMX-resistant *S. maltophilia*, because of a limited number of affected patients. Evidently, *S. maltophilia* is isolated from specimens from different clinical conditions and this bacterium continues to be problematic for patients admitted to ICUs.

Conclusions

The emergence of clinical strains of *S. Maltophilia* resistant to TMP/SMX is a significant cause for alarm, as this antimicrobial agent is the accepted drug of choice for treating infections. The results of this study indicate that minocycline and levofloxacin could be considered a promising alternative treatment. The bacteriostatic action of minocycline and the possibility of resistance development during therapy warrant careful consideration of antimicrobial combinations in the treatment of severe infections, especially in immuno-compromised patients.

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