JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2013. Vol. **7**(2), p. 905-912

Multi-drug Resistance in *Stenotrophomonas maltophilia* Isolated from Nkonkobe Municipality Environment in the Eastern Cape Province, South Africa

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(Received: 02 October 2012; accepted: 14 November 2012)

Stenotrophomonas maltophilia **is increasingly emerging as an opportunistic pathogen of global concern. Trimethoprim-sulphamethoxazole (TMP-SMX) is the generally accepted antibiotic of choice for the treatment of infections caused by this organism, but resistance to the drug is increasingly being reported; hence, the need for alternative therapeutic options. In this study, the antimicrobial susceptibility profile of 110 commensal *S. maltophilia* isolates obtained from Nkonkobe municipality, Eastern Cape Province, Republic of South Africa was investigated. Twenty-one antibiotics including TMP-SMX and the fluoroquinolones; levofloxacin, gatifloxacin and moxifloxacin were included in the antibiotic panel. About 63.4% of the isolates were susceptible to TMP-SMX with a resistance rate of 28.2%. The fluoroquinolones were more effective with susceptibilities ranging from 76% to 94.7%. Levofloxacin was the most effective fluoroquinolone tested. Phenotypic dectection of extended spectrum β-lactamases (ESBLs) showed double disc synergy test (DDST) positivity in 59.5% of the isolates. Isolates exhibited nine different ESBL phenotypes, however, PCR amplification of the *bla* genes revealed four isolates that possessed genes belonging to the *bla*CTX-M group (*bla*CTX-M-1 and *bla*CTX-M-8 groups). ESBL positive isolates appeared more susceptible to the fluoroquinolones compared to TMP-SMX. The fluoroquinolones are a possible alternative treatment option for *S. maltophilia* infections in this environment.**

Keywords: *Stenotrophomonas maltophilia*, Antibiogram, Trimethoprim-sulphamethoxazole, fluoroquinolones, ESBL.

Stenotrophomonas maltophilia, a non-fermentative Gram-negative bacillus, which was initially thought to be a predominantly non-pathogenic environmental organism, is becoming progressively recognised as an important nosocomial organism responsible for significant morbidity and mortality in immunocompromised and debilitated patients[1,2]. The increase in *S. maltophilia* infections has been attributed to advances in cancer treatment, the use of invasive therapeutic devices and the widespread use of broad spectrum antibiotics[3].

S. maltophilia displays a vast array of resistance mechanisms which contribute independently or collectively to its multi-drug resistant status[4]. These include the expression of multiple β-lactamases, inducible efflux pumps, outer membrane impermeability[5] and also the acquisition of integrons, plasmids and transposons which carry various resistance genes[6].

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Trimethoprim-sulphamethoxazole (TMP-SMX) is the recommended treatment of choice for *S. maltophilia* infections, however, resistant strains are increasingly being reported which have been linked to mobile elements that also carry extended spectrum beta-lactamase genes[7,8].

Majority of the studies done on antibiotic resistance have been carried out on clinical and veterinary isolates but there is mounting evidence that resistance phenotypes are being selected in natural environments. The susceptibilities of these environmental isolates remain largely uncharacterised and this study was carried out to determine the antibiotic susceptibility profile of *S. maltophilia* isolates in the environment of the Eastern Cape Province, South Africa.

MATERIALS AND METHODS

One hundred and ten (110) *S. maltophilia* isolates were obtained from the culture collection of Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa for use in this study. The bacteria had been previously isolated from the rhizosphere of plants in Nkonkobe Municipality, Eastern Cape Province, South Africa which is a predominantly rural and agrarian community. Identification was done using standard biochemical methods, confirmed with the API 20NE identification kit and species specific PCR[10].

The antibiogram of the isolates was determined by disc diffusion susceptibility testing following the standardised guidelines outlined elsewhere[11]. A total of 21 antibiotic discs (MAST Diagnostics, Merseyside, United Kingdom) were used and includes: meropenem (30 µg), ampicillin (10 µg), ampicillin-sulbactam (20 µg/10 µg), amoxicillin-clavulanic acid (20 µg/10 µg), aztreonam (30 µg), minocycline (30 µg), kanamycin (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), gatifloxacin (5 µg), moxifloxacin (5 µg), cefuroxime (30 µg), ceftazidime (30 µg), cefpodoxime (10 µg), cefepime (30 µg), cefotaxime (30 µg), colistin sulphate (10 µg) and polymyxin B (300 U), trimethoprim-sulfamethoxazole (TMP-SMX) (1.25 µg/23.75 µg), trimethoprim (5 µg). The use of these various antibiotics from several classes was to provide a baseline description of the antimicrobial susceptibility of *Stenotrophomonas maltophilia* isolates in this important agrarian community for which no such information existed, and could serve as a guide to inform future investigations.

The bacterial suspensions were streaked on freshly prepared Mueller-Hinton agar plates, antibiotics applied and incubated at 35°C for 20-24 h. The zones of inhibition were then measured and interpreted using the Clinical Laboratory Standards Institute (CLSI) recommended *S. maltophilia* zone diameter breakpoints for TMP-SMX, levofloxacin and minocycline. Disc breakpoints for other non-fermentative Gram-negative bacilli or *Pseudomonas aeruginosa* were applied for interpretation of other antibiotics[11].

The multiple antibiotic resistance (MAR) index, when applied to a single isolate, is defined as [a/b], where [a] represents the number of antibiotics to which the isolates was resistant and [b] represent the number of antibiotics against which the isolate was tested[12]. The MAR index of the isolates was determined using this formula.

Phenotypic detection of ESBLs

Phenotypic confirmation of ESBL production was done using the double disc synergy test (DDST) as described by Jarlier *et al.*[13] with modifications. Isolates that showed resistance or intermediate susceptibility to the cephalosporins were included. The inoculum was standardized as previously described and streaked on freshly prepared Mueller-Hinton agar plates. Antibiotic discs (MAST Diagnostics) containing ceftazidime (30 µg), cefotaxime (30 µg), cefpodoxime (10 µg) and cefepime (30 µg) were placed around a central amoxicillin-clavulanic acid (30 µg) disc at a distance of 25 mm centre to centre. Isolates that showed synergy towards the central clavulanate disc were phenotypically confirmed to be ESBL producers.

Extraction of genomic DNA

Genomic DNA was extracted using the method of Alzahrani *et al.*[14] with modifications. The *S. maltophilia* isolates were subcultured on Luria-Bertani agar plates and incubated at 37°C overnight. Three to five colonies of the bacteria was then suspended in 250 µl of sterile nuclease-free water and vortexed to achieve a uniform suspension. The cells were then lysed by heating to 100°C for 15 min on a heating block (Accublock Digital Dry Bath, Labnet). The suspension was centrifuged (Thermo scientific, Haraeus Fresco 17) at 15,000 rpm for 15 min at 4°C to remove cell debris

and the lysate was stored at -20°C until used for the PCR reactions.

PCR amplification

Conventional PCR was used to amplify possible ESBL genes from the isolates that showed DDST positivity. Amplification was carried out with specific primers based on already published sequences of the ESBL enzyme groups [Table 1] as described in the method by Schlesinger *et al.*[15] with modifications to screen for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{IBC}, bla_{PER}, bla_{OXA}, bla_{VEB} and bla_{SFO} genes. All reactions were performed at a final volume of 25 µl containing 12.5 µl PCR master mix 2x (Fermentas), 0.5 µl of each primer (Inqaba Biotec), 3 µl template DNA and 8.5 µl nuclease free water (Fermentas) under the following conditions in a Biorad Mycycler Thermal Cycler: 15 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at the annealing temperature published for each primer set, 1 min at 72°C followed by a further extension for 10 min at 72°C. Ten microlitres of each PCR amplicon was then loaded on 1.5% agarose (Pronadisa, low grade EEE agarose) gel containing 0.5x TBE buffer along with 100 bp DNA ladder (Fermentas, GeneRuler, 0.5 µg/µl). Electrophoresis was carried out at a voltage of 100 V for 75 min. The gel was stained with 0.5 µg ml^{-1} ethidium bromide and the DNA was then visualised with a UV transilluminator and captured with the documentation software Alliance 4.7 (Uvitec).

RESULTS AND DISCUSSION

The antibiogram profile of the *S. maltophilia* isolates assessed is as shown in Table 2. Generally, this current study revealed that the bacterial isolates were inherently resistant to several antibiotic groups including β-lactams, macrolides and aminoglycosides in support of previous reports[5]. TMP-SMX is generally accepted worldwide as the antimicrobial of choice for the treatment of infections caused by *S. maltophilia*. Numerous studies have found the antibiotic to be the most effective amongst those tested[16,17,18] and in this study, 63.4% of the isolates were susceptible to the antibiotic while resistance was observed in 28.2%. This is similar to the results obtained in other studies[5,6,7]. Resistance to TMP-SMX has been attributed to the selection pressure caused by extensive use of the antibiotics in patients with serious debilitating diseases and poor immunity[7]. South Africa has a high burden of HIV/AIDS and TMP-SMX is widely used in affected persons as prophylaxis against opportunistic infections such as *Pneumocystis jirovecii* pneumonia[19]. This may explain the high level of resistance to TMP-SMX in this environment. Studies done suggest that increased use of TMP-SMX may lead to increased resistance in invasive organisms in hospitals as well as the community[20]. The high level of resistance may also reflect the effect of use of antibiotics in agricultural practices. Valdezate *et al.*[5] however suggest that these differences may be due to the use of different methodologies and varying breakpoints in the performance and interpretation of susceptibility tests for *S. maltophilia*.

The presence of TMP-SMX-resistant *S. maltophilia* strains has significant implications. A study by Tsiodras *et al.*[21] investigated the clinical implications of TMP-SMX resistant strains and found that these infections occurred in severely ill patients with extensive exposure to the health care system and often required invasive procedures for effective treatment. Infections were directly associated with severe morbidity. Recent studies done have indicated that resistance genes responsible for TMP-SMX resistance are linked to insertion sequence common region (ISCR) elements[7]. These ISCR elements are thought to be responsible for the mobility and dissemination of many antibiotic resistance genes including ESBLs, carbapenemase genes and aminoglycoside, chloramphenicol and quinolone genes[22-25]. In this study, all the isolates resistant to TMP-SMX showed a high level of resistance to the penicillins, cephalosporins, and the aminoglysoside tested; further confirming the multi-drug resistant phenotype of these isolates.

It has been suggested that the fluoroquinolones may be a suitable alternative treatment option for *S. maltophilia* infections. In agreement with other studies[5,9,18,26], the new fluoroquinolones; levofloxacin, gatifloxacin and moxifloxacin all showed good activity against the isolates with susceptibilities ranging from 88% to 94.7% whereas the susceptibility to ciprofloxacin was 76%. Valdezate *et al.*[18] reported that more than 95% of the isolates tested in their study were susceptible to the newer fluoroquinolones and

Table 1. Primers sequences, annealing temperature, expected product size and references

Primer type and gene family	Sequence	Annealing temp (°C)	Reference	PCR produt size (bp)
TEM	F:5'-TCAACATTTCCGTGTCG-3' R:5'-CTGACAGTTACCAATGCTTA-3'	42	15	860
SHV	F:5'-ATGCGTTATATTCGCCTGTG-3' R:5'-AGATAAATCACCACAATGCGC-3'	47	15	780
CTX-M 1	F:5'-GACGATGTCACTGGCTGAGC-3' R:5'-AGCCGCCGACGCTAATACA-3'	53	38	490
CTX-M 2	F:5'-ATGATGACTCAGAGCATTCG-3' R:5'-TGGGTTACGATTTTCGCCGC-3'	55	39	870
CTX-M 8	F:5'-CTGGAGAAAAGCAGCGGGGG-3' R:5'-ACCCACGATGTGGGTAGCCC-3'	51	40	580
CTX-M 9	F:5'-ATGGTGACAAAGAGAGTGCA-3' R:5'-CCCTTCGGCGATGATTCTC-3'	55	38	870
OXA	F:5'-ACACAATACATATCAACTTCGC-3' R:5'-AGTGTGTTTAGAATGGTGATC-3'	42	41	810
IBC	F:5'-GGGCGTACAAAGATAATTTCC-3' R:5'-GAAGCAACGTCGGCTTGAACG-3'	47	15	940
VEB	F:5'-ACGGTAATTTAACCAGATAGG-3' R:5'-ACCCGCCATTGCCTATGAGCC-3'	46	15	970
SFO	F:5'-GTTAATCCATTTTATGTGAGG-3' R:5'-CAGATACGCGGTGCATATCCC-3'	44	15	940
PER	F:5'-ATGAATGTCATTATAAAAGC-3' R:5'-AATTTGGGCTTAGGGCAGAA-3'	42	42	930

Table 2: Antibiogram of *Stenotrophomonas maltophilia* isolates. S (Susceptible); I (Intermediate); R (Resistant).

Antibiotic class	Antibiotics	S (%)	I (%)	R (%)
Penicillin	Ampicillin	42.6	1.5	55.9
Penicillin/β-lactam inhibitor	Ampicillin-sulbactam	73.0	4.0	23.0
Monobactam	Aztreonam	14.3	27.1	58.6
Carbapenems	Meropenem	87.3	3.8	8.9
Tetracycline	Minocycline	93.7	0	6.3
Aminoglycoside	Kanamycin	40.6	7.2	52.2
Fluoroquinolones	Ofloxacin	89.2	8.1	2.7
	Ciprofloxacin	76.0	21.5	2.5
	Levofloxacin	94.7	4.0	1.3
	Moxifloxacin	88.0	9.3	2.7
	Gatifloxacin	92.3	6.4	1.3
Cephalosporins	Ceftazidime	81.8	5.2	13.0
	Cefuroxime	2.8	2.8	94.4
	Cefepime	58.4	10.4	31.2
	Cefotaxime	5.2	13.2	81.6
	Augmentin	59.7	7.8	32.5
Polymyxins	Colistin sulphate	97.2	0	2.8
	Polymyxin B	97.2	0	2.8
Dihydrofolate reductase inhibitors	Trimethoprim-sulfamethoxazole	63.4	8.4	28.2
	Trimethoprim	2.8	1.4	95.8

Weiss *et al.*[9] in a comparison of seven quinolones found that clinafloxacin, moxifloxacin and trovafloxacin had significantly better *in vitro* activity compared to ciprofloxacin and levofloxacin. They also found that the quinolones were effective against some of the isolates that were resistant to TMP-SMX. This is comparable to the results in this study in which all the TMP-SMX resistant isolates were susceptible to the fluoroquinolones tested except for four TMP-SMX resistant isolates that showed only intermediate susceptibility to ciprofloxacin.

The issue of ESBL production in *S. maltophilia* is a controversial one. Several studies have reported double disk synergy test positivity in clinical isolates of this organism[27,28,29] but Munoz Bellido and Garcia-Rodriguez[30] suggest that these reactions could be explained by other properties of the L2 β-lactamase of *S. maltophilia*. More recent studies, making use of phenotypic and molecular methods have, however, further confirmed the presence of ESBLs in this species[31,32]. In this study, 59.5% of the isolates were positive for ESBL production using the DDST, however, some ESBL phenotypes may have been masked by the effect of the L1 β-lactamase.

A little over a decade ago, investigations into extended-spectrum β-lactamase enzymes almost exclusively revealed TEM and SHV types of ESBLs. However, the dynamics have changed with the CTX-M ESBLs increasingly becoming the predominant enzyme type isolated from Gram-negative organisms and it is being found more frequently in community isolates[33]. The PCR amplification of *bla* genes (Table 1) in this study revealed the presence of *bla*CTX-M ESBLs in four of the *S. maltophilia* isolates. This is similar to results obtained by Al-Naeimi *et al.*[31] and Lavigne *et al.*[34] who reported the presence of CTX-M enzymes in clinical isolates of *S. maltophilia*. In South Africa, several studies have also reported the presence of CTX-M ESBLs in several Gram-negative organisms including *P. aeruginosa* and *E. coli* [35,36,37] but to the best of our knowledge this is the first description of *bla*CTX-M genes in environmental isolates of *S. maltophilia*. Extended-spectrum β-lactamases genes are often plasmid-encoded along with antibiotic resistance genes; thus suggesting the need for further studies targeting the molecular basis of multiple drug resistance observed in these environmental isolates and these are ongoing in our group. Also, about 79% of the isolates tested in this study had MAR index greater than 0.2 (Table 3). This indicates that they originated from a high risk source of contamination which may reflect the effect of the agricultural practices in this area[12].

Table 3. MAR indices of *Stenotrophomonas maltophilia* isolates

MAR index	% of isolates (n=110)
0.06	1.3 (2)
0.10	5.3 (6)
0.19	14.5 (16)
0.21	1.3 (2)
0.24	13.2 (14)
0.25	1.3 (2)
0.29	13.2 (14)
0.31	3.9 (4)
0.33	13.2 (14)
0.38	3.9 (4)
0.43	13.2 (14)
0.45	1.3 (2)
0.48	6.6 (7)
0.50	3.9 (4)
0.62	1.3 (2)
0.67	0.9 (1)
0.70	1.3 (2)

CONCLUSION

Majority of the studies done to evaluate the antimicrobial susceptibility profile of *S. maltophilia* have been carried out on clinical isolates but with mounting evidence on the interactions between human pathogenic bacteria and environmental bacteria, the need for surveillance of these resistance reservoirs cannot be underestimated. The high level of TMP-SMX resistance seen in these environmental isolates further buttresses the need for special attention on these organisms as possible reservoirs of antibiotic resistance determinants. Furthermore, with the ongoing pandemic of HIV/AIDS and the continuous selective pressure exerted on these organisms, community-acquired multidrug resistant infections may increase. TMP-SMX still remains an effective drug for the management of *S. maltophilia* infections if a tight rein is kept on its appropriate use. The fluoroquinolones however

provide suitable alternatives in cases where TMP-SMX is either unsuitable or ineffective. The presence of ESBL genes has significant implications for infection control specialists and microbiologists because laboratories do not routinely screen for the presence of ESBLs in this group of organisms. Their occurrence in these organisms may therefore, be seriously underreported making nosocomial and opportunistic organisms such as *S. maltophilia* an un-monitored reservoir of these genes with the potential to spread them to both community and hospital isolates. Further studies are however required to determine how these environmental isolates acquired the *bla*CTX-M ESBLs. Their presence may indicate the increasing proportion of *bla*CTX-M positive isolates of other Gram-negative bacteria in the community or the transfer of these resistance genes from hospital pathogens to environmental bacteria.

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