Microbes and Infectious Diseases 2022; 3(2): 300-308

Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Emergence of *Stenotrophomonas maltophilia* co-harboring *tetM* and *smqnr* and over-expressing different efflux pumps among clinical isolates from tertiary care hospitals in Alexandria, Egypt

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ARTICLE INFO

Article history:
Received 4 January 2022
Received in revised form 8 February 2022
Accepted 10 February 2022

Keywords:
Stenotrophomonas maltophilia
smqnr
smeABC
smeDEF
smrA

ABSTRACT

Background: *Stenotrophomonas maltophilia (S. maltophilia),* is a remarkable nosocomial pathogen, packed with different intrinsic mechanisms of resistance to most antimicrobials. Trimethoprim/Sulfamethoxazole (SXT) is the treatment of choice for *S. maltophilia* infections. However, different acquired factors may render SXT ineffective. Our aim was to investigate the susceptibility pattern to levofloxacin (LEV) and minocycline (MIN) among SXT non-susceptible isolates, as well as the different expression levels of different efflux pumps. **Methods:** Susceptibility pattern to LEV and MIN was investigated as well as the expression level of different efflux pumps SmeABC, SmeDEF and SmrA and the presence of *smqnr* and *tetM*. **Results:** Among the 19 SXT non-susceptible isolates, 57.89% were susceptible to LEV and 10.52% were susceptible to MIN. It was found that 68.42%, 15.78% and 36.84% of the isolates showed over-expressed SmeABC, SmeDEF and SmrA, respectively. The results showed no significant correlation between over-expression of efflux pumps and resistance to LEV and MIN. Moreover, *smqnr* was detected in 4 out of 8 LEV non-susceptible isolates, while *tetM* was present in 11 out of 17 MIN non-susceptible isolates. **Conclusion:** As far as previously reported, this is the first study dedicated to SXT non-susceptible *S. maltophilia* isolates, that reported the presence of *tetM* and *smqnr* and the over-expression of SmeABC, SmeDEF, and SmrA among clinical isolates in Alexandria, Egypt. The findings emphasize that LEV can be used as a suitable option in managing *S. maltophilia* infections.

Introduction

Stenotrophomonas maltophilia (*S. maltophilia)* is an outstanding opportunistic Gram-negative organism, which possess an arsenal of different intrinsic mechanisms to resist most of the available antimicrobial agents. It was once considered an organism of low clinical value. However, it has become well recognized as a nosocomial pathogen with elevated mortality rates especially among immunocompromised and patients with cystic fibrosis [1-3]. *Stenotrophomonas maltophilia* is intrinsically resistant to many of the commonly used antibimicrobial agents. This is due to different factors including poor membrane permeability, antibiotic deactivating enzymes as well as chromosomally encoded multidrug efflux pumps. Hence, the available therapeutic options against *S. maltophilia* infections are limited [3, 4].

DOI: 10.21608/MID.2022.117532.1237
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In fact, the Clinical and Laboratory Standards Institute (CLSI) (2021) has specified the breakpoints of only a few antibacterial agents to treat infections caused by this opportunistic organism [5]. Trimethoprim/sulfamethoxazole (SXT), levofloxacin (LEV) and minocycline (MIN) are among these agents [5]. Trimethoprim/sulfamethoxazole remains the drug of choice for managing infections caused by *S. maltophilia*. However, resistance to SXT is, continuously, growing due to the acquisition of resistance genes. Alternatively, LEV and MIN are among the few suitable alternate options that can be used to manage *S. maltophilia* infections [6-8].

Different mechanisms have been implicated in *S. maltophilia* resistance to LEV and MIN. In fact, the genome of *S. maltophilia* encodes different multi-drug efflux pumps. The best described among these efflux pumps, is the resistance nodulation division (RND) family (SmeABC and SmeDEF). SmeABC is implicated in acquired resistance to fluoroquinolones, aminoglycosides, and beta-lactams. SmeDEF is deemed the primary determinant of fluoroquinolone resistance in *S. maltophilia.* It is also involved in intrinsic as well as acquired resistance to fluoroquinolones, tetracycline, and chloramphenicol. Another multi-drug efflux pump is the ATP binding cassette (ABC) efflux pump (SmrA), which promotes resistance to fluoroquinolones and tetracycline. [4,9,10]. Additionally, fluoroquinolone resistance can be attributed to a chromosomally encoded SmQnr, which binds to topoisomerases and protects them from fluoroquinolones. Interestingly, mutations in the quinolone resistance determining region (QRDR) of topoisomerases are not responsible for fluoroquinolone resistance among *S. maltophilia* [4, 11]. The aim of this study was to investigate the susceptibility pattern to LEV and MIN among SXT non-susceptible isolates, as well as the different expression levels of different efflux pumps.

Materials and Methods

Sample collection

This was a prospective study, in which SXT non-susceptible *S. maltophilia* isolates were collected from the microbiology laboratories of different hospitals, during a period of eight months, in Alexandria, Egypt. In this study, the inclusion criteria were as following: adult patients, admitted to ICUs of three major hospitals, who suffered from *S. matophilia* infections. Identification of all isolates was performed using conventional biochemical methods, and confirmed by Vitek-2 (bioMérieux, France).

Antibiotic susceptibility testing

Antibiotic susceptibility tests were carried out by disk diffusion method on Mueller–Hinton agar plates according to the CLSI guidelines (2021) [5]. The antibiotic disks that were used for antibiotic susceptibility testing included SXT (1.25/23.75 μg), LEV (5 μg) and MIN (30 μg) All the culture media and antibiotic disks that we used were purchased from Oxoid (Cambridge, UK).

Investigation of the expression level of different efflux pumps

The expression level of the different efflux pumps including RND efflux pumps (*smeB* and *smeF*) and the ATP binding cassette family (*smrA*), were investigated among all the isolates using Real-time PCR, as described previously [12]. The total RNA was extracted from the cell suspensions (log phase cultures), using TRIzol Reagent from Invitrogen (Thermo Fisher Scientific, California, USA). The total RNA concentration was determined using Jenway Genova Nano Micro-Spectrophotometer (TEquipment, NJ, USA). Then, cDNA was obtained by reverse-transcription of the extracted RNA using TOPscript DryMIX (dN18/dN6 plus) (Daejeon, South Korea) and this was performed on T100 Thermal Cycler (Biorad, California, USA). Determination of the relative expression level was performed on 7500 Real Time PCR System, Applied Biosystems (Thermo Fisher Scientific, California, USA) using BioEasy Master Mix (SYBR Green) (Bioer, Hangzhou, China). The primers used are demonstrated in **table (1)**. All the primers that we used, were purchased from Invitrogen (Thermo Fisher Scientific, California, USA). The amplification scheme was: activation for 10 minutes at 95 °C, denaturation for 15 seconds at 95 °C, annealing for 15 seconds at 53 °C, extension for 30 seconds at 72 °C followed by melting curve analysis. The expression level of *smeB*, *smeF* and *smrA* genes was normalized using the *rDNA* housekeeping gene and was compared to that of *S. maltophilia* ATCC 13637 (Oxoid, London, UK). This was calculated according to the formula described previously by **Chang et al.** [12]. According to this formula over-expression of SmeABC, SmeDEF and SmrA was present if n is less than one, where n is the fold difference in gene expression.

Genotypic detection of *smqnr* and *tetM* genes

The isolates that were non-susceptible to LEV were further investigated for the presence of *smqnr* gene, while the isolates that were non-susceptible to MIN were investigated for the presence of *tetM* gene. Boiling method was used for the extraction of the bacterial DNA, as described previously [13]. The amplicon size for *smqnr* was 811 bp while the amplicon size for *tetM* was 406 bp. Detection of both genes was carried out using conventional PCR. The primers used are shown in **table (1)**. The PCR master mix used was DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, California, USA).

Statistical analysis

Data statistical analysis was carried out using IBM SPSS software package version 20.0. (Armonk, IBM Corp, NY, US) The results' significance was evaluated at the level of 5%. The tests that were used included Chi-square test and Fisher's Exact.

Results

Nineteen SXT non-susceptible *S. maltophilia* isolates were collected from different sample sources. Eight isolates were obtained from blood stream infections, 7 isolates from respiratory tract infections and four isolates from wound infections. These different sources are demonstrated in **figure (1)**. The susceptibility pattern of these isolates is detailed in **table (2)**.

Using quantitative real-time PCR, the expression level of the *smeB*, *smeF* and *smrA* genes was investigated and the results are shown in **table (3)**. Also, the different distribution of over-expressed genes among our isolates together with their susceptibility patterns are detailed in **table (4)**. The correlation between the over-expression of efflux pumps and resistance to LEV and MIN are shown in **table (5)** and **table (6)**, respectively.

Using conventional PCR, *smqnr* gene was found in 4 (50%) out of the 8 LEV non-susceptible isolates, while *tetM* was found in 11 (64.7%) out of the 17 MIN non-susceptible isolates.

Figure 1. Distribution of the different sources of the 19 *S. maltophilia* isolates.

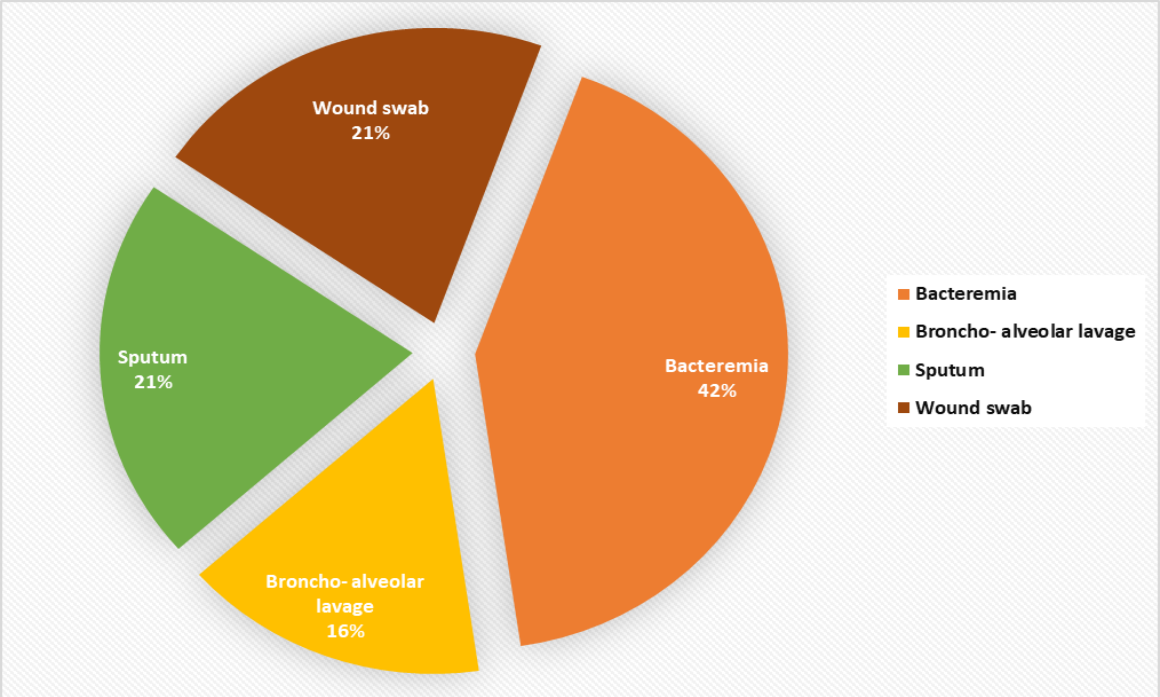

Table 1. Primers used in this study.

Primer	Nucleotide Sequence (5'–3')	Target	Annealing temperature in °C	Reference
smeB (F)	ACCGCCCAGCTTTCATACAG	*smeABC*	53	[12]
smeB (R)	GACATGGCCTACCAGGAACAG			
smeF (F)	TCGTCCAGGCTGACATTCAA	*smeDEF,*	53	[12]
smeF (R)	AACGCGGATCGTGATATCG			
smrA (F)	ACCAACATTCCCACGCTGAA	*smrA*	53	This study
smrA (R)	GGCGATCAGGAAACCGTTGA			
rDNA (F)	TGACACTGAGGCACGAAAGC	*rDNA*	53	[12]
rDNA (R)	CATCGTTTAGGGCGTGGACTA			
smqnr (F)	ACACAGAACGGCTGGACTGC	*smqnr*	56	[29]
smqnr (R)	TTCAACGACGTGGAGCTGT			
tetM (F)	GTGGACAAAGGTACAACGAG	*tetM*	58	[30]
tetM (R)	CGGTAAAGTTCGTCACACAC			

Table 2. Susceptibility pattern of the 19 non-susceptible *S. maltophilia* isolates.

Antimicrobial	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Trimethoprim/Sulfamethoxazole (SXT)	11	57.89	8	42.10	0	0
Levofloxacin (LEV)	6	31.57	2	10.52	11	57.89
Minocycline (MIN)	3	15.78	14	73.68	2	10.52

Table 3. Level of expression of *smeB, smeF* and *smrA* genes among the 19 *S. maltophilia* isolates.

Efflux pump genes	Over-expressed		Not over-expressed	
	No.	%	No.	%
smeB	13	68.42	6	31.57
smeF	3	15.78	16	84.21
smrA	7	36.84	12	63.15

Table 4. The distribution of the over-expression of *smeB*, *smeF* among the different isolates along with their susceptibility patterns.

Isolate	SXT	LEV	MIN	*smeB*	*smeF*	*smrA*
Isolate 1	I	I	I			
Isolate 2	I	S	S	over-expression		
Isolate 3	R	R	R	over-expression		
Isolate 4	R	S	I	over-expression	over-expression	over-expression
Isolate 5	R	S	I			
Isolate 6	I	S	I	over-expression		over-expression
Isolate 7	I	S	I			over-expression
Isolate 8	R	S	I			over-expression
Isolate 9	I	R	I	over-expression		
Isolate 10	I	R	R	over-expression	over-expression	over-expression
Isolate 11	R	S	S			
Isolate 12	I	R	I	over-expression		
Isolate 13	R	S	I	over-expression	over-expression	
Isolate 14	R	S	I	over-expression		
Isolate 15	R	S	I	over-expression		
Isolate 16	R	S	I	over-expression		over-expression
Isolate 17	I	I	I	over-expression		
Isolate 18	R	R	I	over-expression		
Isolate 19	R	R	R			over-expression

S: Susceptible; I: Intermediate; R: Resistant

Table 5. Correlation between the overexpression of efflux pumps and resistance to LEV.

	LEV				***p***
	Non susceptible (n = 8)		**Susceptible (n = 11)**		
	No.	**%**	**No.**	**%**	
smeF	1	12.5	2	18.2	1.000*
smeB	6	75.0	7	63.6	1.000*
smrA	2	25.0	5	45.5	0.633*

P: p value for comparing between the two groups; *Statistically insignificant (*p* value > 0.05)

Table 6. Correlation between the over-expression of efflux pumps and resistance to MIN.

	MIN				***p***
	Non susceptible (n = 17)		**Susceptible (n = 2)**		
	No.	**%**	**No.**	**%**	
smeF	3	17.6	0	0.0	1.000*
smeB	12	70.6	1	50.0	1.000*
smrA	7	41.2	0	0.0	0.509*

P: p value for comparing between the two groups; *Statistically insignificant (*p* value > 0.05)

Discussion

Stenotrophomonas maltophilia is a remarkable nosocomial pathogen, which harbors an array of different intrinsic mechanisms to resist many antimicrobial agents. This renders managing its infections very challenging with extremely limited therapeutic choices. Hence, the aim of this study was to investigate the susceptibility pattern to LEV and MIN among SXT non-susceptible isolates, as well as the different expression levels of different efflux pumps.

Trimethoprim/Sulfamethoxazole is the antimicrobial of choice for *S. maltophilia* infections, other options are LEV and MIN, as reported by different studies [14-16]. In this study, the susceptibility pattern to LEV and MIN among SXT non-susceptible isolates was investigated. It was found that among the 19 isolates that were non-susceptible to SXT, 17 (89.47%) were also non-susceptible to MIN and only 8 (42.10%) were non-susceptible to LEV. Different studies investigated SXT, LEV and MIN susceptibility patterns including **Shortridge et al.** [17] who reported that (99.5%) of their *S. maltophilia* isolates were susceptible to MIN, (95.0%) were susceptible to SXT and (96%) were susceptible to LEV. **Bostanghadiri et al.** [18] found that (2.35%) and (4.71%) of their isolates were resistant to SXT and LEV, respectively, while all their isolates were susceptible to MIN.

Here, we investigated the expression level of different efflux pumps involved in tetracycline and fluoroquinolone resistance among SXT non-susceptible *S. maltophilia* isolates and compared them to their expression level in *S. maltophilia* ATCC 13637. SmeABC, SmeDEF and SmrA belonging to two efflux pumps families: RND and ABC.

Over-expression of SmeABC is thought to confer acquired resistance to fluoroquinolones and other antibiotics such as aminoglycosides and beta-lactams [3]. Thirteen (68.42%) of our isolates over-expressed *smeB*. However, statistically there was no significant correlation between *smeB* over-expression and the resistance to LEV. **Zhao et al.** [19] demonstrated that there was no significant difference in *smeA* over-expression between LEV susceptible and LEV non-susceptible isolates. **Chong et al.** [20] also reported no significant difference between over-expression of *smeB* in both LEV resistant and susceptible isolates. However, **Herrera-Heredia et al.** [21] reported that SmeABC was over-expressed in (74.7 %) of their isolates and it reported an association between over-expression of SmeABC and LEV resistance.

SmeDEF is encoded by the chromosomes, moreover it is highly conserved among *S. maltophilia*. Over-expression of smeDEF is thought to be a key player in resistance to fluoroquinolones. It is implicated in both acquired and intrinsic resistance to fluoroquinolones, tetracycline, trimethoprim/sulfamethoxazole, aminoglycosides, macrolides chloramphenicol and tigecycline [3, 22]. Only 3 (15.78%) of our isolates over-expressed SmeDEF. Statistically, there was no significant correlation between the over-expression to *smeF* and the resistance to LEV or MIN. **Herrera-Heredia et al.** [21] found that (65.9 %) of their isolates over-expressed SmeDEF. However, they found no significant correlation between its over-expression and resistance to LEV or MIN among their isolates. **Chong et al.** [20] reported no significant relation between the over-expression of *smeF* and the resistance to LEV or MIN. **Cho et al.** [23] found no significant correlation between over-expression of SmeDEF and resistance to LEV. On the other hand, **Zhao et al.** [19] showed that there was a significant difference in *smeD* over-expression between LEV susceptible and LEV non-susceptible isolates, also it reported a significant difference between doxycycline susceptible and non-susceptible isolates [19].

El-hamad et al. [10] concluded that SmrA may impact acquired and /or intrinsic resistance. Over-expression of *smrA* is linked to fluoroquinolones and tetracycline resistance. [3] Seven (36.84%) of our isolates over-expressed *smrA*. However, there was no significant correlation between *smrA* over-expressed and resistance to LEV and MIN. **Rizek et al.** [24] reported that only one isolate harbored *smrA*. This isolate was susceptible to both LEV and MIN. **Zhao et al.** [19] reported no significant correlation between over-expression of *smrA* and LEV resistance.

In this study, *smqnr* was found in 4 (50%) out of 8 LEV non-susceptible isolates. **Azimi et al.** [11] found *smqnr* in (52%) of their isolates that were resistant to ciprofloxacin. Another study reported that *smqnr* was present in (62.80%) of their *S. maltophilia* isolates. **Kanamori et al.** [25] reported that was *smqnr* present in (57.5%) of their isolates.

tetM is one of the ribosomal protecting proteins (RPPs), which are considered an important

mechanism for tetracycline resistance, and they are generally present among Gram-negative bacteria. [19,26,27] Here, we investigated the presence of *tetM* among our MIN non-susceptible isolates. Eleven (64.7%) out of 17 non-susceptible isolates carried *tetM*. **Li et al.** [28] reported that *tetM* was found in the two *S. maltophilia* incorporated in their study, however *tetM* was found abundantly among their isolates belonging to different species. To the best of our knowledge, this is the first study dedicated to SXT non-susceptible *S. maltophilia* isolates, that reported the presence of *tetM* and *smqnr* and the over-expression of SmeABC, SmeDEF, and SmrA among clinical isolates in Alexandria, Egypt. This is the first study to report isolates co-harboring *tetM* and *smqnr* and over-expressing SmeABC, SmeDEF and SmrA among *S. maltophilia* in Egypt.

Conclusion

The findings emphasize that LEV can be used as a suitable option in managing *S. maltophilia* infections, and that further studies are needed to shed more light on the resistance mechanisms to antibiotics other than SXT.

Acknowledgment

The authors thank Pharos University in Alexandria, Medical Research Insitute and the Medical Technology Center for their technical support.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare no conflict of interest.

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