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RESEARCH ARTICLE

Prevalence of trimethoprim/sulfamethoxazole resistance genes among *Stenotrophomonas maltophilia* clinical isolates in Egypt

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ABSTRACT

Stenotrophomonas maltophilia is an important multidrug resistant nosocomial pathogen. Trimethoprim/sulfamethoxazole (TMP/SMX) is considered the drug of choice for treatment of *S. maltophilia* infections, thus emerging resistance to TMP/SMX poses a serious threat. In the present study we aimed to investigate the frequency of TMP/SMX resistance genes (*sul1, sul2, dfrA*), and to evaluate their relatedness with integron 1 *(int1),* and insertion sequence common regions *(ISCR)* among 100 *S. maltophilia* from different clinical isolates in Egypt. Isolates were identified biochemically and confirmed by VITEK2. Detection of *sul1*, *sul2*, and *dfrA* genes, *int1* and *ISCR* elements was performed by PCR. Among the 16 TMP/SMX resistant isolates, *sul1* gene was detected in all of them, and it was associated with *int1* gene presence in all resistant isolates. The *sul2* gene was detected in 6 out of 16 resistant isolates (37.5%), and only 2 of the 16 resistant isolates (12.5%) harboured *dfrA* gene. *ISCR* was detected in 10 of the resistant isolates (62.5%) and in 4 of them it was associated with the presence of *sul2* gene. Among the 84 TMP/SMX sensitive isolates, *sul1* gene was detected in 15 (17.8%), *int1* in 16 (19%) and *ISCR* in 6 (7.1%). None of the susceptible isolates had *sul2* or *dfrA* genes. These findings point out an increasing frequency of TMP/SMX resistance genes among *S. maltophilia* clinical isolates in our region, so the adoption of prudent use of *S. maltophilia* antimicrobial agents and the establishment of a surveillance system are desperately needed.

KEYWORDS

Stenotrophomonas maltophilia, trimethoprim/sulfamethoxazole, integron 1, *sul1*, *sul2*, *ISCR*

INTRODUCTION

Stenotrophomonas maltophilia is an opportunistic pathogen that is considered as the third most common non-fermenting Gram-negative bacilli responsible for a broad range of serious healthcare-associated infections especially in immunocompromised patients [1, 2]. The treatment of *S. maltophilia* infections is challenging because this pathogen is known to be resistant to a wide range of antibiotics due to both intrinsic and acquired resistance mechanisms [3, 4]. Trimethoprim/sulfamethoxazole (TMP/SMX) is a combination of two antimicrobial drugs, which act synergistically and block microbial synthesis of folic acid, so it has bactericidal effect. TMP/SMX has been the first line of treatment and the most effective antimicrobial agent in *S. maltophilia* infection based on previous *in vitro* susceptibility data and favorable clinical results [5]. Although other drugs can be used for the treatment of *S. maltophilia* infections such as levofloxacin, ceftazidime, ticarcillin-clavulanate, tetracycline and tigecycline; these alternatives are usually used in combination with TMP/SMX [6].

A lot of studies from different geographic regions have reported the increased incidence of resistance to TMP/SMX in *S. maltophilia* which limit the available treatment options of infections caused by this pathogen thus, increasing the burden on the health care system

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Table 1. Primers for the detection of target genes

Primer	Nucleotide sequence	Amplicon size (bp)	Annealing temperature	Reference
sul1	Forward ATGGTGACGGTGTTCGGCATTCTGA Reverse CTAGGCATGATCTAACCCTCGGTCT	840	54°C	[10]
sul2	Forward GAATAAATCGCTCATCATTTTCGG Reverse CGAATTCTTGCGGTTTCTTTCAG	810	52°C	[10]
dfrA	Forward TTGTGAAACTATCACTAATGGTAG Reverse CTTGTTAACCCTTTTGCCAGA	480	50°C	[9]
int1	Forward GCCTGTTCGGTTCGTAAGCT Reverse CGGATGTTGCGATTACTTCG	Variable	56°C	[8]
ISCR	Forward GCGAGTCAATCGCCCACT Reverse CGACTCTGTGATGGATCGAA	Variable	52°C	[10]

[7–9]. TMP/SMX resistance is mediated by the acquisition of resistance genetic determinants as integrons, transposons and plasmids [9]. The *sul* genes encoding dihydropteroate synthases are known to be responsible for TMP/SMX resistance furthermore, *sul1* gene has been reported to be associated with class 1 integrons, while *sul2* is mostly found on the plasmid carried on *ISCR* elements [10]. Moreover, the *dfrA* gene encodes dihydrofolate reductase enzyme, which is also located in the class 1 integrons gene cassettes, and it has also been associated with TMP/SMX resistance [11].

Due to the lack of local information in our hospitals on the prevalence of TMP/SMX resistance and related mechanisms in *S. maltophilia*, we aimed to investigate the occurrence of the genes encoding resistance to TMP/SMX, and to evaluate their relatedness with *int1*, and *ISCR* among 100 *S. maltophilia* different clinical isolates from hospitalized Egyptian patients.

METHODS

Bacterial isolates

This study was carried out during a period of one year (from December 2019 to December 2020). During this period a total of 100 clinical isolates of *S. maltophilia*, collected from different types of clinical specimens from microbiological laboratories of both Alexandria main university and medical research institute hospitals, Alexandria University, Egypt. Isolates were identified biochemically by standard biochemical methods and then all suspected isolates for *S. maltophilia* were confirmed by VITEK 2 automated instrument ID System (bioMérieux, France). The identified isolates were preserved at –80°C on Luria Bertani (LB) broth with 20% glycerol for further investigations. For bacterial restoration, one loopful was streaked over blood agar and incubated at 37°C.

Antimicrobial susceptibility testing

Isolates were tested for their susceptibility to various antimicrobial agents by disc diffusion method according to Clinical laboratory standards institute (CLSI) recommendations [12]. The following antimicrobial discs were used: Trimethoprim-sulfamethoxazole (1.25/23.75 µg), levofloxacin (5 µg) and Minocycline (30 µg) Ceftazidime (30 µg), Ticarcillin/clavulanate (75/10 µg), Tetracycline (30 µg). All culture media and antibiotic discs were from Oxoid (Oxoid Ltd; Basingostok; Hampshire, England). Susceptibility to TMP/SMX for all isolates was confirmed by automated MIC susceptibility testing by VITEK2- AST with interpretation carried out according CLSI guidelines as follows: Isolates with MIC to <2/38 µg/ml were defined as TMP/SMX susceptible while resistant isolates had MIC >4/76 µg/mL.

Detection of trimethoprim-sulfamethoxazole resistant genes by PCR

Bacterial DNA was extracted from *S. maltophilia* isolates by boiling method [13]; shortly 3–4 colonies of fresh overnight cultures were emulsified in sterile distilled water to make a heavy suspension. The bacterial suspension was incubated for 15 min in a boiling water bath, followed by rapid cooling on ice for 5 min, and centrifugation at 14,000 rpm for 15 min. The clear supernatant was used as a DNA template. This stock DNA extract was diluted by 10 folds, in Tris-EDTA (TE) buffer and then used as a template for PCR amplification. PCR was carried out on Veriti Thermal Cycler (Applied Biosystems, CA, USA) using primers listed in (Table 1) for amplification of *sul1, sul2, dfrA,* and for assessing the presence of class 1 integrons (*int1*) and *ISCR* in

each strain. All primers were supplied by Biosearch Technologies. Each PCR reaction was performed in a 25 µl reaction mixture containing 12.5 µl 2X Dream Taq™ Hot Start Green master mix (Thermo Fisher), 10 pmol of each primer, and 5 µl of DNA extract. A negative control was prepared by the addition of the same contents with water placed instead of the DNA extract.

Amplification of *sul1, sul2* and *dfrA* genes was performed according to the following parameters; initial denaturation at 95°C for 4 min followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at the annealing temperature of each primer at 52°C and 1 min extension at 72°C with a final extension step at 72°C for 10 min [10]. The same thermal parameters were used for amplifications of *int1* and *ISCR* genetic elements with elongation of the extension step in the 35 cycles to 3 min. PCR products were separated by electrophoresis in 1.5% agarose gels with ethidium bromide staining for 45 min and visualized under UV transillumination.

Statistical analysis

Statistical analysis was performed using the SPSS version 26 software (SPSS Inc., Chicago, IL, USA). Qualitative data are described as numbers and percentages. Pearson Chi-square, Fisher Exact, and Monte Carlo tests were used for comparison between groups. Results with $P < 0.05$ were considered statistically significant.

Ethics statement

This study was approved by the Ethics Committee of the medical research institute, Alexandria University. No informed patient consent was required as bacterial isolates were collected from clinical samples send to the microbiology laboratory for routine culture and sensitivity.

RESULTS

Bacterial isolates and antimicrobial susceptibility

Totally, 100 clinical isolates of *S. maltophilia* were included in the present study. Maximum numbers of isolates were from respiratory samples (40%); 27 from sputum and 13 from broncho-alveolar lavage followed by blood (38%), and pus (22%).

The results of antimicrobial susceptibility and (MICs) of *S. maltophilia* isolates showed that ceftazidime and ticarcillin/clavulanate exhibited the highest resistance of 32% and 24%, respectively, whereas 12% of isolates were resistant to tigecycline, 6% to levofloxacin, and only 4% were resistant to Minocycline. As regards to trimethoprim-sulfamethoxazole 16% of isolates were resistant by MIC method.

Characterization of TMP/SMX resistance genes and their relatedness to class 1 integrons and ISCRs

Table 2 shows the distribution of TMP/SMX resistance genes among the *S. maltophilia*. All the 16 (100%) TMP/SMX resistant isolates harbored *sul1* gene and were simultaneously *int1* positive. Meanwhile, six isolates (37.5%) had *sul2* gene, *dfrA* gene was detected only in two resistant isolates (12.5%) and *ISCR* gene elements in ten (62.5%) of these TMP/SMX resistant isolates. As shown in Table 3 in TMP/SMX resistant isolates, 4/16 (25%) isolates contained only *sul1* and *int1* genes, while the remaining of the resistant isolates 12/16 (75%) showed co-occurrence of additional resistance genes.

An interesting finding is that *sul1* gene was detected in 15 (17.8%) of the TMP/SMX sensitive isolates and 6 (7.1%) of these *sul1* carrying sensitive isolates were class 1 integron-negative, whereas 7 (8.3%) of *sul1*-negative sensitive isolates carried class 1 integron. There was statistically significant association ($P < 0.001$) between the presence of *sul1* and *int1*, and between *sul2* and *ISCR* among our isolates, as shown in Table 4.

Table 2. Distribution of *sul1*, *sul2*, *dfrA*, *int1*, *ISCR* among 16 TMP/SMX resistant isolates and 84 TMP/SMX susceptible isolates

	TMP/SMX Resistant isolates ($n = 16$)	TMP/SMX sensitive isolates ($n = 84$)	Total $N = 100$	FEp
Gene	No (%)	No (%)		
sul1	16 (100%)	15 (17.8%)	31	FEp < 0.001*
sul2	6 (37.5%)	0 (0%)	6	FEp < 0.001*
dfrA	2 (12.5%)	0 (0%)	2	FEp = 1
int1	16 (100%)	16 (19%)	32	FEp < 0.001*
ISCR	10 (62.5%)	6 (7.1%)	16	FEp < 0.001*

FEp:Fisher Exact test.
*Statistically significant at $P \leq 0.05$.

Table 3. Distribution of *sul1*, *sul2*, *dfrA*, *int1*, *ISCR* among the 16 TMP/SMX resistant isolates

No of positive isolates	Isolate number	Gene				
		sul1	*sul2*	*dfrA*	*int1*	*ISCR*
6	3,8,11,4,59,62	+	−	−	+	+
4	20,26,71,77	+	−	−	+	−
4	38,40,89,91	+	+	−	+	+
2	23,74	+	+	+	+	−
Total	16	16	6	2	16	10

Table 4. The relation between *sul1* gene and *int1* as well as between *sul2* gene and *ISCR* elements in all strains of this study

		int1		χ^2 (P)
		Positive	*Negative*	
sul1	Positive	25 (78.1%)	6 (8.8%)	$\chi^2 = 48.8$ $P < 0.001$*
	Negative	7 (21.9%)	62 (91.2%)	
		ISCR		
		Positive	*Negative*	
sul2	Positive	4 (25.0%)	2 (2.4%)	$\chi^2 = 12.1$ $P < 0.001$*
	Negative	12 (75.0%)	82 (97.6%)	

* Statistically significant when P value ≤0.05.
* χ^2 Chi square test.

DISCUSSION

Over the last few years, there has been a significant increase in the incidence of *S. maltophilia* infections especially in immunocompromised and hospitalized patients [14]. In the current study, the majority of *S. maltophilia* isolates were obtained from respiratory samples followed by blood which agreed with many previous studies [15, 16]. In contrast, in other literatures most of the *S. maltophilia* isolates have been reported from blood and less percentage from respiratory samples [17–19].

Treatment of *S. maltophilia* infection is problematic, owing to the intrinsic antibiotic-resistant nature of this pathogen. Moreover, some strains have acquired resistance to different antibiotics, which further limit the available treatment options [20]. Among the 100 *S. maltophilia* clinical isolates included in the present study, the highest susceptibility was to minocycline and levofloxacin as they exhibited susceptibility of 96% and 94%, respectively. These findings were in agreement with previous literature as in Bostanghadiri et al., Kaur et al., and Neela et al. which suggest these two antibiotics as a suitable alternative for treating *S. maltophilia* infections [17, 18, 21].

On the other hand, a significant percentage of our isolates were resistant to ceftazidime and ticarcillin/clavulanate (32% and 24%, respectively). Similarly, Bostanghadiri et al. revealed that among 164 isolate of *S. maltophilia*, ceftazidime resistance was 36.58% [17] whereas, Neela et al. and Kaur et al. reported a higher percentage of ceftazidime resistance among their isolates which reached 56.3% and 70.7%, respectively [21, 18]. This high rate of resistance to these antibiotics may be explained by the acquisition of inducible β lactamases.

Inappropriately, resistance to TMP/SMX is increasing nowadays and it is very alarming because it is the drug of choice in treatment of *S. maltophilia* infections due to its good efficacy and clinical outcomes [22].

As regards to susceptibility pattern of *S. maltophilia* isolates 16% of isolates in the present study were TMP/SMX resistant. Nearly similar TMP/SMX resistance rates were reported by several previous studies as Çıkman et al. Wang et al., and Kaur et al. who reported resistance rate of 20.3%, 20.5%, and 22.6% respectively [16, 15, 18]. In other reports TMP/SMX resistance rate was considerably lower compared to our results such as in Bostanghadiri et al., Neela et al. and Chung et al. stated that 4%, 3.04% and 1.5% of their isolates were TMP/SMX resistant [17, 21, 23].

In the present study, class 1 integron genes were detected in all the 16 TMP/SMX resistant *S. maltophilia* isolates and all these resistant isolates also harbored *sul1* gene. These results are similar to those of previous studies; Hu et al. reported out of 116 TMP/SMX-resistant isolates *int1* was detected in 83.6% and *sul1* gene was detected in 81% isolates [9]. Lower rates were revealed by Kaur et al. out of 24 TMP/SMX resistant isolates *int1* and *sul1* genes were detected in 20.8% and 50% respectively [18]. In study by Malekan et al., *int1* and *sul1* genes were detected in 14% and 26% of 27 TMP/SMX resistant isolates, respectively [24]. Interestingly, some researchers have reported the detection of the *sul1* gene in some TMP/SMX susceptible *S. maltophilia* isolates but at a lower percentage than in resistant isolates [9, 19]; among our 84 TMP/SMX sensitive isolates, *sul1* gene was detected in 17.8% and *int1* in 19% in six of these sensitive isolates *sul1* gene was detected without *int1*, whereas the *sul1* gene was absent in seven *int1* positive sensitive isolates, These findings suggest either that class I integrons in these isolates have lost the *sul1* gene region or that this gene is carried on another genetic context in these strains which agrees with previous reports suggesting unusual structures of the 3′- conserved region of class 1 integrons [25].

The *sul2* gene was detected in 6 (37.5%) of TMP/SMX resistant isolates while none of the TMP/SMX susceptible isolates harbored this gene. Although higher rates of *sul2* gene positive in the TMP/SMX resistant *S. maltophilia* clinical isolate was reported by previous researchers [11, 18, 24], other previous studies, did not detect *sul2* gene among their TMP/SMX resistant isolates [10, 26]. *ISCR* gene elements were detected in 10 (62.5%) TMP/SMX resistant isolates and was associated with *sul2* gene in 4 of these isolates. Also, in 7.1% of the sensitive isolates *ISCR* gene elements were detected.

The *dfrA* gene was detected only in 2 (12.5%) TMP/SMX resistant isolates and none of the TMP/SMX susceptible isolates had this gene. Hu et al. reported higher rate (49.1%) of *dfrA* gene among their TMP/SMX resistant isolates [9].

All our TMP/SMX resistant *S. maltophilia* isolates were concomitantly positive for more than one of the studied resistance genes in agreement with numerous previous researchers which suggest that resistance mechanism is a multifactorial process [8, 9, 24].

The presence of *sul1* was significantly associated ($P < 0.001$) with class 1 integrons among isolates in the present study. Similarly, previous studies have reported this association [19], whereas on the contrary others failed to detect this finding [18]. Additionally, there was statistically significant association ($P < 0.001$) between *sul2* and *ISCR*, which facilitate further rapid dissemination of these resistance genes among *S. maltophilia* isolates.

CONCLUSIONS

In conclusion, the results of this study revealed an increasing dissemination of TMP/SMX resistance genes among *S. maltophilia* clinical isolates in our region and an alarming tendency of decreased TMP/SMX susceptibility, so the imprudent empirical selection of TMP/SMX may be dissatisfactory in treatment of *S. maltophilia* infection and the use of this antibiotic should be limited to infections caused by susceptible strains. These findings also emphasize the necessity of considering the appropriate preventive measures to control the rapid dissemination of these resistance genes among *S. maltophilia* isolates.

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