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ASSOCIATION OF SUL GENES AND CLASS 1 INTEGRON WITH TRIMETHOPRIM-SULFAMETHOXAZOLE RESISTANCE IN *STENOTROPHOMONAS MALTOPHILIA* CLINICAL ISOLATES IN ZAGAZIG UNIVERSITY, EGYPT

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ABSTRACT

Background: *Stenotrophomonas maltophilia* (*S. maltophilia*) is an intrinsically drug resistant opportunistic pathogen associated with serious infections in humans. Acquired resistance to trimethoprim-sulfamethoxazole (SXT,co-trimoxazole), the main stay of therapy against *S. maltophilia*, has made its treatment more problematic. **Objectives:** This work aimed to determine the occurrence of SXT resistance among *S. maltophilia* isolated from Zagazig University Hospitals in Egypt and to assess the association of *sul* genes and integron1 with SXT-resistant isolates. **Material and Methods:** Thirty-two *S. maltophilia* isolates were identified in this study during the period from 2013 to 2015. Screening of SXT-resistant isolates was done by Kirby-Bauer method. Minimum inhibitory concentration(MIC) values for SXT were determined by agar dilution. *S. maltophilia* isolates were tested for the presence of *sul1*, *sul2*, *sul3*, and *int 1* genes by multiplex polymerase chain reaction. **Results:** Amongst the 32 *S. maltophilia* isolates, 12(37.5%) were resistant to SXT. All SXT-resistant isolates were found to harbor *sul1* gene and integron1. One of these isolates had *sul2* gene (1/12,8.3%). Meanwhile, *sul3* gene was not detected in any of the SXT-resistant isolates. Only 2 of the 20 SXT-susceptible isolates was found to yield positive PCR results for *sul1* gene, one of them gave positive result for class 1 Integron. The association of *sul* genes and Integron1 with resistance to SXT had a statistically significant difference($P < 0.0001$). **Conclusion:** Our study indicated a high frequency of SXT resistance among clinical *S. maltophilia* isolates from Zagazig University Hospitals, in which *sul* genes and class 1 integron were found to have a major role.

Keywords: *Stenotrophomonas maltophilia*; Sulphamethoxazole-trimethoprim-resistant; Multiplex PCR; *sul* genes; Integron 1

ASSOCIATION DE SUL GENS ET INTEGRON DE CLASSE 1 AVEC LA RESISTANCE DE LA TRIMETHOPRIME – SULFAMETHOXAZOLE EN ISOLATS CLINIQUES DE *STENOTROPHOMONAS MALTOPHILIA* A L'UNIVERSITE DE ZAGAZIG, L'EGYPTE.

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RESUME

Contexte: *Stenotrophomonas maltophilia* est un pathogène opportuniste résistant à la drogue intrinsèquement associée à des infections graves chez l'homme. Résistance acquise à triméthoprime – sulfaméthoxazole (SXT, co- trimoxazole), le pilier de la thérapie contre *S. maltophilia*, a fait son traitement plus problématique.

Objectifs : Ce travail visait à déterminer l'apparition de la résistance de SXT chez *S. maltophilia* isolés des hôpitaux universitaires Zagazig et d'évaluer l'association de gènes et intégron 1 avec des isolats résistants à SXT.

Matériel et Méthodes: Trente – deux isolats de *S. maltophilia* ont été identifiés dans cette étude pendant la période de 2013 à 2015. Le dépistage des isolats SXT a été fait par la méthode Kirby – Bauer. Les concentrations minimales inhibitrices (MIC) pour SXT ont été déterminées par dilution gélose. Les isolats *S. maltophilia* ont été testés pour la présence des gènes de *sul1*, *sul2*, *sul3*, par réaction en chaîne polymérase multiplex.

Résultats : Parmi les 32 isolats *S. maltophilia*, 12 (37,5%) étaient résistants à SXT. Tous les isolats résistants à SXT ont été trouvés d'abriter le gène *sul1* et intégron 1. L'un de ces isolats a eu le gène *sul2* (1/12,8,3%). Cependant, le gène *sul3* n'a pas été détecté dans aucun des isolats résistants à SXT. Deux seulement des vingt des isolats sensibles ont été trouvés pour obtenir des résultats positifs de PCR pour le gène *sul1*, l'un d'entre eux a donné des résultats positifs pour la classe d'intégron 1. L'association des gènes de *sul* et intégron 1 avec une résistance à SXT avait une différence statistiquement significative ($P < 0,0001$).

Conclusion: Notre étude a indiqué une fréquence élevée de résistance à SXT chez *S. maltophilia* clinique des hôpitaux universitaires Zagazig dans lesquels les gènes *sul* et intégron 1 ont été trouvés d'avoir un rôle majeur. **Mots clés:** *Stenotrophomonas maltophilia* ; Sulphaméthoxazole – triméthoprime – résistant; Multiplex PCR; *sul* gènes ; intégron 1.

INTRODUCTION

Stenotrophomonas maltophilia (*S. maltophilia*) is a glucose non fermentative, aerobic, motile Gram negative bacillus. It was first isolated in 1943 and named *Bacterium booker*. In 1961, it was re classified as a member of the genus *Pseudomonas*, then *Xanthomonas* in 1983 and later *Stenotrophomonas* in 1993 (1). It is commonly found in various environments such as water, soil, plants as well as in hospital settings (2).

These bacteria typically colonize areas of the body without causing infection. However, in severely ill, hospitalized patients, *S. maltophilia* can cause a wide range of serious infections, including nosocomial pneumonia, bacteremia, pulmonary infections, urinary tract infections, wound infections, skin and soft tissue infections, meningitis, and endocarditis, particularly with those having impaired immune system (1,2). This is facilitated by the organism's ability to survive on almost any humid surface, its tendency to form biofilm, and its employment of several mechanisms that confer resistance to a number of antimicrobial agents (3).

Factors that increase the risk for *S. maltophilia* infection include admission to an intensive care unit, prolonged hospitalization, HIV infection, cancer, cystic fibrosis, neutropenia, recent surgery, trauma, mechanical ventilation, and previous therapy with broad-spectrum antibiotics (4). *S. maltophilia* has high-level intrinsic resistance to many antibiotics owing to its multidrug-efflux pumps and low outer membrane permeability, which makes its infections difficult to manage (5). In addition to being intrinsically drug-resistant pathogen, it can acquire antibiotic resistance by horizontal transfer of resistance genes located on plasmids, transposons and integrons (5). This has made the World Health Organization to classify *S. maltophilia* as one of the leading multidrug resistant organisms (MDROs) in hospital settings (6).

Trimethoprim/sulfamethoxazole (SXT, co-trimoxazole) is considered the first-line agent recommended for the treatment of *S. maltophilia* (7). However, SXT resistance in *S. maltophilia* has been widely increasing over recent years (8). This constitutes a great clinical problem, as the range of

effective antibiotic agents is even more limited in infections caused by co-trimoxazole-resistant *S. maltophilia* (9). Resistance to co-trimoxazole can result from mutations in the chromosomal dihydropteroate synthetase (DHPS) gene or more frequently from the

acquisition of an alternative DHPS gene (*sul*), whose product has a lower affinity for sulfonamides (10).

The *sul1* gene is mostly found linked to other resistance genes in class 1 integrons, while *sul2* is usually located on small plasmids. *Sul3*, shares an amino acid identity of ~40% with previously known resistant enzymes and it is a plasmid borne resistance gene. The genetic localization of *sul* genes on efficient mobile genetic structures probably contributes to the widespread of sulfonamides resistance (11). As no much information is currently available regarding the frequency of SXT resistance among *S. maltophilia* isolates in our hospital, this study aimed to determine the occurrence of SXT resistance among *S. maltophilia* isolated from Zagazig University Hospitals, Egypt and to assess the association of *sul* genes and integron 1 with SXT-resistant isolates.

MATERIAL AND METHODS

Study Design and Patient Selection

Across-sectional study was carried out from June 2013 to August 2015 during which samples were collected according to the site of infection from patients admitted to Zagazig University Hospitals. The study was conducted in Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Egypt. This study was approved by the local institutional review board (Zagazig University IRB). Urine, sputum, endotracheal aspirates (ETA), blood and pus were collected from patients located in different medical wards, surgical wards and ICUs according to the standard microbiological methods. Informed consent was obtained from the patients.

Cultivation and Presumptive Identification of *Stenotrophomonas* Isolates

Samples were grown on blood and MacConkey agar except urine samples, which were grown on CLED (Oxoid, UK). Blood samples were collected in blood culture bottles containing brain-heart infusion broth and then subcultured onto agar plates (Blood and MacConkey agar). Non-lactose fermenting colonies were identified initially by Gram stain, catalase test, oxidase test, then confirmed to be *S. maltophilia* by API20NE (Bio-Mérieux, Marcy L'Etoile, France).

Phenotypic Antibiotic Susceptibility Test

The Kirby-Bauer disc diffusion method was employed to determine the antibiotic susceptibility pattern of the isolates using antibiotic discs (Oxoid, UK) on Mueller Hinton agar. All isolates were tested against gentamicin (10µg), amikacin (30µg), levofloxacin (5µg), ciprofloxacin (5µg), imipenem (10µg), trimethoprim/sulphamethoxazole (SXT) (1.25/23.75µg), piperacillin/tazobactam (100/10µg), ceftazidime (30µg) and ticarcillin/clavulanate

(75/10µg), tigecycline (30µg) and colistin (10µg). The antimicrobial susceptibilities were categorized in accordance with the 2012 Clinical Laboratory Standard Institute (CLSI) for *S. maltophilia*. For agents without published CLSI criteria for *S. maltophilia*, the relevant criteria for non-*Enterobacteriaceae* were used (12).

Determination of SXT Minimum Inhibitory Concentration

Only isolates that showed resistance to co-trimoxazole (irrespective of resistance to other antibiotics) were selected for minimum inhibitory concentration (MIC) determination. MIC values for co-trimoxazole were determined by E test according the E-test reading guide: where the edge of the inhibition ellipse intersects the side of the strip (Bio-Mérieux, Marcy L'Etoile, France).

Assessment of SXT Resistance Genes

DNA extraction

A single colony was inoculated into Mueller-Hinton broth and incubated for 20 hours at 37°C. After centrifugation at 10000×g for 10 minutes (min), each pellet was washed three times in 750µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and then resuspended in 500µl TE buffer. The solution was boiled for 20 min and centrifuged at 10000×g for 10min, and the supernatant was then used as a crude DNA extract in PCR. Extracted DNA was stored at 20°C until further processing.

sul1, *sul2* and integron 1 detection

Multiplex PCR amplification of *sul1*, *sul2* and integron 1 was conducted as described by Kernetal.(13) Amplification of *sul1* was performed using the forward primer *sul1f* (5'-CGG CGT GGG CTA CCT GAA CG-3') and reverse primer *sul1r* (5'-GCCGATCGCGTGAAGTCCG-3') (433bp). *sul2* was identified using the forward primer *sul2-F* (5'-GCG CTC AAG GCA GAT GGC ATT-3') and the reverse primer *sul2-B* (5'-GCG TTT GAT ACC GGC ACC CGT-3') (293bp). Integron 1 was amplified by using Int-F (5'-GCC ACT GCG CCG TTA CCA CC-3') and Int-B (5'-GGC CGA GCA GAT CCT GCA CG--3') (898bp). Additionally, the 16SrRNA gene (auniversal bacterial gene) was amplified in each reaction to serve as an internal positive control using the forward primer 16S-F (5'-GCG GAC GGG TGA GTA ATG T-3') and reverse primer 16S-B (5'-TCA TCC TCT CAG ACC AGC TA-3') (200bp).

The PCR mixture contained 5µl of template DNA, 5µl of 10×PCR buffer, 10µl of dNTP mix, 4µl of MgCl₂ 2;0 .25µl of AmpliTaq DNA polymerase, 2.5µl of each primer 16S-F, 16S-B (40µM), *sul1-F*, *sul1-B*, *sul2-F*, *sul2-B*, Int-F and Int-B (2µM) and 5.75µl distilled water. Amplification was carried out by heating for 5 min at 94°C, followed by 30 cycles of 94°C for 15s, 69°C for

30s and 72°C for 60s, followed by one cycle at 72°C for 7 min using Biometra T gradient thermal cycler(Germany).

sul3 detection

Different reaction conditions were used to amplify *sul3* (14). PCR was performed using a 25µl reaction of 2µl of boiled lysate, 1×PCR buffer, 3mM MgCl₂, 4mM dNTPs, 1.5U of *Taq* polymerase, 0.4µM *sul3F* primer (5'-GAG CAA GAT TTT TGG AAT CG-3') and 0.4µM *sul3R* primer (5'-CAT CTG CAG CTA ACC TAG GGC TTT GGA-3') (569bp). Mixtures were centrifuged for 30s at 3000 rpm. Cycling conditions were 98°C for 1min followed by 35 cycles of 98°C for 30s, 51°C for 30s and 72°C for 1min. A final extension was performed at 72°C for 5 min.

Amplicon detection by agarose gel electrophoresis

Ten µl of each amplified DNA & 1500 molecular weight marker (Invitrogen, USA) were separated on 2% agarose gel containing 0.3mg/ml of ethidium bromide. The bands were visualized using UV transilluminator (312nm), photographed & analyzed.

Statistical analysis

Data was analyzed using EPI-INFO 6 for data processing and statistics. Numerical data were expressed as mean and standard deviation. Qualitative data were expressed as frequency and percentage. Chi square test was used to examine the relation between qualitative variables. P value <0.05 was considered significant.

RESULTS

A total of 32 non-duplicate clinical isolates of *S. maltophilia* were obtained from 300 patients (32/300, 10.6%) admitted to different wards and ICUs at Zagazig University Hospitals, between 2013 and 2015. Isolates were obtained from 18 male (56.25%) and 14 female (43.75%) with a male/female ratio of 1.3:1. The mean age of patients was 64.8±9.65 years (range 48 years to 87 years). The most frequent site of isolation was the respiratory tract (68.7%); including ETA (46.87%) & sputum (21.87%) followed by blood (18.75%), wound (9.4%) then urine (3.12%) (**Table1**).

The antimicrobial activities of 11 selected antibiotics against 32 *S. maltophilia* isolates are presented in **Table2**. All isolates were sensitive to tigecycline and resistant to imipenem. Twelve isolates (37.5%) were resistant to SXT.

The *sul* genes and integron1 were tested in all the 32 *S. maltophilia* isolates. All of the 12 SXT-resistant isolates harbored *sul1* gene and were positive for integron 1. One isolate among the resistant isolates (1/12) also carried *sul2* gene. *sul3* gene, on the other hand, had not been detected in any of the resistant

isolates. Only 2 of the 20 SXT-susceptible isolates was found to yield positive PCR results for *sul1* gene, one of them gave positive result for Integron 1. The association of *sul* genes and Integron 1 with resistance to SXT had a statistically significant difference ($\chi^2=23.9$, $P<0.0001$) (Table 3 & Fig.1).

TABLE (1): DISTRIBUTION OF *S.MALTOPHILIA* ISOLATES IN DIFFERENT SAMPLES

Specimen	No.(%)
ETA	15(46.9)
Sputum	7(21.9)
Pus	6(18.75)
Blood	3(9.4%)
Urine	1(3.12)
Total	32(100)

TABLE(2): ANTIMICROBIAL ACTIVITIES AGAINST *S.MALTOPHILIA* ISOLATES

Antibiotic	Sensitive		Resistant	
	No.	%	No.	%
TG	32	100	0	0
CT	27	84.4	5	15.6
LEV	26	81.25	6	18.75
SXT	20	62.5	12	37.5
TIC-CL	20	62.5	12	37.5
CAZ	20	62.5	12	37.5
TZP	17	53	15	47
CIP	15	47	17	53
AK	13	40.6	19	59.4
GM	13	40.6	19	59.4
IPM	0	0	32	100

TG: tigecycline, CT: colistin, LEV: Levofloxacin, SXT: trimethoprim/sulphamethoxazole, TIC-CL: ticarcillin clavulanic acid, CAZ: ceftazidime, TZP: piperacillin/tazobactam, CIP: ciprofloxacin, AK: amikacin, CN: gentamicin, IPM: imipenem.

TABLE (3): ASSOCIATION BETWEEN PRESENCE OF *SUL* GENES, INTEGRON 1 AND TRIMETHOPRIM/SULFAMETHOXAZOLE (SXT) SUSCEPTIBILITY

SXT Susceptibility	MICrange(mg/L)	No.ofisolates	<i>sul1</i> No.(%)	<i>sul2</i> No.(%)	<i>sul3</i> No.(%)	Integron1 No.(%)
Susceptible	≤0.06-2	20	2(10)	0(0)	0(0)	1(5)
Resistant	4-128	12	12(100)	1(8.3)	0(0)	12(100)

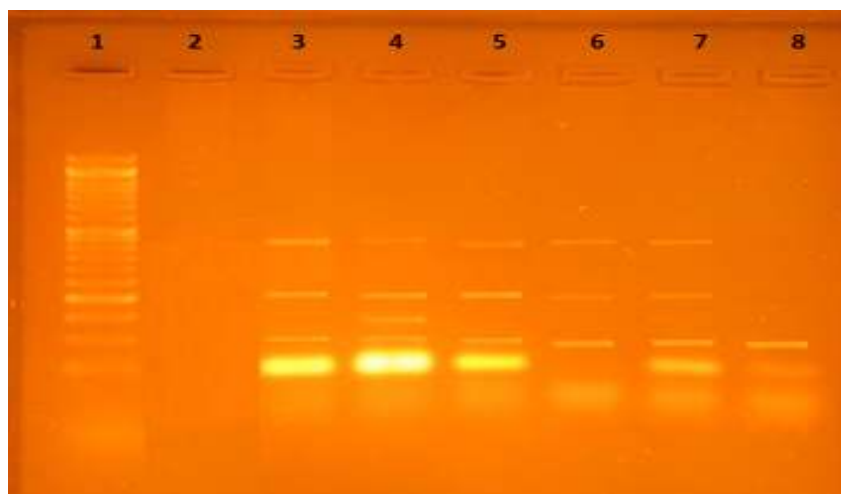


FIGURE (1): GEL ELECTROPHORESIS RESULTS OF MULTIPLEX PCR ON CLINICAL ISOLATES OF *S.MALTOPHILIA*. LANE 1: MOLECULAR WEIGHT MARKER 100 BP, LANE 2: NEGATIVE CONTROL, LANE 3-8: amplicons of *S. maltophilia* strains (Lanes 3, 5, 6 & 7: *sul1*, *int 1* positive, Lane 4: *sul1*, *sul2*, *int 1* positive, Lane 8: negative).

DISCUSSION

S. maltophilia is an emerging multidrug resistant opportunistic pathogen. Its intrinsic or acquired resistance to most antibiotics and its ability to colonize the surfaces of medical devices have made it a potentially dangerous pathogen in hospital settings(15). In our study, 32 consecutive non-duplicate *S. maltophilia* isolates were obtained from 300 patients between June 2013-August 2015, with a percentage of 10.6%. Our findings are in accordance with Samonis *et al* (16) who identified *S. maltophilia* in 10% of the studied samples and with an Egyptian study that revealed *S.maltophilia* in 9.7% of examined clinical samples (17). However, other studies reported a lower percentage; Nseiretal.(18) identified *S. maltophilia* isolates in 2% of the clinical samples during a three-year study period. This difference may be attributed to different patient population with different underlying risk factors and diseases.

In the present study, the frequency of *S.maltophilia* isolation from respiratory specimen (endotracheal aspirate and sputum), blood, wound and urine were 68.7, 18.75, 9.4 and 3.12% respectively. This comes in agreement with Thabit *et al.* (17) who reported in an Egyptian study, that *S. maltophilia* was isolated from

respiratory specimens (endotracheal swabs and sputum), wound, blood and urine with frequencies of 71.43%, 17.14%, 8.57% and 2.86% respectively. Naeem *et al.* (19) reported that 59% of *S. maltophilia* isolates were from respiratory specimen followed by wound (16.22%), blood (14.87%) and urine (4.06%). Also, Samonis *et al.*(16) reported that the main type of infection associated with *S. maltophilia* was respiratory tractinfection (54.4%) followed by blood stream infections (16.2%), skin and soft tissue infections (10%) and lastly urinary tract infection (4.4%).

The variation in the percentages in different studies may be attributed to the patients involved in the studies whether from the intensive care units (ICUs) or from other hospital wards. Most of the patients included in our study were ICU patients, which led to higher occurrence of infections. Overall, the previous results in addition to ours agreed that although *S. maltophilia* may cause many types of human infections, the respiratory tract represents the most common site affected.

The management of *S. maltophilia* infections represents a great challenge to clinicians due to problems with *in vitro* susceptibility testing, lack of

clinical trials to determine optimal therapy, and its intrinsic resistance to a plethora of antimicrobial agents, which severely limits the effectiveness of commonly used empiric antimicrobial therapies (15).

In our study, *S. maltophilia* isolates were tested against 11 antibiotic to determine their antibiotic sensitivity using the disc diffusion method. The most effective antibiotics *in vitro* were tigecycline, colistin, levofloxacin, SXT, then ticarcillin-clavulanic and ceftazidime, which showed the highest sensitivity (62.5%) among the β -lactams used in this study. Similar results were obtained by Samonis *et al.* (16) who reported that colistin and tigecycline could be considered as new therapeutic options against *S. maltophilia* infections. This is also in line with Zhanel *et al.* (20) who stated that tigecycline displayed good *in vitro* activity against MDR isolates of *S. maltophilia*. Chung *et al.* (8) reported that tigecycline and levofloxacin have shown good *in vitro* activity against clinical isolates of *S. maltophilia*. Other variable findings regarding colistin activity against *S. maltophilia* isolates were reported (21). This could be explained by differences in the susceptibility testing methods.

Levofloxacin, as one of the new fluoroquinolones, was found to be moderately effective against *S. maltophilia*, in our study, with 81.25% susceptibility ratio. Similar data from worldwide SENTRY studies has revealed 83.4% sensitivity ratio of *S. maltophilia* to levofloxacin during the period 2003–2008 (22) which was decreased to 77.3% in 2011 (23). Lower susceptibility rates ranging from 64–69.6% have been reported in Canada (20), China (24,25), and Korea (8). However, it exhibited better potency against *S. maltophilia* than ceftazidime or ticarcillin-clavulanate in our study, which comes consistent with Chang *et al.* (3) who reported the same finding. Trimethoprim/sulfamethoxazole has been considered as the mainstay of therapy against *S. maltophilia* infections. This is primarily based on *in vitro* susceptibility data rather than clinical studies. However, increasing resistance to trimethoprim/sulfamethoxazole has been reported by several studies and has been mostly related to the horizontal spread of mobile genetic elements carrying resistance genes (16).

In our study, 37.5% of *S. maltophilia* isolates showed SXT resistance. This comes much higher than a previous Egyptian study done in Mansoura city, which revealed SXT-resistance only in 4.55% of their isolates (26). This also comes in contrast to Chung *et al.* (8) who stated that resistance rates vary geographically but are generally less than 10%. This difference could be attributed to increased usage of

SXT in our local setting. However, high and various rates of resistance to SXT have been reported in patients with cancer, cystic fibrosis and in several countries, including Taiwan, Japan, Korea, Thailand, Spain, Mexico, Saudi Arabia, Turkey, and Canada (16–78.8%) (3). The *sul* genes and integron 1 were tested in all the 32 *S. maltophilia* isolates, in our study. All of the 12 SXT-resistant isolates harbored *sul1* gene. One isolate among them was additionally positive for *sul2* gene. All of the 12 SXT-resistant isolates had positive results for integron 1 as well. On the other hand, *sul3* gene has not been detected in any of our isolates.

Several investigators have reported that *sul1* genes associated with class 1 integrons are the major mechanism of SXT resistance in *S. maltophilia*. In a survey of 55 *S. maltophilia* isolates (30 sensitive and 25 resistant) by PCR, Toleman *et al.* (27) found that 17 of 25 resistant isolates possessed the *sul1* gene and class 1 integron. Similarly Chang *et al.* (28) reported that 26 out of 100 (26%) *S. maltophilia* isolates were resistant to SXT, with 81% *sul1*-positive and carrying class 1 integron. Liaw *et al.* (29) reported an increased class 1 integron presence in *S. maltophilia* isolates (15 out of 17, 88%), with 73% (n=11) carrying the *sul1* gene. Song *et al.* (30) reported that none of the isolates without *sul1* had a class 1 integron. These data along with our data underline the high prevalence of class 1 integron in SXT-resistant clinical isolates of *S. maltophilia*. Only 2 of the 20 SXT-susceptible isolates were found to yield positive PCR results for *sul* gene, one of them had integron 1. This is in line with other researchers who reported the presence of *sul* genes in SXT susceptible *S. maltophilia* isolates (30,31). However, others reported absence of *sul* genes in SXT-susceptible isolates. (27,32)

It is worth mentioning that the co-presence of a class 1 integron gene cassette and the *sul1* gene in *S. maltophilia* can further lead to the development of multi-drug resistance and may act as a potential source for the dissemination of resistance. This indeed confirms the importance of strict application of infection control measures in order to decrease the incidence of infections caused by this serious world wide intrinsically drug-resistant pathogen.

Conclusion

In conclusion, this study highlighted the widespread of co-trimoxazole resistance among *S. maltophilia* isolated from Zagazig University Hospitals, which was much associated with *sul1* gene and class 1 integron. This necessitates continuous surveillance of antimicrobial drug resistance and careful epidemiological monitoring of co-trimoxazole

resistance, which has the potential to spread by

means of mobile genetic elements.

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