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ORIGINAL ARTICLE

Comparative Study between SmeDEF Multidrug Efflux Pump and CTX-M ESBL as Resistance Determinants in Nosocomial Isolates of *Stenotrophomonas maltophilia*

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***S. maltophilia*, SmeDEF efflux pump, CTX-M ESBL, MDR**

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ABSTRACT

Background: *Stenotrophomonas maltophilia (S. maltophilia) is an important opportunistic pathogen that is usually associated with hospital acquired infections. Increased antibiotic resistance among those isolates is a real life threatening problem. Efflux pump and ESBL are two main causes of resistance in S. maltophilia.* ***Objectives:*** *The aims of this study were to isolate S. maltophilia from different sites of nosocomial infections among patients in Mansoura University Hospitals (MUHs), to determine multidrug resistant (MDR) isolates, to characterize those isolates as regarding presence of SmeDEF efflux pump and CTX-M ESBL and to compare the prevalence of both determinants as a cause of resistance in those isolates.* ***Methodology:*** *The study was conducted on 220 S. maltophilia isolates from nosocomialy infected patients which were subjected to antimicrobial susceptibility testing to determine the MDR isolates that were phenotypically tested for presence of efflux pump mediated antibiotic resistance and ESBL production by microdilution test and double disk synergy test respectively followed by detection of SmeDEF and CTX-M genes by PCR in the phenotypically positive isolates.* ***Results:*** *Antibiotic susceptibility testing of the 220 S. maltophilia isolates revealed that 179 (81.4%) of them were MDR, of which 43 (24%) were positive for efflux pump system and 147 (82.1%) were positive for ESBL production by phenotypic tests. PCR revealed that SmeDEF gene was detected in 38 (21.2%) of the MDR S. maltophilia isolates, where as CTX-M gene was found to be harbored by only 12 (6.7%) of those isolates.* ***Conclusion:*** *SmeDEF efflux pump was found to be a more prevalent cause of multiple antibiotic resistance in S. maltophilia nosocomial isolates than CTX-M ESBL; this may be helpful in improving the patient outcome if the exact cause of resistance is kept in mind during designation of the patient treatment regimen.*

INTRODUCTION

Stenotrophomonas maltophilia is one of the most important Gram negative bacteria associated with opportunistic infection particularly in health care setting. The high morbidity and mortality rates recorded in patients infected with this pathogen is an alarming particularly in immunocompromised cases [1]. Infections caused by *S. maltophilia* are ranged from urinary tract or repiratory tract infections to bacteremia [2]. Virulence factors and predisposing conditions for *S. maltophilia* are only presented in few studies [3].

The isolation rate of *S. maltophilia* from different pathological samples of patients suffering from nosocomial infections is increasing as it has been reported to be the second causative agent of nosocomial infections caused by Gram nagative bacteria after *Pseudomonas aeruginosa* [4]. The major problem of those isolates is the selection of multidrug resistant mutants together with their important property of being intrinsically resistant to different antibiotic classes [5].

Multidrug resistance in *S. maltophilia* may be caused by decreased permeability of the outer membrane and efflux pump system of different antibacterial agents [6] however, The detailed mechanisms of antimicrobial resistance in *S. maltophilia* is not clear up till now [7].

The efflux pumps systems are specific proteins in the bacterial membrane which cause excretion of antibiotics outside the cell [8]. Many types of efflux system are responsible for antibiotic resistance mainly in Gram negative bacteria [9] of which RND (resistance nodulation division) type efflux pump mechanism determined by the SmeDEF genes has been recognized in clinical isolates of *S. maltophilia* as a major cause of their unresponsiveness to different antibiotics groups [10]. SmeDEF efflux system is usually associated with resistance to different antibiotic classes including fluoroquinolones, tetracycline, macrolides and chloramphenicol [11].

The prevalence of ESBL among clinical isolates of Gram bacteria as a significant cause of resistance is increasing all over the world [12]. Previous reports

mentioned CTX-M as an important determinant for ESBL in those isolates [13]. The presence of CTX-M in *S. maltophilia* isolates was only determined in few researches that supply those bacteria with more potent resistant determinant and ensure more difficulties in dealing with this organism particularly in clinical setting [14,15,16].

To our knowledge, efflux pump mechanisms and ESBL production in *S. maltophilia* is not compared as resistance mechanisms at any one of previous studies. This study was aiming at isolation of *S. maltophilia* from different sites of nosocomial infections among patients in MUHs, determination of MDR isolates, characterization of those isolates as regarding presence of SmeDEF efflux pump mechanism and CTX-M ESBL by phenotypic and genotypic methods and comparing the prevalence of both determinants as a cause of resistance in those isolates.

METHODOLOGY

Study design:

The present work was conducted over a period of 28 months starting from October, 2014 to January, 2017. During that period 220 *S. maltophilia* were isolated from nosocomialy infected patients [17] admitted in different departments MUHs. Comparative study was performed on those isolates as regarding presence of SmeDEF and CTX-M resistance determinants. The protocol of this study was accepted by the ethical committee in the Faculty of Medicine, Mansoura University, code number R/17.03.44 .

Clinical samples:

Different samples were collected from all studied patients with nosocomial infections under complete aseptic condition including; blood, urine, sputum, endotracheal aspirate, wound discharge, medical devices and cerebrospinal fluid.

Microbiological studies:

The collected samples were processed and examined in Microbiology Diagnostic and Infection Control Unit in the Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University. Urine samples were cultured on CLED media where as other samples were cultured on macConkey's agar, chocolate agar and blood agar media.

Identification of S. maltophilia isolates [18]:

S. maltophilia isolates were identified by their characteristic shape on different culture media as they produced large, smooth, glistening colonies with uneven edge and they developed faint lavender-green color with characteristic ammonia odor on blood agar. Gram stained smears were done from the suspected bacterial colonies revealing the Gram negative bacilli. Further identification of the isolates was performed using different biochemical reactions as they were oxidase negative, DNase positive and they developed positive motility testing. Identification was confirmed by (API) 20 E analytical profile index *(Bio-merieux SA, Montalieu Vercica and France).*

Preservation of the selected isolates:

The isolated *S. maltophilia* were inoculated on slopes of nutrient agar and incubated at 37°C for 24 hours, after that the slopes were kept at 4°C. Passage of the isolates was done every 2-3 weeks. Before doing any experiment, subculture was performed twice to let isolates restore their viability.

Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing was performed for all selected *S. maltophilia* isolates according to the recommendation of CLSI, 2014 [19]. Isolates that were found to be resistant to three or more antimicrobial classes were defined as MDR [20] and subjected to further testing.

Phenotypic detection of efflux pump mediated antibiotic resistance:

Ciprofloxacin was used as an example of antibiotics affected by presence SmeDEF efflux pump system [21] for determination of its minimum inhibitory concentration (MIC) against the studied MDR isolates using the microdilution test. The test was done in duplicate with and without efflux pump inhibitor. Broth of Muller Hinton (50 µL) was poured into sterile microdilution plate wells, after that 50 µL of the tested antibiotic with adequate concentration was added to the first row of the plate wells, then serial dilutions were done. After that, 10 µL of PβNA (Phe-arg-beta-naphthylamide, 200 µg/mL) (Sigma) and 40 µL suspension of bacterial isolates were added to each well so that a concentration of 20 µg/mL PβNA was obtained. Isolates showed four-fold decrease in the MIC of the tested antibiotics with addition of inhibitory agent PβNA were reported to be positive for presence of efflux pump system [22].

Phenotypic detection of ESBL by double disk synergy test:

Multidrug resistant *S. maltophilia* isolates were also examined for ESBL production by double disk synergy test, increased diameter of inhibition zone around any of cephalosporin or aztreonam disks due to synergy of clavulanate in co-amoxiclav disk means positive results for ESBLs production [23].

Detection of SmeDEF multidrug efflux pump and CTX-M ESBL by PCR:

PCR assays were conducted for all phenotypically positive isolates for efflux pump and ESBL production.

- ***DNA extraction [24]:***

DNA was extracted from all tested isolates by suspending 4 to 5 colonies of 12 hours incubated bacterial cultures on agars of Mueller Hinton in 500 µl distilled water, after that the mixture was heated for 10 minutes at 100 °C then the suspension was frozen for 5-10 minutes. Finally, centrifugation was done for 5

minutes at 19000 rpm. The used DNA template was taken from the supernatant.

- ***PCR techniques [25]:***

PCR reaction was performed with 25 µl total volume consisting of 2.5 µl 1× Taq DNA polymerase solution with 0.2 µl 1U Taq DNA polymerase, 2.5 mM $MgCl_2$, 2.5 µl deoxynucleotide triphosphate (200 mM), 2 µl forward and reverse primers (15 pmol), 5 µl template DNA and 13 µl deionized water. PCR products were electrophoresed in 2% agarose gel using #SMO373, 50 base pair DNA Ladder (Thermo Scientific Inc.) to measure the detected bands size.

- ***Primers used and cycling conditions:***

Primers used for detection of SmeDEF gene were 1 (5'-CCAAGAGCCTTTCCGTCAT-3') and 2 (5'-TCTCGGACTTCAGCGTGAC-3'), band size 150 base pair, cycling conditions were 94°C for 90 seconds, then 35 cycles of 30 seconds at 94°C, 60 seconds at 58°C, 90 seconds at 72°C and final extension step at 72°C for 10 minutes [5], whereas primers used for detection of CTX-M gene were 1(5′-CGCTTTGCGATGTGCAG-3′) and 2 (5′ ACCGCGATATCGTTGGT-3′), band size 550 base pair [26], cycling conditions were 95°C for 5 minutes, then 35 cycles of 1 minutes at 95°C, 30 seconds at 60°C, 1 minutes at 72°C and final extension step at 72°C for 5 minutes [27].

Analysis of Data:

Statistical Package of Social Science (SPSS), software version 17 was used for entry and statistical analysis of data. Description of qualitative data was done as numbers and percentages.

RESULTS

Two hundred and twenty *S. maltophilia* isolates were detected among the studied clinical samples, the distribution of those isolates was as follows; 123 (55.9%) from sputum and endotracheal aspirate, 46 (20.9%) from blood, 37 (16.8%) from wounds, 11 (5%) from urine and 3 (1.4%) from medical devices. Intensive care units (ICUs) were the most common sites from which *S. maltophilia* had been isolated, 142 isolates (64.5%) followed by surgical wards, 47 isolates (21.4%) and medical wards, 31 isolates (14.1%). Out of the detected 220 *S. maltophilia*, 128 (58.2%) were isolated from male patients, 132 (60%) were isolated from patients above 60 years and 146 (66.4%) were found to be present in patients with history of prolonged hospitalization (>10 days).

Antibiotic susceptibility testing of the 220 *S. maltophilia* isolates (table 1) showed that 179 (81.4%) of them were MDR. Most of the isolates recorded high resistance to the majority of examined antibiotics, highest resistance was recorded for meropenem (93.2%), ceftriaxone (92.3%), imipenem (91.4%) and piperacillin (87.7%). On the other hand, chloramphenicol, sulfamethoxazole/trimethoprim and levofloxacin were found to be the most effective antibiotics against examined isolates.

Microdilluion test of ciprofloxacin against the examined 179 MDR isolates showed that 43 (24%) of them recorded four fold decrease in the MIC of that antibiotic after addition of the efflux pump inhibitory agent (PβNA) indicating presence of efflux pump system in those isolates (19.5% of all *S. maltophilia* isolates). On the other hand, 147 (82.1%) of the MDR isolates were found to be positive for ESBL production by double disk synergy test representing 66.8% of all isolated *S. maltophilia*. Of the previous positive isolates, 14 (6.4% of all *S. maltophilia* isolates) were found to be positive for both efflux pump and ESBL presence. The detailed results of both phenotypic tests were described in table 2.

PCR assays that were conducted for all phenotypically positive isolates for efflux pump and ESBL (figure 1 and 2) revealed that SmeDEF gene was detected in 38 (88.4%) of the examined 43 efflux pump positive isolates representing 21.2% of the MDR *S. maltophilia* isolates, where as CTX-M gene was found to be harbored by only 12 (8.2%) of all examined 147 ESBL positive isolates representing 6.7% of the MDR *S. maltophilia* isolates. Of the 14 isolates that were positive for both efflux pump and ESBL, only one harbored both SmeDEF and CTX-M genes (table 3).

Table 1: Antibiotic sensitivity pattern of the detected *S. maltophilia* isolates.

Antibiotics	*S. maltophilia* (number=220)	
	Sensitive(%)	Resistant(%)
Amoxicilline/Clavulinic acid	58(26.4%)	162(73.6%)
Piperacillin	27(12.3%)	193 (87.7%)
Sulfamethoxazole/Trimethoprim	163(74.1%)	57(25.9%)
Piperacillin/Tazobactam	30(13.6%)	190(86.4%)
Imipenem	19(8.6%)	201(91.4%)
Meropenem	15(6.8%)	205(93.2%)
Gentamicin	37(16.8%)	183(83.2%)
Amikacin	31(14.1%)	189(85.9%)
Ceftriaxone	17(7.7%)	203(92.3%)
Ciprofloxacin	56(25.5%)	164(74.5%)
Levofloxacin	133(60.5%)	87(39.5%)
Aztreonam	42(19.1%)	178(80.9%)
Cefotaxime	52(23.6%)	168(76.4%)
Ceftazidime	105(47.7%)	115(52.3%)
Chloramphenicol	173(78.6%)	47(21.4%)
Tobramycin	58(26.4%)	162(73.6%)

Table 2: Results of phenotypic tests used for detection of efflux pump and ESBL production in MDR *S. maltophilia* isolates.

MDR *S. maltophilia* isolates (number=179)			
Positive for efflux pump alone n (%)	**Positive for ESBL alone n (%)**	**Positive for both efflux pump and ESBL n (%)**	**Negative for both efflux pump and ESBL n (%)**
29 (16.2%)	133 (74.3%)	14 (7.8%)	3 (1.7%)

Table 3: PCR results of SmeDEF and CTX-M genes in the examined phenotypically positive *S.maltophilia* isolates.

MDR *S. maltophilia* isolates (number=179)	**Isolates positive for efflux pump alone (n=29)**	Positive for SmeDEF gene. n(%)	26(89.7%)
		Negative for SmeDEF gene. n(%)	3(10.3%)
	Isolates positive for ESBL alone (n=133)	Positive for CTX-M gene. n(%)	11(8.3%)
		Negative for CTX-M gene. n(%)	122(91.7%)
	Isolates positive for both efflux pump and ESBL (n=14)	Positive for SmeDEF gene. n(%)	11(78.6%)
		Positive for CTX-M gene. n(%)	0(0.0%)
		Positive for both SmeDEF and CTX-M genes. n(%)	1(7.1%)
		Negative for both SmeDEF and CTX-M genes. n(%)	2(14.3%)

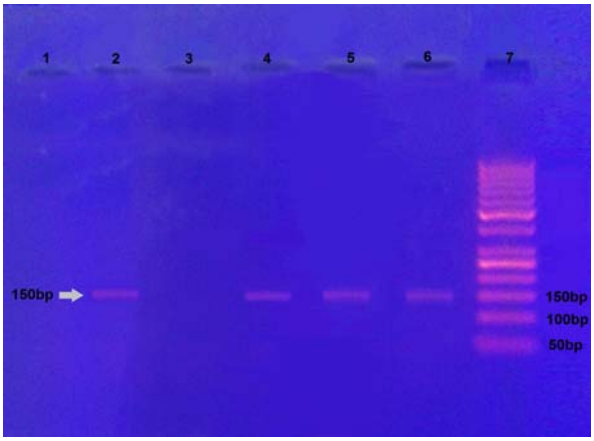

Fig. 1: PCR results of SmeDEF gene in the examined efflux pump positive isolates: Lane 7 shows the molecular size marker #SMO373, lane 2,4,5,6 show the 150 base pair bands from PCR positive isolates.

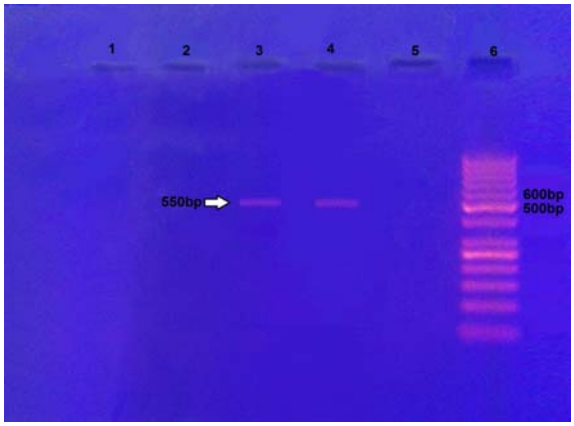

Fig. 2: PCR results of CTX-M gene in the examined ESBL positive isolates: Lane 6 shows the molecular size marker #SMO373, lane 3 and 4 show the 550 base pair bands from PCR positive isolates.

DISCUSSION

S. maltophilia is one of the most important non fermentative Gram negative bacteria that associated with different types of infections mostly those acquired in hospitals. increased resistance rate was recorded for those bacteria limiting their therapeutic options [11]. Many studies were performed to detect the actual mechanisms of resistance in this organism, but there isn't any one compare the presence of specific determinants as causes of resistance in certain group of those bacteria [28], so we tried to get in the depth of resistance causes of this organism and we chose two of the most important mechanisms to be compared, efflux pump system and ESBL production.

The present study was conducted on 220 *S. maltophilia* nosocomial isolates that mostly detected in respiratory specimens (55.9%) indicating its strong association with respiratory tract infecion than other types of infection. Previous studies confirm this association by observing that respiratory tract was the most common site of infection from which *S. maltophilia* had been isolated [2, 4, 29, 30, 31], on the other hand few studies stated that bacteremia was recorded as the most common type of infection from which *S. maltophilia* has been isolated [32, 33] and reported that blood stream infecion caused by *S. maltophilia* is usually of nosocomial source in 76% of cases [34]. The elevated isolaion rate S. *maltophilia* from ICUs than other hospital wards indicates the importance of this organism in being mostly associated with cases suffering from sever illness that need special effort in designation of the treatment regimen.

The examined *S. maltophilia* isolates recorded high degree of resistance to various classes of examined antibiotics, 81.4% of them were classified as MDR isolates which support the previous finding of other studies that reported the majority of their examined isolates as MDR (96.7%) [31]. Although most of the isolates (74.1%) were found to be sensitive to sulfamethoxazole/trimethoprim, the remainig 25.9% non responsive isolates should raise our attention to the problem of presence of certain isolates that developed resistance even to the first antibiotic of choice used in their management. The observed resistance to sulfamethoxazole/trimethoprim was also documented by previous researches as an increasing problem among clinical isolates of *S. maltophilia* [4, 30, 31, 35, 36] that highlights the importance of the interest in searching for causes of resistance.

The mechanisms that are usually involved in the antimicrobial unresponsiveness of clinical isolates of *S. maltophilia* are mainly the intrinsic resistance that mostly caused by β-lactamase production [37] and mulidrug efflux pump system [38]. Acquired resistance may be also occurred in those isolates either by plasmides, transposons or integrons [39].

All of MDR *S. maltophilia* were subjected to two phenotypic tests for detection of efflux pump system and ESBL producion, 24% of those isolates (19.5% of all *S. maltophilia* isolates) were found to be positive for presence of efflux pump as they recorded a decrease of the MIC of the tested antibiotic after addition of the efflux pump inhibitory agent. Several studies were performed on *S. maltophilia* to check the presence of this system as a cause of resistance in those isolates, of which a performed Chinese study in 2012 recorded a prevalence of efflux pump system in the examined isolates that approximates our results [29], also 16.7% of *S. maltophilia* was reported to be positive for efflux pump in a previous study performed in Saudi Arabia which is near to our results, the authors also observed that bacteria as one of the most common Gram negative bacteria showing an elevated prevalence of this resistance mechanism among their studied isolates [25].

Double disk synergy test revealed that 82.1% of the MDR isolates were positive for ESBL (66.8% of all isolated *S. maltophilia*), which was higher than the prevalence that was observed in previous studies at which 55% of the examined MDR *S. maltophilia* isolates were ESBL producers [35], however higher prevalence of ESBL producing *S. maltophilia* was reported in other research performed in Saudi Arabia (83.3%) [32] cofirming the great association between ESBL production and the natural resistance that usually observed in those bacterial isolates.

Presence of Efflux pump and ESBL in *S. maltophilia* as important markers of resistance in those bacteria that are commonly isolated from nosocomialy infected patients particularly in our locality necessitate an extra effort to be done inorder to determine which one of both mechanisms is more common, so we intend in this study to determine the presence of SmeDEF and CTX-M genes as two important determinants of both studied resistance mechanisms aiming at improvement of the patient outcome if we could actually target the more prevalent one.

PCR revealed that SmeDEF gene was detected in 21.2% of the studied MDR *S. maltophilia* isolates, where as only 6.7% of those isolates showed positive PCR results for CTX-M gene, previous studies documented higher prevalence of SmeDEF gene among clinical strains of *S. maltophilia*, 31% and 33% respectively [4, 5]. The great role SmeDEF mulidrug efflux system in the acquisition of resistance in clinical strains of *S. maltophilia* was also approved recently by certain studies at which the authors reported that 57.5% and 100 % of their studied isolates were found to be respectively positive for that efflux system [6, 29].

Presence of CTX-M gene among *S. maltophilia* was tested in few studies, this gene was detected in (4/12) 33.3% of ESBL producing *S. maltophilia* that were isolated in previous study performed in France, although the four strains were isolated from the same patient [15], this prevalence is more than that reported in

the present study. Supporting the observation of low CTX-M gene prevalence among our *S. maltophilia* isolates, other studies recorded absence of CTX-M gene among the examined *S. maltophilia* [40], and reported presence of other genetic determinants conferring resistance to beta lactams like TEM gene [25].

CONCLUSION

By comparing the prevalence of SmeDEF and CTX-M genes among our studied isolates (21.2% and 6.7% of the MDR *S. maltophilia* isolates respectively) we could achieve a final conclusion of higher prevalence of SmeDEF gene among the examined nosocomial *S. maltophilia* isolated from our hospitals being a more prevalent cause of gaining the MDR criteria in those isolates than the CTX-M gene. This emphasize the important role of multidrug efflux pump as a major cause of antibiotic unresponsiveness in *S. maltophilia* acqiured by hospital infection particularly that determined by SmeDEF determinant and put the ESBL determined by new CTX-M genitic determinant in the second position after it. These findings could be an additional tool for designation of treatment regimen of *S. maltophilia* at which MDR is the most important obstacle facing the medical staff in dealing with infections caused by those isolates.

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