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Detection and Characterization of *Stenotrophomonas maltophilia* Strains Isolated from Egyptian Hospitals

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Abstract

Stenotrophomonas maltophilia is an emerging multidrug resistant organism with an increasing incidence in hospital acquired infections particularly in developing countries. Despite intensive surveillance and preventive measures, treatment of invasive *S. maltophilia* infections is a great challenge because of the inherent multidrug resistance of this organism, which has been shown to increase mortality rates among many other nosocomial infections causing pathogens. In this study a total of 50 *S. maltophilia* isolates were isolated from different clinical specimens including 7blood, 3 urine,8 sputum,2 wound, 8nasal, 11 endotracheal tube and 11nasogastric tube swaps collected from 5 hospitals in Cairo and Giza (National Cancer Institute, Al-Kasreleiny Hospital, Al-Zahraa Hospital, Al-Demerdash Hospital, Al-Galaa Hospital). These isolates were identified as *S. Maltophilia* biochemically, by API20E for suspected isolates and genetically by detection of 23S rRNA gene. All isolates show positive results and confirmed for the presence of the gene.

The antibiotic susceptibility patterns for the isolated *S. maltophilia* were also evaluated. The detection of the presence of *L1 enzyme gene* responsible for production of Metallo-β-lactamases was also done. We observed that only forty six out of fifty isolates (92%) were positive to the Metallo-β-lactamases gene L1. They were also evaluated for the effectiveness of drug combination of Trimethoprim/sulfamethoxazole, levofloxacin and ciftazidime against 10 isolates. Regarding the combination of levofloxacin and ciftazidime, 4 of the 10 strains showed synergy with a percentage of 40%. While 6 strains showed additive results with a percentage of 60%. For the combination of Trimethoprim/sulfamethoxazol and ceftazidime, 3 of the 10 strains showed additive with a percentage of 30%. While 7 strains showed in difference results with a percentage of 70%. For the combination of Trimethoprim/sulfamethoxazol and levofloxacin, 2 of the 10 strains showed additive with a percentage of 20 %. While 8 strains showed indifference results with a percentage of 80%.

The antibacterial effect of the 3 combinations on *S. maltophilia* showed mostly additive results. No significant differences between the three combinations that can give a priority for a combination over the other. Or over a single drug use.

Keywords: *Stenotrophomonas maltophilia, HAI, Egyptian Hospitals*

Introduction

Hospital- Acquired Infection (HAI) or nosocomial infection is defined as a localized or systemic condition resulting from an adverse reaction to an infectious organism or its toxin that develops in a patient 48 hours or more after entrance to the hospital and was not incubated at the admission time [1].

A surveillance program conducted in Egypt showed high rates of ICU-onset HAIs, and a high resistance pattern of organisms causing HAIs, representing a major risk to patient safety [2]. *S. maltophilia* is a Gram-negative bacterium that is widespread in the environment

and that has become important in the last years as an emerging opportunistic pathogen associated with nosocomial colonization and infection. *S. maltophilia* is frequently isolated from clinical specimens and is implicated in catheter-related bacteremia and septicemia, urinary and respiratory tract infections [3].

S. maltophilia is an emerging nosocomial pathogen. In a surveillance performed from 1997 to 1999 in the Asia-Pacific, Europe, Latin America, Canada, and the United States regions, *S. maltophilia* was the third most frequently isolated non-fermentative gram-negative *bacilli*, following *Pseudomonas aeruginosa* and *Acinetobacter* species [4]. The treatment of *S. maltophilia* infections is problematic, as isolates are resistant to many clinically useful antibiotics. A number of laboratories have begun to address the molecular bases for the broad antibiotic resistance and for virulence in *S. maltophilia* [3]. Multidrug resistance, which is engendered by a diversity of resistance mechanisms, leaves insufficient alternatives for treatment in some patients [5]. For this reason there are not many options available for treatment and control of this pathogen in hospitals in Egypt. Combination therapy is one of the most successful methods for treatment as it reduces toxicity and resistance to the antibiotics used. The aim of the work is detection of *P. aeruginosa* isolates from intensive care units and cancer patients in Egypt. Moreover, explore their antimicrobial susceptibility patterns and their major resistance mechanisms. Also, determine the best option for treatment of such pathogen.

Materials and Methods

Bacteriological Examination

A total of 50 *S. maltophilia* isolates were isolated from different clinical specimens including 7 blood, 3 urine, 8 sputum, 2 wound, 8 nasal, 11 endotracheal tube and 11 nasogastric tube swaps collected from 5 hospitals in Cairo and Giza (National Cancer Institute, Al-Kasreleiny Hospital, Al-Zahraa Hospital, Al-Demerdash Hospital, Al-Galaa Hospital). *S. maltophilia* isolates identified biochemically by using catalase test, motility test and Dnase test [6]. Ten suspected isolated were confirmed by API 20E test kit (BioMèrieux, France).

DNA Extraction

DNA was extracted from the bacterial colonies using the QIAmp DNA mini kit (Qiagen Inc.) according to the manufacturer's recommendation. The DNA concentration and purity were determined by measuring the absorbance at 260 nm and by calculating the ratio of absorbance at 260 nm to that at 280 nm using a spectrophotometer (U.V-VIS.), U.V 2500 (Labomed. Inc.) [7]. DNA was used directly or stored at -20^{0}C for future use.

Detection of *S. maltophilia* group specific23S rRNA gene and L1 gene responsible for production of Metallo-β-lactamases responsible for Carbapenem resistance by polymerase chain reaction assay:

Amplification and detection of *S. maltophilia* group specific gene was done according to the method previously described by Gallo., *et al.* [8]. All oligonucleotides were synthesized in Bio Basic Inc. (Canada). The sequences of the primers used for detection of *S. maltophila* group specific gene"23S rRNA are: 5'-GCTGGATTGGTTCTAGGAAAACGC-3' and 5'-ACGCAGTCACTCCTTGCG- 3'. The sequences of the primers used for detection of the *L1* gene are: 5'ACCATGCGTTCTACCCTGCTCGCCTTCGCC-3' and 5'-TCAGCGGGCCCCGGCCGTTTCCTTGGCCAG- 3' [9].

The PCR was performed in a total volume of 25 µl reaction mixtures containing 150 - 200 ng of DNA as template, 0.5 µM of each primer and 1x of PCR master mix (Taq Master/High yield, Jena Bioscience) which provides 2.5 units per reaction of DNA polymerase, 0.2 mM of each deoxynucleotide triphosphate, 1xPCR buffer (with 1.5 mM-$MgCl_2$). The amplification cycles were carried out in a programmable heating block, (Primus Thermal Cycler, MWG Biotech, Germany). Reaction conditions were optimized to be 94°C for 3 minutes as initial denaturation, followed by 30 cycles of 95°C for 30seconds, (58°C for 45 seconds and72°C for 45 minute for detection of *S. maltophila* and 53°C for 30 seconds and 72°C for 1 minute for *L1* gene). A final extension step at 72°C for 5 minutes was followed. Negative control (no template) and positive control (reference strain) were included. Amplification products were electrophoresed in 2% agarose gel in 0.5x TBE (Tris-borate-EDTA) at 70 Volts for 60 min and visualized under ultraviolet light. To assure that the amplification products were of the

expected size, a 1500 bp DNA ladder was run simultaneously as a marker. Presence of 278 bp and 876 bp DNA fragment indicated positive sample of *S. maltophila* group specific and *L1* gene, respectively.

Antimicrobials Susceptibility Testing

A total of 50 *S. maltophila* isolates were tested for their sensitivity to various antimicrobial agents by disc diffusion method according to Clinical laboratory standards institute [10]. Isolates were cultured on Mueller-Hinton agar and tested for their susceptibility to12 antimicrobial agents [10].

The following antimicrobial discs were used: ceftazidime (30 µg), levofloxacin (5 µg) and trimethoprim/sulfamethoxazole (1.25/ 23.75 µg). All antimicrobial discs were purchased from Oxoid Chemical Co. UK.

Assessment of combination therapy by checkerboard assay for treatment of multi-drug resistant bacterial isolates [11]:

The MICs of ceftazidime, levofloxacin and trimethoprim/sulfamethoxazole for the bacterial isolates were determined by the broth microdilution method, according to the CLSI [10]. Five strains were resistant to ceftazidime, levofloxacin and Trimethoprim/ sulfamethoxazol. And five strains were susceptible to Trimethoprim/sulfamethoxazol only and resistant to ceftazidime and levofloxacin. The synergy study for levofloxacin and ceftazidime, trimethoprim/sulfamethoxazole and ceftazidime and trimethoprim/sulfamethoxazol and levofloxacin combinations was performed for ten isolates, using the checkerboard method. The checkerboard test was performed on Ninety-six well microtiter plates were prepared with doubling dilutions of trimethoprim/sulfamethoxazol for trimethoprim/sulfamethoxazole and ceftazidime, trimethoprim/sulfamethoxazol and levofloxacin combinations. Doubling dilutions of Levofloxacin for levofloxacin and ceftazidime, trimethoprim/sulfamethoxazol and levofloxacin combinations. And doubling dilutions of ceftazidime for levofloxacin and ceftazidime, trimethoprim/sulfamethoxazole and ceftazidime combinations. The initial bacterial inoculum was adjusted to 10^6 CFU/mL. The plate was incubated for 18 h at 35^0C. The fractional inhibitory concentration index (FICI) was calculated using the concentrations in the first non-turbid (clear) well in each row and column along the turbidity/non-turbidity interface and then averaged. The results were then classified as: synergy for Σ FIC ≤ 0.5; additive for Σ FIC between 0.5 and 1.5; and indifference for values of Σ FIC between 1.5 and 2; Antagonism was linked to values above 2 [12-14].

Statistical Methods

Statistical analysis of results from checkerboard assay was performed using the chi-square test. Differences were considered significant when $p \leq 0.05$. The data was coded and entered using the statistical package SPSS version 15 (IBM, New York, United States).

Results

Fifty isolates of *S. maltophila* were identified and confirmed by different morphological, biochemical tests. All isolates reacted positively to motility, catalase and dnase tests. 10 suspected isolates were confirmed by API 20E system showed 90.32 % identification. All isolates were confirmed by PCR assay using S. maltophila specific primers. Peaks for positive samples appeared at (278 bp) as shown in figure 1.

Fifty isolates were tested for antibiotics resistance patters Seventy two percent were sensitive to trimethoprim/sulfamethoxazol followed by levofloxacin (68%), then ceftazidime (20%). PCR reactions for confirmed *S. maltophila* isolates were done for detection of L1 gene that is responsible for carbapenem resistance. 46 isolates show positive results and confirmed for the presence of the gene by showing a band on 876 bp, as illustrated in figure 2.

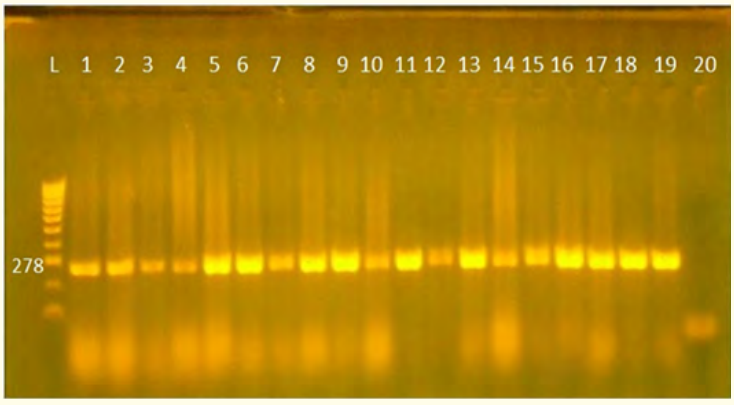

Figure 1: *PCR amplification with 23S rRNA gene. primers. A 278-bp of 23S rRNA gene. Lane (L), DNA molecular size marker (l500 bp ladder), Lanes (1-18) show positive result with positive bands of 278 bp. Lane (19) Positive control and Lane (20) negative control.*

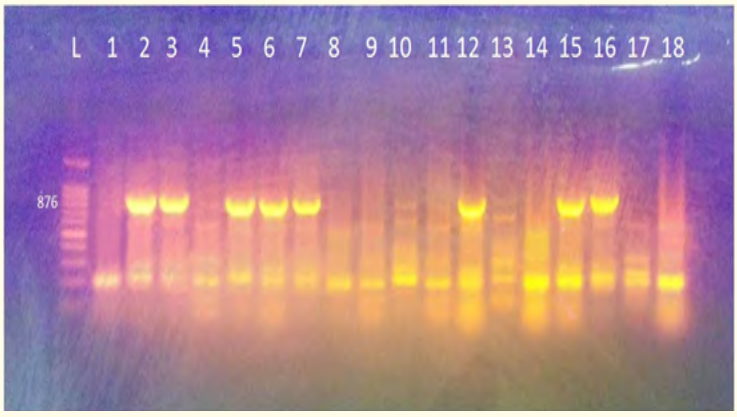

Figure 2: *PCR amplification with L1 gene primers. A 876-bp of L1 gene. Lane (L), DNA molecular size marker (l500 bp ladder) and Lanes (2-3), (5-7), (12) and (15-6) show positive results with positive bands of 876 bp. Lanes (1), (4), (8-11), (13-4) and (17-18) show negative results.*

MIC determination of ceftazidime, levofloxacin and trimethoprim/sulfamethoxazole against *S. maltophilia:*

10 strains of *S. maltophilia* of different susceptibility profiles were used in this test. Five strains were resistant to ceftazidime, levofloxacin and Trimethoprim/ sulfamethoxazol. And five strains were susceptible to Trimethoprim/sulfamethoxazole only and resistant to ceftazidime and levofloxacin. The MICs obtained for each antibiotic are shown in tables 1-3.

MICs results (MIC done in triplet) by broth macrodilution for ceftazidime (CAZ) for 10 *S. maltophilia* strains shows no sensitive isolates, 20 % intermediate and 80 % resistant. MIC Interpretive Criteria (≤ 8 = Sensitive, 16 = Intermediate and ≥ 32 = Resistant) [10].

While MICs results (MIC done in triplet) by broth macrodilution for Levofloxacin (LEV) for 10 *S. maltophilia* strains showed 20% sensitive, 30% intermediate and 50 % resistant as Shown in table 2. MIC Interpretive Criteria (≤ 2 = Sensitive, 4 = Intermediate and ≥ 8 = Resistant) [10].

While MICs results (MIC done in triplet) by broth macrodilution for Trimethoprim /sulfamethoxazol (SXT) for 10 *S. maltophilia* strains showed 50% sensitive and 50 % resistant as shown in table 3.

No. of Isolates	MIC Ug/ml				Interpretation
	A	B	C	Median	
1	128	64	128	128	R
1	512	512	512	512	R
1	512	256	512	512	R
3	64	32	64	64	R
1	128	256	256	256	R
1	128	128	128	128	R
2	16	16	32	16	I

Table 1: *MICs results by broth macrodilution of ceftazidime (CAZ) for 10 S. maltophilia strains (a and b and c: MIC done in triplet).*

No. of Isolates	MIC Ug/ml				Interpretation
	A	b	C	Median	
2	16	16	16	16	R
2	16	8	16	16	R
1	8	8	16	8	R
1	4	4	4	4	I
1	2	4	4	4	I
1	8	4	4	4	I
1	1	1	0.5	1	S
1	0.5	1	0.5	0.5	S

Table 2: *MICs results by broth macrodilution of Levofloxacin (LEV) for 10 S. maltophilia strains (a and b and c: MIC done in triplet.*

No. of Isolates	MIC Ug/ml				Interpretation
	A	b	C	Median	
1	8/ 152	4/76	8/152	8/152	R
1	16/304	16/304	32/608	16/304	R
2	4/76	8/152	4/76	4/76	R
1	8/152	16/304	16/304	16/304	R
1	1/19	1/19	1/19	1/19	S
1	0.5/9.5	0.25/4.75	0.25/4.75	0.25/4.75	S
2	1/19	0.5/9.5	0.5/9.5	0.5/9.5	S
1	1/19	2/38	2/38	2/38	S

Table 3: *MICs results by broth macrodilution of Trimethoprim /sulfamethoxazol (SXT) for 10 S. maltophilia strains (a and b and c: MIC done in triplet).*

Checkerboard Results

Table (4-6) shows the FICs calculated for all the *S. maltophilia* strains using the 3 combinations of antibiotics. For the combination of levofloxacin and ciftazidime 4 of the 10 strains showed synergy with a percentage of 40%. While 6 strains showed additive results with a percent of 60%. For the combination of Trimethoprim/sulfamethoxazol and ceftazidime 3 of the 10 strains showed additive effect with

a percentage of 30%. While 7 strains showed indifferent results with a percentage of 70%. For the combination of trimethoprim/sulfamethoxazol and levofloxacin 2 of the 10 strains showed synergy with a percent of 20%. While 8 strains showed additive results with a percent of 80%.

The antibacterial effect of the 3 combinations on *S. maltophilia* showed mostly additive results. No significant differences between the three combinations that can give a priority for a combination over the other. Or over a single drug use.

Number of isolates	Percent	Effect
4	40%	Synergism
6	60%	Addition

Table 4: *FICs calculated for all the S. maltophilia strains using combinations of levofloxacin and ceftazidime.*

Number of isolates	Percent	Effect
3	30%	Addition
7	70%	Indifference

Table 5: *FICs calculated for all the S. maltophilia strains using combinations trimethoprim/sulfamethoxazole and ceftazidime.*

Number of isolates	Percent	Effect
2	20%	Synergism
8	80%	Addition

Table 6: *FICs calculated for all the S. maltophilia strains using combinations of trimethoprim/sulfamethoxazol and levofloxacin.*

Discussion

HAIs (Hospital-Acquired Infections) lead to high mortality and remain a major problem in health care centers in the world. The highest rates of HAIs are observed in ICUs (intensive care units), which are also the ward in that the most severely ill patients are treated [15].

S. maltophilia is an emerging multidrug opportunistic pathogen. It's intrinsic resistance to most antibiotics and colonize the surfaces of medical devices a potentially dangerous pathogen settings [16]. In this study a total of 50 *S. maltophilia* clinical isolates were collected from clinical different specimens from ICU patients. The antimicrobial susceptibility testing of the isolates was done using the Kirby Bauer disk diffusion method following the definition of the Clinical and Laboratory Standards Institute (2013) [10] using antibiotics containing discs. The antimicrobial susceptibility testing of *S. maltophilia* isolates revealed that 72% were sensitive to trimethoprim/sulfamethoxazol followed by levofloxacin (68%), then ceftazidime (20%). These results nearly resembles that presented by Church., *et al.* [17], where (17%) were resistant to trimethoprim/sulfamethoxazole, (26%) for ceftazidime and (28%) for levofloxacin. While, a study conducted in Egypt by Morsi., *et al.* [18] that revealed that (37.5%) of S. maltophilia isolates were resistant to trimethoprim/sulfamethoxazole, which may be due to excessive use of SXT among MDR bacteria in that hospital.

Carbapenems have been the drug of choice for the treatment of infections caused by Multi drug resistant Gram-negative bacilli [19]. However, carbapenem resistance has been observed frequently in *S. maltophilia*. Resistance to carbapenem is mainly due to carbapenem hydrolyzing enzymes-carbapenemase [20]. The carbapenemases found are mostly metallo-β-lactamases (MBL), including *L1* gene [21].

Different families of metallo-β-lactamases (MBL) have been reported from several geographical regions so far. One of The most commonly reported family is VIM (for Verona Integron-encoded metallo-β-lactamase, first isolated in Italy). VIM 2 producing Pseudomonas strains have been reported worldwide, in different geographical areas [22]. PCR analysis using specific primers for *L1* gene confirmed the presence of the metallo β lactamase gene [21]. The present study revealed that (92%) of carbapenem resistant *S. maltophilia* isolates were

positive to *L1* gene which is responsible for carbapenem resistance in *S. maltophilia* isolates. This finding was supported by results of previous study by Walsh., *et al.* [21] demonstrating the presence of *L1* gene (95%) as the most dominant MBL implicated in carbapenem resistant *S. maltophilia*.

Due to the lack or inefficiency of infection control programs in many hospitals, random/extensive use of antibiotics and many other reasons, resistance highly emerged within these pathogens and they became known as highly resistant microorganisms [23]. Carbapenem resistant *S. maltophilia* strains are nowadays widely spread.

S. maltophilia is a non-fermenting, aerobic Gram-negative rod that has been more frequently isolated as a nosocomial pathogen over the past decade. Because of the low pathogenicity of this organism, patients infected with *S. maltophilia* usually have underlying immunodeficiency or history of long-term or multiple hospitalizations, invasive devices (e.g., indwelling catheters), immunosuppressive chemotherapy and/or broad-spectrum antibiotic exposure [24].

Treatment of *S. maltophilia* infections presents a significant challenge, as the organism is typically resistant to most commonly used antimicrobial drugs. *S. maltophilia* produces at least two clinically important inducible β lactamases: an L1 zinc-dependant carbapenemase, which is not inhibited by clavulanic acid and an L2 cephalosporinase that is inhibited by clavulanic acid [25]. The L1 β-lactamase hydrolyzes most β lactam drugs including carbapenems (imipenem and meropenem) and only aztreonam is relatively resistant to hydrolysis. Consequently, aztreonam may serve as a competitive inhibitor of the L1 enzyme [26]. With the combination of ticarcillin/clavulanate, the L2 enzyme is expected to be irreversibly inhibited by clavulanate and the L1 enzyme partially inhibited by aztreonam. In addition, changes in outer membrane characteristics and low numbers of membrane porin channels limit the penetration of several antibiotics to their site of action [27,28].

Currently the drug of choice for *S. maltophilia* infections is the combination trimethoprim/ sulfamethoxazole (TMP/SMX). This is based primarily on in vitro susceptibility data and case reports; however, resistance to this drug appears to be increasing [29].

With this in mind, we designed a study to examine several antimicrobial combinations for activity against *S. maltophilia* and assess these combinations for additive and/or synergistic effects.

This study revealed that (40 %) of *S. maltophilia* isolates shows synergistic effect to levofloxacin and ciftazidime combination and (60%) shows an additive effect. While, (20%) of *S. maltophilia* isolates shows synergistic effect to trimethoprim/ sulfamethoxazol and levofloxacin combination and (80%) shows an additive effect. On the other hand, (20%) of *S. maltophilia* isolates shows additive effect to trimethoprim/ sulfamethoxazol and ceftazidime combination and (80%) shows an indifferent effect. According to our results, ceftazidime can be synergistic with levofloxacin.

These results shows some differences with the results obtained by Juhasz., *et al*. [30] where (75%) of *S. maltophilia* isolates shows synergistic effect to levofloxacin and ciftazidimecombination and (25%) shows an additive effect. While, (25%) of *S. maltophilia* isolates shows synergistic effect to trimethoprim/ sulfamethoxazol and levofloxacin combination and (75%) shows an additive effect. On the other hand, all S. maltophilia isolates shows additive effect to trimethoprim/ sulfamethoxazol and ceftazidime combination. This difference may be due to the genetic diversity of *S. maltophilia* isolates in hospitals among different countries.

Combination therapy is recommended for severe invasive infections, for immunocompromised patients and for empirical therapy in areas with high frequency of local resistance against SXT [31]. Combinations can be more active than monotherapy and can reduce the risk of developing antibiotic resistance during treatment, but superiority of combination therapy is not proved [32].

S. maltophilia infections are often polymicrobial where the use of a combination therapy may be also advantageous [33]. Against biofilm growth *S. maltophilia* isolates combinations were in vitro effective too [34]. In case of extremely drug-resistant *S. maltophilia* infections, combination therapy can be useful (maybe the only) therapeutic alternative. Several studies tested in vitro antibiotic combinations on SXT-susceptible *S. maltophilia* isolates, but only few ones focused on SXT-resistant ones [35].

Another advantage for combination therapy is the delay in emergence of bacterial resistance and specifically the rapidly developing resistance toward Colistin [23]. It must be mentioned that not only synergy is considered as an advantage for the therapy but also additive result is by itself beneficial, because even a miniature raise in the antibacterial activity using the combination therapy may help clinical success and recovery.

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