ORIGINAL ARTICLE

A one year single-center experience on Stenotrophomonas maltophilia strains in Alexandria, Egypt

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ABSTRACT

Background & objectives: Stenotrophomonas maltophilia (S. maltophilia) emerged as an important opportunistic nosocomial multidrug resistant pathogen. This study aimed to investigate S. maltophilia strains to determine their virulence factors, antimicrobial resistance pattern, detect integrons and reveal their genotypic relatedness.

Methodology: S. maltophilia clinical isolates were subjected to antimicrobial susceptibility testing using the VITEK-2 compact system (BioMérieux), Biofilm production, hemolytic, protease and lipase activity were tested. Polymerase Chain Reaction (PCR) for integrase 1 and 2 genes and Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR were done. Results: S. maltophilia constituted 0.8% of gram-negative non-fermenters. All strains were biofilm producers. All strains were susceptible to trimethoprim/sulfamethoxazole. Class 1 integron was detected in five (35.7%) strains. ERIC PCR showed high genetic diversity between the strains. Conclusions: Although multiple virulence factors were detected, strains were still susceptible to the recommended antimicrobials. ERIC PCR was found to be valuable in detecting genetic diversity among S. maltophilia strains being easy, rapid, cheap and available technique.

INTRODUCTION

Stenotrophomonas maltophilia (S. maltophilia) is a low-virulence opportunistic pathogen that has been identified as an emerging hospital pathogen. Infections caused by S. maltophilia have increased over the years due to increased high-risk patients e.g. patients with immune suppression, hematological malignancies, ICU admission. It is an aerobic biofilm producing gram-negative bacterium, has several virulence potentials, that grows in wet environment like ventilation tubes, catheters and endoscopes. Pulmonary infections are the most common followed by blood stream infections then skin and soft tissue infections, and urinary infection.

Identification of gram negative non fermenters remain a challenge that needs an accurate and rapid technique to be conclusive. Matrix –assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has rapidly emerged as a routine method to identify difficult pathogens as an effective and economic way.

The extraordinary innate and acquired resistance to numerous antimicrobials, including carbapenems, aminoglycosides, and cephalosporins, makes treatment of S. maltophilia infections problematic. It is a multidrug resistant (MDR) bacterium since it has limited therapeutic choices. The existence of class 1 and 2 integrons, which are mobile elements capable of carrying a large number of gene cassettes responsible for various antimicrobial resistance mechanisms among bacteria, is one major molecular mechanism leading to its antibiotic resistance.

Strain typing is important in surveillance for healthcare associated infections as well as investigating outbreaks. Various molecular typing methods are available including multilocus sequence typing (MLST), PCR fingerprinting and pulsed field gel electrophoresis.

The goal of this study was to investigate the clinical strains of S. maltophilia in order to detect their virulence factors, antimicrobial resistance patterns, detect the presence of class 1 and 2 integrons and their relationship to antibiotic resistance, and look at their strain diversity and genotypic relatedness. This information was then utilised to compare the pathogenicity capacities of various strains.

METHODOLOGY

A prospective observational study was conducted on S. maltophilia strains collected from different healthcare associated infections (HAI) defined according to CDC definition, and submitted to Diagnostic Medical Microbiology laboratory, Alexandria University Main Hospital (AUMH), Egypt, throughout a period of one year starting from April 2020 to March 2021. The study was
approved by Medical Research Ethics Committee of Alexandria Faculty of Medicine. Laboratory workup was performed on strains collected through routine clinical work and patient identifiable information was anonymized, no written or verbal informed consent to participate in this study from patient was necessary. The authors had no contact or interaction with the patients.

**Isolation and identification of S. maltophilia strains**

All clinical specimens were processed according to standard operating procedures SOPs of AUMH lab. Specimens were inoculated onto Blood agar and MacConkey’s agar plates (Oxoid, UK), incubated aerobically at 37°C for 24-48 hours. Preliminary identification of *Stenotrophomonas* strains was performed according to the standard microbiological techniques which included: growth as non-lactose fermenting colonies on MacConkey’s agar, non-fermenter on triple sugar iron agar, and negative for oxidase, citrate, urease, and indole, while positive for both ornithine decarboxylation, and motility tests. The organisms that were presumptively identified by conventional methods as *Stenotrophomonas* spp. were further verified by the MALDI-TOF MS as previously described.

**Antimicrobial susceptibility testing (AST)**

The susceptibility of all strains of *S. maltophilia* to different antimicrobial agents was tested by the VITEK 2 compact automated antibiotic susceptibility system (BioMérieux) according to the manufacturer’s instructions. The test was performed using VITEK AST-GN222 Cards (BioMérieux, Inc., Durham, NC, USA).

**Testing virulence factors**

*Biofilm formation assay*

The test was performed using the quantitative microtiter plate test as described before. The optic density (OD) was read at 540 nm. The cut off value was defined as three standard deviations above the mean optical density of control (ODc). Classification of strains were performed according to the following criteria: non biofilm producer (OD ≤ ODc), weak biofilm producer (ODc ≤ OD ≤ 2 x ODc), moderate biofilm producer (2 x ODc ≤ OD ≤ 4 x ODc) and strong biofilm producer (4 x ODc ≤ OD). *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 were included as positive and negative controls for biofilm production respectively.

*Hemolytic activity test*

It was tested by streaking of purified single colony on 5% sheep blood agar plates then incubated at 37°C for 24 hours. A colony with a hemolytic zone was classed as a hemolysin-positive strain.

*Protease activity test*

Pure cultures were streaked onto Mueller–Hinton (MH) agar plates (Oxoid, UK) containing 3% skimmed milk and incubated at 37°C for 24 hours. After incubation, the plates were observed for clear zones around bacterial colonies, indicating positive results for hydrolysis of casein.

*Lipase activity test*

Pure cultures were streaked onto Tryptic soya agar plates (Oxoid, UK) supplemented with 1% Tween 80 and incubated at 37°C for 24 hours. After incubation, the presence of a turbid halo around the colony indicated a positive result.

**Detection of integrase 1 and integrase 2 genes using conventional polymerase chain reaction (PCR)**

DNA extraction was done using boiling method. The specific primers used for PCR amplification of integrase 1 gene (IntI1) were 5-CCTCCCGACGATGATG-3 and 5-TCCACGCTCGTCAGGC-3 and for integrase 2 gene (IntI2) were 5-ATGTCTAACAGTCCATTTTTAATT-3, and 5-GTAGCACAAGTGTGACGAAATG-3. The Thermal cycle parameters were as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1.5 min, followed by a final elongation step at 72°C for 7 min.

**Molecular genotyping of clinical strains by Enterobacterial Repetitive Intergenic Consensus region PCR (ERIC-PCR)**

Characterization of the genetic profiles of the clinical strains was performed using DNA polymorphism analysis after amplification using PCR with primers for conserved sequences from the Enterobacterial Repetitive Intergenic Consensus region (ERIC-PCR). ERIC 1 (5-ATGTAAGCTCTGGGATTCAC-3) and ERIC 2 (5-AAGTAAGTGAAGGGGTAGAG-3) were used. Different strains were identified when a difference of 2 or more DNA bands between ERIC-PCR patterns was detected, subtypes were considered when a difference by only 1 band was present.

**Statistical analysis**

The statistical analysis was performed using Statistical Package for Social Sciences (SPSS ver. 22, IBM Corp., USA). ERIC PCR dendrogram was generated based on neighbor joining method using the Phoretix 1D total lab TL120 software. Strains were categorized to be of the same pattern if the similarity between them reaches values of ≥80%.

**RESULTS**

*S. maltophilia* isolation and identification

6824 culture positive HAI were delivered to AUMH lab during the study period. Out of all culture positive cases, 4718 (69.1%) were gram-negative, 1328 (19.5%) were gram-positive organisms, and 778 (11.4%) were fungi. Out of all gram-negative isolates (4718), 2991 isolates (63.4%) were identified as Enterobacteriales, while 1727 (36.6%) were identified as non-fermentative Gram-negative bacilli. *S. maltophilia* accounted for
0.3% of all gram-negative isolates and for 0.8% of gram-negative non-fermentative, constituting only 14 strains. (Table 1)

Table 1: Distribution of S. maltophilia strains among non-fermenters gram negative bacilli.

<table>
<thead>
<tr>
<th>Type of isolates</th>
<th>Number of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter spp.</td>
<td>926</td>
<td>53.6%</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>764</td>
<td>44.2%</td>
</tr>
<tr>
<td>Burkholderia spp.</td>
<td>23</td>
<td>1.33%</td>
</tr>
<tr>
<td>S. maltophilia</td>
<td>14</td>
<td>0.81%</td>
</tr>
<tr>
<td>Total</td>
<td>1727</td>
<td>100%</td>
</tr>
</tbody>
</table>

The highest isolation rate was from blood specimens (12; 85.7%), followed by urine and sputum specimens (one isolate each; 7.1%). Six isolates (42.8%) were recovered from intensive care units (ICU), while 8 isolates (57.2%) were from other hospital Departments (Oncology, Cardiology, General Surgery and Internal Medicine). All patients were elderly with immunocompromised conditions as presence of invasive devices, recent surgeries, malignancies, ICU admission.

**Antimicrobial susceptibility pattern**

All strains (100%) were susceptible to the following antibiotics using the VITEK2 compact system: trimethoprim-sulfamethoxazole (TMP/SMX), minocycline (MIN), levofloxacin (LEV), chloramphenicol (CL) and ticarcillin/clavulanic acid (TIC/CA). Regarding the susceptibility to CAZ, nine (64.2%) strains were susceptible, one (7.1%) was intermediately susceptible, while four strains (28.5%) were resistant.

**Virulence profile**

According to microtitre plate biofilm production, all strains were biofilm producers; eight strains (57.1%) were strong biofilm producers, while six (42.8%) were moderate biofilm producers. Regarding other virulence factors, all 14 strains (100%) were positive for hemolytic activity, eight strains (57.1%) showed positive protease activity while seven strains (50%) were positive for lipase activity. (Figure 1)

**Class 1 and 2 integrons detection by PCR**

Class 1 integrase gene, IntI1, (280 bp) was detected in five (5/14; 35.7%) strains, while class 2 integrase, IntI2, was negative in all tested strains (Figure 2).
Relation between virulence factors, antimicrobial profile and integron genes

Out of the 14 *S. maltophilia* strains, four (28.6%) strains tested positive for all phenotypic virulence factors and all were strong biofilm producers. Only one of these four strains was resistant to CAZ. Three out of four CAZ resistant strains were strong biofilm producers while the last was moderate producer and all expressed at least two other virulence factors (hemolytic, protease, or lipase activity). The intermediately susceptible isolate to CAZ was moderate biofilm producer and produced hemolytic and protease activities. Two strains having class 1 integron were resistant to CAZ (Table 2).

Table 2: Virulence factors, antimicrobial susceptibility pattern and class 1 integron among the fourteen *S. maltophilia* isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Hemolytic activity</th>
<th>Protease activity</th>
<th>Lipase activity</th>
<th>Biofilm formation</th>
<th>Antibiotic susceptibility</th>
<th>Class 1 integron</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Strong</td>
<td>Susceptible</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Strong</td>
<td>Susceptible</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Moderate</td>
<td>Susceptible</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Moderate</td>
<td>Susceptible</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Moderate</td>
<td>Susceptible</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Strong</td>
<td>Susceptible</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Moderate</td>
<td>Susceptible</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Strong</td>
<td>Ceftazidime resistant</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Strong</td>
<td>Susceptible</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Moderate</td>
<td>Intermediately susceptible to Ceftazidime</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Strong</td>
<td>Ceftazidime resistant</td>
<td>Positive</td>
</tr>
<tr>
<td>12</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Strong</td>
<td>Susceptible</td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Moderate</td>
<td>Ceftazidime resistant</td>
<td>Positive</td>
</tr>
<tr>
<td>14</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Strong</td>
<td>Ceftazidime resistant</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Typing results by ERIC PCR

Fourteen genotypes were identified for the 14 clinical strains by ERIC PCR. A dendrogram was generated based on the “Neighbour joining” method in the Phoretix TotalLab TL120 software. A visual analysis of the dendrogram showed heterogeneous profiles, in which the degree of similarity varied from 20%-100%. A cluster analysis was performed using the same software. Thirteen major clusters labeled A-M were defined sharing an average ~80% of genotype similarity. Clusters H include 2 strains representing 14.2% of the total strains. (Figure 3).

Fig. 3: (A) Agarose gel stained with ethidium bromide. Lane M: 100-1000 bp DNA ladder. Lane 1-14: show some genotypes obtained by ERIC PCR. (B) ERIC PCR dendogram shows 13 clusters from closely related isolates sharing an average ~80% of genotype similarity.
DISCUSSION

*S. maltophilia* has recently emerged as a threatening nosocomial pathogen having wide variety of virulence factors, intrinsic antimicrobial resistance to a wide range of commonly used drugs, including β-lactams, carbapenems, and aminoglycosides.1

In the present study, *S. maltophilia* isolates represented 0.3% of total isolates, and 0.8% of non-fermenter gram-negative bacilli. The frequency of occurrence ranged from 0.8 to 1.4% in SENTRY studies during 1997-2003 that increased to 1.3-1.68% in the following decade.14 Closely reported rate was described by a recent Indian study, which reported that *S. maltophilia* represented 0.4% from non-fermenter gram-negative isolates.15

*S. maltophilia* bloodstream infections, once considered infrequent, are increasing as a HAI due to higher incidence of immunocompromised population (hematological cancers, transplantation), invasive procedures and surgical interventions allowing bacteria to invade the host with crude mortality rate of 40-50%.16 Indeed, all bloodstream strains in the present study were isolated from elderly patients having comorbidity or immunocompromised condition.

*S. maltophilia* is a pathogen with multiple virulence factors important in pathogenicity and antimicrobial resistance. Biofilm formation, which is an extracellular polymeric substance, is a very important virulence factor. It acts as a diffusion barrier, evades immune response, enhance persistence and have efflux mechanisms making the organism multidrug resistant with limited options.17 All our strains were biofilm producers. Biofilm production was detected in majority of strains if not all in previous studies.18,19

Additionally, all strains had haemolysin activity, and about 50% of the strains produced lipase and protease. These extracellular enzymes inactivate the immune system, degrade serum and tissue proteins increasing the pathogenicity of infection. Alcaraz et al. and Flores-Treviño S reported that all strains were positive for studied virulence factors.20,21

*S. maltophilia* is a multidrug resistant pathogen with limited treatment options due to its intrinsic and acquired resistance to different antibiotics. TMP/SMX remains the most effective antimicrobial agent in treatment, besides its antimicrobial effect it also has biofilm inhibitory properties.22 Other treatment options include CAZ, TIC/CA, fluoroquinolones, and tetracyclines. In the current study, all strains (100%) were susceptible to TMP/SMX, MN, LEV, CL, and TIC/CA. Worldwide, considerable resistance rates have been reported. According to The SENTRY Antimicrobial Surveillance Program, the sensitivity rates of TMP/SMX decreased from 97.2% in (2001–2004) to 95.7% in (2013–2016), with variable rates among different geographical regions.23 Resistance rates in Western countries ranged from 2–10%, however, higher resistance levels were reported in selected countries (Spain: 27%, Turkey: 10–15%, Taiwan: up to 25%, China: 30–48%) and may be even higher in immunocompromised patients (20–80%).16 Error! Bookmark not defined. A study from Egypt reported resistance to TMP/SMX to be 8.6%.24 In the SENTRY study, overall resistance to LEV was higher than TMP/SMX (11%) while susceptibility to MN was higher (99%).25 The 100% susceptibility rates to TMP/SMX, MN, LEV, CL, and TIC/CA reported in the present study, together with the relatively low CAZ resistance rate was an interesting finding, however, this low antimicrobial resistance profile needs to be continuously monitored to ensure future effectiveness.

In the present study, IntI1 gene (class 1 integron) was detected in five strains (35.7%), while IntI2 gene (class 2 integron) was not detected in any isolate. A lower rate (8.5%) was reported by Kaur Pet et al.26 Ebrahim-Saraie et al.27 Hu et al.28 reported higher rates, 54.5% and 66.7% respectively, and both reported that all strains were negative for class 2 integron. Our data suggests that the presence of IntI1 gene (class 1 integron) does not confirm presence of resistant gene cassettes which was also reported by Hu et al.27

Several typing schemes have been used to study the epidemiology of *S. maltophilia* and associated hospital outbreaks. Phenotypic methods have been known by their poor discriminative power because of a low inter-strain variability. Various Molecular genotyping techniques have been successfully used to investigate the epidemiology of *S. maltophilia*. Several methods have high discriminatory power but are costly, time consuming and not available routinely. ERIC PCR which relays on chromosomal repetitive sequence polymorphism represents an ideal technique for epidemiological typing as it is easy rapid relatively cheap and applicable.29 It can be implemented in the routine microbiology lab in developing countries where resources to expensive equipment and their consumables are challenging.

In our study, 13 heterogeneous ERIC profiles were observed among 14 clinical strains, demonstrating no genetic correlation between them. High genomic diversity within the species of *S. maltophilia* have been observed from data obtained from several epidemiological surveys in developed countries.18,21 Using ERIC PCR, Alcaraz E et al.20 detected 52 ERIC fingerprints out of 63 strains, also Kaur et al.25 reported high diversity. *S. maltophilia* is ubiquitously found in the environment colonizing humid surfaces due to biofilm formation which could then facilitate unrelated transmission from different sources.29 Careful infection prevention and control practices especially environmental disinfection and hand hygiene are
mandatory to identify foci colonized with *S. maltophilia* which may be widely distributed facilitating transmission by direct contact.

With the increasing number of *S. maltophilia* isolates producing HAIs around the world, it's more important than ever to keep track of clonal relatedness, antibiotic resistance profiles, and virulence factor expression in clinical and environmental strains. This study has some limitations. First, only a few strains were investigated due to the low frequency of *S. maltophilia* in our hospital. Second, because it is a single-center study, the results cannot be generalized. Third, while our in vitro evidence supports the use of trimethoprim-sulfamethoxazole, minocycline, or levofloxacin as first-line therapy options for *S. maltophilia* infections, more clinical trials are needed to assess their clinical efficacy.

**CONCLUSIONS**

The current study highlighted the importance of *S. maltophilia* in healthcare associated bloodstream infections. Although, the strains possessed several virulence factors, they were still susceptible to the recommended antimicrobials. ERIC PCR was found to be valuable in detecting genetic diversity among *S. maltophilia* strains. It is an easy, rapid, cheap and available technique. The high genomic diversity observed among *S. maltophilia* strains indicates the need for appropriate epidemiological evaluation during nosocomial outbreaks. To our knowledge, this is the first epidemiological study for the clonal relatedness of *S. maltophilia* clinical strains in our country.

**Authors’ contributions**

This work was carried out in collaboration between all authors. M.A.M., M.M.R. contributed to the study conception and design. M.A.M. supervised all microbiological and molecular laboratory work. M.A.H. performed all laboratory work, collected data, wrote the manuscript draft. All authors analyzed and interpreted data, reviewed and approved the final manuscript.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

**REFERENCES**


