Stenotrophomonas maltophilia in Mexico: antimicrobial resistance, biofilm formation and clonal diversity

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Stenotrophomonas maltophilia is an important multidrug-resistant nosocomial pathogen associated with high mortality. Our aim was to examine antimicrobial susceptibility, biofilm production and clonal relatedness of clinical isolates of S. maltophilia. S. maltophilia isolates were collected between 2006 and 2013 from two tertiary care hospitals in Mexico. Antimicrobial susceptibility was evaluated by the broth microdilution method. PCR was used to determine the presence of β-lactamase genes L1 and L2. Biofilm formation was assessed with crystal violet staining. Clonal relatedness was determined by PFGE. Among the 119 collected S. maltophilia isolates, 73 (61.3 %) were from the respiratory tract. Resistance levels exceeded 75 % for imipenem, meropenem, ampicillin, aztreonam, gentamicin and tobramycin. Resistance to trimethoprim-sulfamethoxazole was 32.8 %. L1 and L2 genes were detected in 77.1 % (91/118) and 66.9 % (79/118) of isolates, respectively. All S. maltophilia strains were able to produce biofilms. Strains were classified as weak (47.9 %, 57/119), moderate (38.7 %, 46/119), or strong (13.4 %, 16/119) biofilm producers. A total of 89 distinct PFGE types were identified and 21.6 % (22/102) of the isolates were distributed in nine clusters. This is the first study in Mexico to reveal characteristics of clinical isolates of S. maltophilia. Clonal diversity data indicate low cross-transmission of S. maltophilia in a hospital setting. The high antibiotic resistance underscores the need for continuous surveillance of S. maltophilia in hospital settings in Mexico.

INTRODUCTION

Stenotrophomonas maltophilia, a non-fermentative, Gram-negative, rod-shaped bacterium is abundant in the environment with a wide geographical distribution. This bacterial species has been isolated from aqueous sources,
both in and out of clinical settings (Brooke, 2012). Infections caused by Stenotrophomonas maltophilia have a high attributable mortality rate (37.5%) (Falagas et al., 2009), depending on the initial clinical condition of patients (Paez & Costa, 2008). Over the last decade, S. maltophilia has emerged as an important nosocomial pathogen especially in patients with cystic fibrosis, malignancies, neutropenia, central venous catheters, a prolonged length of stay or a treatment history of broad-spectrum antibiotic use (VanCouwenbergh et al., 1997; Senol, 2004).

Infections associated with S. maltophilia include respiratory tract infections such as pneumonia, acute exacerbations of chronic obstructive pulmonary disease, plus bacteraemia, urinary tract infections, soft tissues infections and endophthalmitis (Brooke, 2012). Treatment of S. maltophilia infections is complicated by the fact that isolates exhibit resistance to many antimicrobial agents including macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines and polymyxins (Senol, 2004; Nicodemo & Paez, 2007; Brooke, 2012). Multiple mechanisms provide antibiotic resistance in S. maltophilia. Resistance to β-lactams in some clinical isolates is due to expression of two inducible β-lactamases, L1 and L2, although not all clinical isolates of S. maltophilia express β-lactamases even after exposure to β-lactam agents (Nicodemo & Paez, 2007). L1 metallo-β-lactamase is a clavulanic acid-resistant metalloenzyme that hydrolyses penicillins, cephalosporins and carbapenems (Walsh et al., 1994; Nicodemo & Paez, 2007). L2 serine-β-lactamase is a cephalosporinase that hydrolyses penicillins, cephalosporins and aztreonam; it is inhibited by clavulanic acid (Walsh et al., 1997; Nicodemo & Paez, 2007). Expression of these β-lactamases is determined by genes which are highly polymorphic in S. maltophilia (Avison et al., 2002; Nicodemo & Paez, 2007).

S. maltophilia can form biofilms on abiotic and biotic surfaces such as glass, plastics and host tissues (Pompilio et al., 2011; Brooke, 2012). Biofilms have been associated with approximately 65% of hospital-acquired infections (Potera, 1999; Brooke, 2012). Recent evidence indicates that the incidence of S. maltophilia infections will increase as the at-risk populations increase. Thus, there is a need for effective control strategies including appropriate use of antibiotics in hospital settings (Senol, 2004). Although S. maltophilia is an important nosocomial pathogen, little is known about the epidemiology of this organism in hospital settings in Mexico. Therefore, we intended to determine the resistance rate of S. maltophilia in Mexico as no report has been published before. The results may help us provide information for appropriate use of antibiotics in hospital settings. As well, it is important to determine if there is cross-transmission of S. maltophilia in the hospital setting, for which we evaluated clonal relatedness of isolates. Finally, biofilms have been associated with approximately 65% of hospital-acquired S. maltophilia infections and are strongly associated with drug resistance; thus it is important to determine the biofilm production of clinical isolates.

Our aim was to examine antimicrobial susceptibility, biofilm production and clonal relatedness of clinical isolates of S. maltophilia.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (µg ml⁻¹)</th>
<th>Percentage of isolates*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>50 %</td>
</tr>
<tr>
<td>Amikacin</td>
<td>≤16–&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>≤4–&gt;16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>≤8–&gt;16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>≤8–&gt;16</td>
<td>16</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>≤8–&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>≤4–&gt;16</td>
<td>16</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≤0.5–&gt;2</td>
<td>2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>≤2–&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Imipenem</td>
<td>≤4–&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>≤1–&gt;4</td>
<td>1</td>
</tr>
<tr>
<td>Meropenem</td>
<td>≤4–&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>≤4–&gt;8</td>
<td>8</td>
</tr>
<tr>
<td>Olofoxin</td>
<td>≤1–&gt;8</td>
<td>1</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>≤4–&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>TMP-SMX</td>
<td>≤2–&gt;2</td>
<td>≤2</td>
</tr>
</tbody>
</table>

*Value of 100% corresponds to 119 isolates.
Clone

A
B
C
D
E
F
G
H
I

Clinical isolate

13-336
13-341
13340
13337
14518
13-333
13343
13297
13333
14278
13334
11704
8071
3772
13396
11388
13-333
13-1112
13339
11352
3381
13331
2656
14464
13-338
14819
13342
14022
13-450
12378
13886
13-742
13752
14510
13092
11527
14064
12893
12029
13738
14023
13397
14512
14511
12892
12608
6474
13-337
14615
14193
3797
13-63
13388
13336
13739
13296
14063
13340
13885
13737
13-128
13277
12021
12043
3382
8368
12044
14808
14514
14209
13-801
10739
11361
13-605
13-167
14922
14961
14513
14455
14431
13-449
14131
13278
13318
12092
13521
11452
8373
3546
3388
12437
13-352
13-334
13-330
14626
13963
14079
14175
12286
10971
13093
13081

Similarity (%)
METHODS

Culture and identification of isolates. S. maltophilia isolates were collected from 2006–2013 at two tertiary care hospitals in Mexico: the Hospital Universitario ‘Dr José Eleuterio González’ in the state of Nuevo León and the Hospital Civil de Guadalajara and Instituto de Patología Infectiosa y Experimental ‘Dr Francisco Ruiz Sánchez’ in the state of Jalisco. All patients attending the clinic who presented with an infection with S. maltophilia were included in the study. The local ethics committee approved the study. One isolate per patient was assayed. Data collected in the study were the site of origin, the type of ward, the type of clinical sample and the age and gender of each patient.

Isolates were identified at the species level by using Sensititre panels (TREK Diagnostic Systems) according to the manufacturer’s instructions. Species identification was confirmed by PCR amplification of a 134 bp fragment of the 16S rRNA gene (Rios-Licea et al., 2010). Briefly, reaction mixtures contained 1 × PCR buffer, 2 mM MgCl2, 0.2 mM concentration of each deoxyribonucleotide triphosphate, 200 nM concentration of each primer (forward: 5'-GATCCTGGCT CAGAGTGAC G-3' ; reverse: 5'-CCCAAGCAC AGTAGTCC G-3'), 1 U AmpliTaq polymerase (Bioline USA) and 200 ng DNA. PCR was initiated by denaturation for 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, with a final extension for 5 min at 72 °C. All S. maltophilia isolates were stored at −70 °C until use. S. maltophilia ATCC 13637 was used as the reference strain.

Antimicrobial susceptibility. Susceptibility testing was performed by the broth microdilution method. Sensititre plates were obtained (TREK Diagnostic Systems) and were used according to the manufacturer’s instructions. Tested agents included amikacin, ampicillin, aztreonam, cefepime, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, meropenem, norfloxacin, oloxacan, tobramycin and trimethoprim-sulphamethoxazole (TMP-SMX). Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2012). For agents without specific CLSI criteria for S. maltophilia, relevant criteria for non-Enterobacteriaceae were used. Multidrug-resistant isolates were defined according to the European Centre for Disease Prevention and Control and the Centers for Disease Control and Prevention definitions as micro-organisms that were resistant to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012).

Amplification of β-lactamase genes. The presence of β-lactamase genes L1 and L2 was determined in all S. maltophilia isolates. Primers and PCR conditions were as described previously (Furushita et al., 2005). Briefly, the reaction mixture contained 1 × PCR buffer, 2 mM MgCl2, 0.2 mM concentration of each deoxyribonucleotide triphosphate, 200 nM concentration of each primer (L1 forward: 5'-CACACCTGGGC AGATCGCCAC-3' and L1 reverse: 5'-CCCGCAT- CCG GTTGGCCC-3'; L2 forward: 5'-CGATTTCTGC AGTTCAGT-3' and L2 reverse: 5'-CGTGATCCTC ATCCGATC-3'). 1 U AmpliTaq polymerase and 200 ng DNA. PCR was initiated by denaturation for 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 65 °C for L1 or 55 °C for L2 and 30 s at 72 °C, with a final extension for 5 min at 72 °C. Expected PCR products for L1 and L2 were 459 and 870 bp, respectively.

Biofilm formation assay. Biofilm formation was determined by crystal violet staining as previously described (Di Bonaventura et al., 2004; Pompilio et al., 2011). All isolates were tested in triplicate in two different experiments conducted on different days. The turbidity of overnight cultures was adjusted to match that of a 1.0 McFarland standard. The cultures were then diluted 1:100 in 200 μl tryptic soy broth and were inoculated into the wells of a flat-bottomed polystyrene plate (Falcon). After 24 h incubation at 37 °C, plates were washed three times with sterile PBS (pH 7.3). Adherent biofilms were fixed for 1 h at 60 °C, stained for 5 min at room temperature with 200 μl Hucker-modified crystal violet and then rinsed in standing water and allowed to dry. Biofilm samples were destained with 250 μl 33 % glacial acetic acid for 15 min and the OD was read at 492 nm (OD492). The low cut-off was chosen according to criteria described by Christensen et al. (1985) which included samples approximately three standard deviations above the mean OD of control wells.

The results were subtracted from the negative control (OD492 = 0.1243) and expressed as means. According to biofilm production, strains were classified as follows: no biofilm producer (OD492<0.0); weak biofilm producer (>0.0  OD492 ≤0.1); moderate biofilm producer (0.1 <OD492 ≤0.4) ; strong biofilm producer (OD492 >0.4).

Clonal diversity by PFGE. The genetic relatedness of isolates was studied by PFGE as previously described (Denton et al., 1998). DNA was digested with the restriction enzyme XbaI and electrophoresis was performed on a CHEF-DR III instrument (Bio-Rad Laboratories) for 20 h at 14 °C with 5–35 s of linear ramping at 6 V cm⁻¹ with an included angle of 120°. Band patterns were compared after ethidium bromide staining. Interpretation was based on the criteria of Tenover et al. (1995).

Statistical analyses. Statistical analyses were performed using the SPSS 20.0 statistical software package (IBM Corporation). PFGE band patterns were generated with the Labworks 4.5 software package with 1% tolerance. Similarity coefficients were generated from a similarity matrix calculated with the Jaccard coefficient.

RESULTS

Study population

During the 7-year study period, 119 S. maltophilia isolates were collected including 85 (71.4 %) isolates from Nuevo León and 34 (28.6 %) from Jalisco. The majority of the isolates were from the respiratory tract (73, 61.3 %) followed by blood (19, 16 %) and wounds (14, 11.8 %). Most were patients (68, 57.1 %) from an intensive care unit. None of the patients had cystic fibrosis. Among the 106 isolates obtained from patients whose age and gender were known, the age range was <1–96 years (mean of 39.8 years). In addition, the majority of patients were men (69, 65.1 %).

Antimicrobial susceptibility

The MIC ranges, MIC50, MIC90 and the percentages of isolates resistant, intermediate or susceptible to the 16 antimicrobial agents are shown in Table 1. Based on CLSI interpretive criteria, isolates were highly resistant to imipenem (100 %), meropenem (92.4 %), ampicillin (88.2 %), aztreonam (88.2 %), gentamicin (78.2 %) and tobramycin (75.6 %). Interestingly, we detected 32.8 % resistance for...
TMP-SMX. The majority of the isolates were multidrug-resistant (96.7%, 115/119).

β-Lactamase genes
L1 and L2 were detected in 77.1% (91/118) and 66.9% (79/118) of isolates, respectively. Of the isolates positive for the L1 gene, 100% (91/91) were imipenem resistant and 92.3% (84/91) were meropenem resistant. All of the isolates positive for the L2 gene [100% (79/79)] were imipenem resistant and 91.1% (72/79) were meropenem resistant.

Biofilm production
All S. maltophilia strains were able to form biofilms. Almost half (47.9%, 57/119) of the isolates were weak biofilm producers, 38.7% (46/119) of isolates were moderate biofilm producers and 13.4% (16/119) of isolates were strong biofilm producers.

Clonal diversity
Isolates (n=102) were typified by PFGE, revealing 89 distinct types. PFGE patterns of 17 isolates were not obtained, despite three attempts. The analysed isolates showed restriction patterns containing 8–17 bands. Approximately one-fifth (21.6%, 22/102) of the isolates were distributed in nine clusters (clones A–I) with identical PFGE patterns (Fig. 1). Clones A (n=3), B (n=3) and H (n=3) were clusters of isolates obtained from Jalisco. Clones C (n=2), D (n=2), E (n=2), F (n=2), G (n=3) and I (n=2) were clusters of isolates obtained from Nuevo León. The percentage of similarity among analysed isolates ranged from 75% to 100%. Also, 7 of 39 (17.9%) isolates resistant to TMP-SMX were clones of A (1), C (2), D (1), E (2) and F (1). The remaining 67 isolates had different PFGE types.

DISCUSSION

Based on in vitro susceptibility rates, TMP-SMX is the recommended drug for treatment of S. maltophilia infections (Denton & Kerr, 1998; Brooke, 2012). However, increasing resistance to TMP-SMX has been reported in several studies. Results from the SENTRY Antimicrobial Surveillance Program in 2004 showed 3.8% resistance to TMP-SMX for S. maltophilia (Fedler et al., 2006). Results from the same programme in 1997–1999 showed up to 10% resistance across Europe (Gales et al., 2001). The resistance rate reported for Latin America was 4.5% (Farrell et al., 2010). Although resistance rates of S. maltophilia to TMP-SMX have been reported to vary geographically (Valdezate et al., 2001; Farrell et al., 2010; Chung et al., 2012; Neela et al., 2012; Samonis et al., 2012; Wu et al., 2012), they were generally less than 20%. In our study, we used a daily dose of TMP-SMX of 15 mg kg$^{-1}$ (based on TMP) and we detected a high resistance rate of 32.8%. Unfortunately, no other reports from Mexico are available to compare these results. This important result indicates that TMP-SMX, the recommended drug for the treatment of infections caused by S. maltophilia, may not be the best choice for this particular population (patients who accessed these hospitals and had S. maltophilia infection resistant to TMP-SMX).

Other therapeutic alternatives should be considered such as levofloxacin, which showed a susceptibility rate of 95.8% in this study and which has also been reported to be a highly effective antibiotic against S. maltophilia (Wu et al., 2013).

Resistance of S. maltophilia against TMP-SMX is geographically different or varies in different areas of the world. High resistance rates to TMP-SMX have been reported in isolates obtained from patients in Asian countries such as Taiwan and Korea (Wang et al., 2004; Rhee et al., 2013) and also from cystic fibrosis patients (Cantón et al., 2003; San Gabriel et al., 2004; Valenza et al., 2008), albeit high susceptibility against TMP-SMX in S. maltophilia isolates from cystic fibrosis patients has been reported in Turkey (Nazik et al., 2007) and Germany (Goncalves-Vidigal et al., 2011). Nevertheless, none of our patients had cystic fibrosis.

Mexican S. maltophilia isolates analysed in this study showed high resistance to most antibiotics studied (>75%). However, susceptibility test results for agents other than TMP-SMX should be treated with caution as there are no data to support a relationship between susceptibility testing results and clinical outcome with S. maltophilia infection (www.eucast.org).

Resistance to antibiotics in S. maltophilia occurs through several mechanisms, including intrinsic β-lactamase expression (Nicodemo & Paez, 2007) and acquired expression through integrons, transposons and plasmids (Toleman et al., 2007). Intrinsic resistance to β-lactams is associated with the expression of the inducible β-lactamases L1 and L2, although not all clinical S. maltophilia isolates express β-lactamases (Nicodemo & Paez, 2007). However, expression of L1 and L2 may not be ruled out in these isolates, as challenging S. maltophilia with β-lactams can induce L1 and L2 expression (Avison et al., 2002). Furthermore, several reports indicate the heterogeneity of β-lactamase production in S. maltophilia, with the presence of additional enzymes besides L1 and L2 (Cullmann & Dick, 1990; Paton et al., 1994).

High genetic diversity among S. maltophilia isolates has been reported (Berg et al., 1999; Valdezate et al., 2004; Gülmez & Hasçelik, 2005; Schaumann et al., 2008; Neela et al., 2012; Tanimoto, 2013), although occurrences of outbreaks within hospital settings have also been described (Crispino et al., 2002; Abbasi et al., 2009; Goyot et al., 2013). Most S. maltophilia isolates analysed in our study showed unrelated PFGE patterns and were considered genetically diverse. These findings suggest that most isolates were acquired independently rather than as a consequence of cross-transmission.
Antimicrobial resistance rates have also been reported to vary among clusters of *S. maltophilia* isolates (Rhee et al., 2013). Despite the identification of nine clones in this study, the number of isolates belonging to each cluster was very low. Therefore, comparing antimicrobial resistance rates among the clones was difficult. Nonetheless, 17.9% (7/39) of the TMP-SMX-resistant isolates were clustered. Consequently, expanded surveillance of *S. maltophilia* in Mexico and additional studies to determine clonal relatedness and antimicrobial resistance rates are warranted.

In summary, this work represents the first epidemiological study of antibiotic resistance and clonal relatedness of *S. maltophilia* clinical isolates in Mexico. Clonal diversity detected in this study indicates low cross-transmission of *S. maltophilia* in the hospital setting. The high resistance to some antibiotics and moderate resistance to TMP-SMX detected in this study accentuate the need for continuous surveillance for *S. maltophilia* in hospital settings in Mexico.

**REFERENCES**


