Distinct groups and antimicrobial resistance of clinical *Stenotrophomonas maltophilia* complex isolates from Korea

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One hundred and twenty-one isolates of *Stenotrophomonas maltophilia* complex were collected from seven Korean hospitals. Species and groups were identified using partial gyrB gene sequences and antimicrobial susceptibility testing was performed using a broth microdilution method. Based on partial gyrB gene sequences, 118 isolates were identified as belonging to *S. maltophilia* complex, including *S. maltophilia*, *S. pavanii*, *Pseudomonas beteli*, *P. geniculata* and *P. hibisciola*. The *S. maltophilia* isolates were further divided into three groups, I to III. *S. maltophilia* groups II and III were clustered into clade A with *S. pavanii* and *P. beteli*. *S. maltophilia* group I was clustered into clade B with *P. geniculata* and *P. hibisciola*. For all *S. maltophilia* complex isolates, the resistance rate to trimethoprim/sulfamethoxazole (TMP/SMX) was very high (30.5%). Antimicrobial resistance rates varied among species or groups of *S. maltophilia* complex. Isolates of clade A showed significantly lower antimicrobial resistance rates than those of clade B; while 25% of clade A isolates were multidrug resistant, 46% of clade B isolates were multidrug resistant (*P* = 0.001). The finding of high antimicrobial resistance rates, particularly to TMP/SMX, among *S. maltophilia* complex isolates from Korea, and the existence of distinct groups among the isolates, with differences in antimicrobial resistance rates, suggests consideration of alternative agents to TMP/SMX to treat *S. maltophilia* infections and indicates the importance of accurate identification for appropriate selection of treatment options.

**INTRODUCTION**

*Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*) is a non-fermentative Gram-negative bacillus which is ubiquitous in the environment (Brooke, 2012). It has emerged as one of the important opportunistic pathogens causing nosocomial infections. Although *S. maltophilia* was previously thought to be less pathogenic, infection by this bacterium is now known to be associated with significant morbidity and mortality, especially in immunocompromised patients (Looney et al., 2009). However, it has been difficult to isolate *S. maltophilia* as it is frequently associated with polymicrobial infections or grows slowly in the host. Because several previously proposed species are recognized to be closely related to *S. maltophilia*, it might be referred to as ‘*S. maltophilia* complex’ including *Stenotrophomonas pavanii*, *Stenotrophomonas africana*, *Pseudomonas geniculata*, *Pseudomonas hibisciola* and *Pseudomonas beteli* (Svensson-Stadler et al., 2012). *S. maltophilia* is intrinsically resistant to several antibiotics commonly used to treat nosocomial infections, which can represent a therapeutic challenge and delay in administration of appropriate antibiotics (Brooke, 2012). It is notable that multidrug-resistant (MDR) *S. maltophilia* isolates have been recovered with increasing frequency in recent years in certain parts of the world (Garazi et al., 2012; Nicodemo et al., 2004; Tan et al., 2008). Trimethoprim/sulfamethoxazole (TMP/SMX) is recommended as the first-line therapy against *S. maltophilia* infections. However, resistance to TMP/SMX is increasing (Toleman et al., 2007; Wu et al., 2012).

*S. maltophilia* isolates from environmental and clinical sources form several genomic groups based on genotypic analyses (Berg et al., 1999; Minkwitz & Berg, 2001). While 16S rRNA groups have been identified based on the
sequences of a variable region of the 16S rRNA gene (Minkwitz & Berg, 2001), four phylogenetic groups based on the smeD–smeT intergenic sequences were also revealed (Gould et al., 2006). In addition, nine genomic groups of the genus Stenotrophomonas were proposed based on gyrB restriction fragment length polymorphism (RFLP) analysis (Coenye et al., 2004a). The latter study found that the majority of isolates from cystic fibrosis patients grouped into two genomic groups, indicating the existence of particular S. maltophilia subgroups of different ecological origin and clinical importance. However, multilocus sequence typing (MLST) analysis did not support the evidence for particularly virulent genogroups, although several genogroups were identified in that study (Kaiser et al., 2009).

The present study reports the genetic diversity and antimicrobial resistance of S. maltophilia complex isolates from Korea. We classified the S. maltophilia complex isolates into several groups based on partial gyrB gene sequences and showed that the antimicrobial resistance rates varied markedly between groups.

METHODS

S. maltophilia isolates. A total of 121 isolates tentatively identified as S. maltophilia were included in this study. They were collected from seven tertiary-care hospitals in Korea from 2007 to 2011 and were identified conventionally using VITEK2 systems in the hospitals’ clinical microbiology laboratories. Among them, 85 isolates were from blood, and the others were from sputum (nine isolates), urine (eight isolates), endotracheal aspirate (five isolates), transtracheal aspirate (five isolates), bile (three isolates), pericardial fluid (two isolates), pus (two isolates) and ear discharge (one isolate). The source of one isolate was unknown.

Species identification and grouping. To identify the isolates of Stenotrophomonas spp., we tried to determine the partial gyrB gene sequence of all isolates using primers XgyrB1F (5’-ACGAGTACAA-CCCAGACAA-3’) and XgyrB1R (5’-CCCATCARGTGCTGAAGAT-3’), which amplified one of the variable regions of the gyrB gene, region 2 (Svensson-Stadler et al., 2012). We obtained unambiguous 726 bp sequences from 118 isolates. The gyrB sequences of 33 reference strains, which were reported previously (Svensson-Stadler et al., 2012), were retrieved from the GenBank database and were included in the analysis. For species identification and grouping, a phylogenetic tree based on the 151 determined and retrieved gyrB gene sequences of 33 reference strains including the type strain, CCUG 58667. S. maltophilia group I could be further separated into two subgroups, I-1 (32 isolates) and I-2 (seven isolates). P. geniculata, which was related to S. maltophilia group I, included 10 isolates. One S. maltophilia reference strain, CCUG 54442, was classified as P. geniculata. Thirty-seven isolates belonged to S. maltophilia group II, which included only one reference strain, CCUG 58019. This S. maltophilia group II could be further classified into three subgroups, II-1 (28 isolates), II-2 (six isolates) and II-3 (two isolates). Seven isolates and one reference strain (CCUG 35078) were identified as S. maltophilia group III. S. pavani clustered with S. maltophilia groups II and III and included 18 isolates. Two P. beteli and one P. hibisciola were also identified. One isolate, SMC1008-158, was closely related to ‘S. africana’. S. maltophilia group II, S. pavani, S. maltophilia group III and P. beteli formed one clade (clade A), and S. maltophilia group I, P. geniculata, P. hibisciola and ‘S. africana’ formed another clade (clade B) (Fig. 1). Three isolates, K01-43, 08-B-253 and B0811-107, could not be classified to a particular group based on gyrB gene sequences, although they were considered to belong to the S. maltophilia complex (Fig. 1).

In vitro antimicrobial susceptibility testing. In vitro susceptibility testing was performed with 118 isolates identified as S. maltophilia complex in this study, according to CLSI guidelines (CLSI, 2009). The broth agar dilution method was used for ceftazidime, levofloxacin, piperacillin/tazobactam and tigecycline; the agar dilution method was used for TMP/SMX. For the combination of piperacillin/tazobactam, a constant amount of tazobactam (4 mg l⁻¹) was added to piperacillin. The interpretive criteria used were those established in CLSI standard M100-S21 (CLSI, 2011). Regarding tigecycline, interpretive criteria were defined based on the USA-FDA breakpoint criteria for Enterobacteriaceae (susceptible ≤ 2 mg l⁻¹; intermediate 4 mg l⁻¹; resistant ≥ 8 mg l⁻¹). For piperacillin/tazobactam, the criteria of Acinetobacter spp. in the CLSI guidelines were used (susceptible ≤ 16/2 mg l⁻¹, intermediate 32/4 to 64/4 mg l⁻¹, resistant ≥ 128/4 mg l⁻¹). Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as control strains. The MICs of control strains were within the acceptable QC ranges. An MDR isolate was defined as one showing resistance to two or more antimicrobial agents.

Statistical analysis. Data were analysed using SPSS 11.0 for Windows 2000. Categorical data were tested using χ² analysis. Differences were considered statistically significant at P<0.05 for all tests.

RESULTS

Based on partial gyrB gene sequences (726 bp), 118 isolates were identified as belonging to S. maltophilia complex, such as S. maltophilia, S. pavani, P. beteli, P. geniculata, P. hibisciola and ‘S. africana’ (Fig. 1). We could not obtain unambiguous gyrB gene sequences from three isolates, which were excluded from further analyses. Isolates showing gyrB gene sequence divergence of <4.0% and forming one cluster in the gyrB gene tree were identified as belonging to the same species or group of S. maltophilia complex (Svensson-Stadler et al., 2012). Remarkably, the S. maltophilia isolates, including 10 reference strains, did not cluster into one group. Thus, we classified S. maltophilia isolates into three groups. S. maltophilia group I included 39 isolates and six S. maltophilia reference strains including the type strain, CCUG 58667. S. maltophilia group I could be further separated into two subgroups, I-1 (32 isolates) and I-2 (seven isolates). P. geniculata, which was related to S. maltophilia group I, included 10 isolates. One S. maltophilia reference strain, CCUG 54442, was classified as P. geniculata. Thirty-seven isolates belonged to S. maltophilia group II, which included only one reference strain, CCUG 58019. This S. maltophilia group II could be further classified into three subgroups, II-1 (28 isolates), II-2 (six isolates) and II-3 (two isolates). Seven isolates and one reference strain (CCUG 35078) were identified as S. maltophilia group III. S. pavani clustered with S. maltophilia groups II and III and included 18 isolates. Two P. beteli and one P. hibisciola were also identified. One isolate, SMC1008-158, was closely related to ‘S. africana’. S. maltophilia group II, S. pavani, S. maltophilia group III and P. beteli formed one clade (clade A), and S. maltophilia group I, P. geniculata, P. hibisciola and ‘S. africana’ formed another clade (clade B) (Fig. 1). Three isolates, K01-43, 08-B-253 and B0811-107, could not be classified to a particular group based on gyrB gene sequences, although they were considered to belong to the S. maltophilia complex (Fig. 1).

Among 83 isolates from blood, S. maltophilia group II was the most frequently identified (30 isolates, 36.1 %), followed by S. maltophilia group I (24 isolates, 28.9 %), S. pavani (13 isolates, 15.7 %), P. geniculata (seven isolates, 8.4 %) and S. maltophilia group III (five isolates, 6.0 %). Thus, 81.1 % of the 37 S. maltophilia group II were isolated from blood.

For all S. maltophilia complex isolates, the resistance rates to TMP/SMX and levofloxacin were the highest (30.5 %) among the five antimicrobial agents tested (Table 1).
**Fig. 1.** Phylogenetic grouping of 118 clinical isolates and 33 reference strains of *S. maltophilia* complex inferred from partial *gyrB* gene sequences (726 bp). Only reference strains and three unclassified isolates (K01-43, 08-B-253, and B0811-107) are represented in the tree. Species and groups of *S. maltophilia* complex are indicated. This tree was constructed by the neighbour-joining method. *X. campestris* ATCC 33913T was used as an outgroup.
Antimicrobial resistance rates varied among the groups of the S. maltophilia complex (Table 2). Overall, isolates of clade A showed significantly lower antimicrobial resistance rates than those of clade B except for levofloxacin. While the TMP/SMX resistance rates of S. maltophilia group II, S. pavanii and S. maltophilia group III of clade A were 16.2%, 33.3% and 42.9%, respectively, 28.2% and 60.0% of S. maltophilia group I and P. geniculata isolates of clade B were resistant to TMP/SMX, respectively ($P=0.203$). In addition to TMP/SMX, ceftazidime resistance rates were also significantly lower in groups of clade A ($P<0.001$). Tigecycline-resistant isolates were found in S. maltophilia group II (four isolates, 10.8%), S. maltophilia group I (four isolates, 10.3%) and P. geniculata (three isolates, 30.0%), but no tigecycline-resistant isolates were found in S. pavanii and S. maltophilia group III ($P=0.003$). Although levofloxacin and piperacillin/tazobactam resistance rates did not significantly vary among the groups, they were also high or low in certain groups. Levofloxacin resistance rates were very low in S. pavanii and S. maltophilia group III isolates (5.6% and 0%, respectively), and S. maltophilia group II and S. maltophilia group III of clade A and S. maltophilia group I of clade B showed very low piperacillin/tazobactam resistance rates (5.4%, 0% and 5.1%, respectively). The MDR rates were also significantly higher in S. maltophilia group I and P. geniculata of clade A (41.0% and 60.0%, respectively) ($P=0.001$).

Although S. maltophilia groups I and II isolates could be further divided into two and three subgroups, respectively, antimicrobial resistance rates were not significantly different among the subgroups (data not shown). Among 83 isolates from blood, antimicrobial resistance rates were not significantly different from the others: 30.1% to ceftazidime, 32.5% to levofloxacin, 32.5% to TMP/SMX, 14.5% to tigecycline and 12.0% to piperacillin/tazobactam. One isolate, SMC1102-153, belonging to P. geniculata, showed resistance to all five antimicrobial agents tested in this study.

Table 1. Activity of antimicrobial agents against S. maltophilia complex isolates

<table>
<thead>
<tr>
<th>Agent</th>
<th>MIC (mg l$^{-1}$)</th>
<th>No. of isolates (%)</th>
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<tbody>
<tr>
<td></td>
<td>MIC$_{50}$</td>
<td>MIC$_{90}$</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>TMP/SMX</td>
<td>2/38</td>
<td>16/304</td>
</tr>
<tr>
<td>P/T</td>
<td>16/4</td>
<td>128/4</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>MDR</td>
<td></td>
<td>38</td>
</tr>
</tbody>
</table>

MIC$_{50}$ and MIC$_{90}$ of TMP/SMX were 2/38 and 16/304 mg l$^{-1}$, respectively. The resistance rate to ceftazidime was 28.0%. Of the 118 S. maltophilia complex isolates, 11.0% and 11.9% displayed resistance to piperacillin/tazobactam and tigecycline, respectively. We identified 38 MDR isolates (32.2%).

DISCUSSION

The most notable finding in this study is the high resistance rate to TMP/SMX. Worldwide surveillance studies showed relatively low resistance rates varying from 4% to 10% (Brooke, 2012; Farrell et al., 2010). A recent report from a Korean hospital also documented a low resistance rate of 6% to TMP/SMX using the agar dilution method (Chung et al., 2012). Thus, the high TMP/SMX resistance rate of 30.5% noted in our study might be unexpected. However, it has been reported that 17.5% of S. maltophilia isolates were resistant to TMP/SMX in Taiwan (Wu et al., 2012), and a study from Germany reported that only 34.4% of S. maltophilia isolates from sputum of cystic fibrosis patients were susceptible to TMP/SMX (Valenza et al., 2008). The difference in the TMP/SMX resistance rate between different studies in Korea may be due to the different hospitals, isolation period and number of isolates. In addition, most of our isolates were from blood, which might show higher resistance rates. The high TMP/SMX resistance rate found in this study is of concern because the preferred treatment option of S. maltophilia infections is TMP/SMX (Brooke, 2012). Thus, continuous surveillance of antimicrobial resistance in S. maltophilia is recommended. In contrast to the high resistance to TMP/SMX, piperacillin/tazobactam and tigecycline showed potent activities against S. maltophilia complex isolates. This suggests that tigecycline would be an alternative option for treatment of S. maltophilia infections in regions of high resistance to TMP/SMX. Tigecycline has been considered as an alternative therapeutic option, mainly as a component of combination therapy (Looney et al., 2009).

To date, the genus Stenotrophomonas includes 13 described species (www.bacterio.cict.fr/s/stenotrophomonas.html). However, ‘S. africana’ was revealed to be a later synonym of S. maltophilia (Coenye et al., 2004b; Kaiser et al., 2009). S. pavanii, which was characterized recently and was proposed as a new species based on DNA–DNA hybridization results (Ramos et al., 2011), showed very high similarities with S. maltophilia strains in 16S rRNA and gyrB gene analyses (Svensson-Stadler et al., 2012). In addition, Pseudomonas beteli, P. geniculata and P. hibisciola were found not to belong to the genus Pseudomonas (Anzai et al., 2000). Instead, they were undifferentiated from S. maltophilia strains in 16S rRNA gene analysis (>99.2% similarities), and were clustered with S. maltophilia strains in gyrB gene analysis (Fig. 1) (Svensson-Stadler et al., 2012). Thus, it is logical that they are grouped into the S. maltophilia complex.

In this study, S. maltophilia complex isolates showed genetic diversity based on gyrB gene sequences. High
genetic diversity of *S. maltophilia* complex has been reported in several studies using pulsed-field gel electrophoresis and enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) (Song et al., 2010; Valdezate et al., 2004). Studies based on MLST demonstrated that *S. maltophilia* isolates were heterogeneous (Cho et al., 2012; Kaiser et al., 2009). In this study, the partial *gyrB* gene sequence showed >91.5% identity among *S. maltophilia* complex isolates. They could be classified into several groups. In previous studies, several *S. maltophilia* subgroups have been identified based on 16S rRNA gene, L1 and L2 β-lactamase gene sequences, and the sequences of the *smeD-smeT* intergenic region (Gould et al., 2004, 2006). The 16S rRNA gene sequence variation of *S. maltophilia* group I isolates in this study was the same as that of K279a, a representative strain of phylogenetic group A (Gould et al., 2004, 2006). *S. maltophilia* type strain ATCC 13637T (CCUG 5866T) was designated as phylogenetic group A (Gould et al., 2006). In the *gyrB* gene tree in this study, strains K279a and CCUG 5866T belonged to *S. maltophilia* group I. In addition, *S. maltophilia* group II isolates showed the same 16S rRNA gene sequence variation as N531, a representative strain of phylogenetic group B. The 16S rRNA gene sequences of *P. geniculata* and *P. hibisciola* showed the characteristics of phylogenetic group C (Gould et al., 2004). Thus, overall groupings based on 16S rRNA and *gyrB* genes may be congruent. However, we also found incongruence between groupings based on the 16S rRNA gene and the *gyrB* gene. For example, isolate B0906-096 belonged to *S. maltophilia* group II in this study, but showed a close relationship with *P. geniculata* in 16S rRNA gene analysis (data not shown).

In a previous study, the phylogenetic groups could be identified, but the authors could not find significant differences between phylogenetic groups in terms of their β-lactam resistance and β-lactamase expression (Gould et al., 2006). However, in this study we identified significant differences of resistance rates to some antimicrobial agents. Table 2 shows that high MDR rates in this study are mainly due to *S. maltophilia* group I and *P. geniculata* isolates. This means that accurate identification of *Stenotrophomonas* species is needed to select appropriate antimicrobial agents. Although antimicrobial resistance mechanisms were not investigated in this study, efflux pumps such as SmeABC and SmeDEF are overexpressed in resistant isolates (Brooke, 2012; Liaw et al., 2010). In addition, a class 1 integron was associated with MDR in *S. maltophilia* (Liaw et al., 2010). Whether high resistance in certain species or groups is intrinsic or acquired is unclear and requires study.

To summarize, in this study we identified distinct groups among *S. maltophilia* complex isolates. High antimicrobial resistance rates, particularly to TMP/SMX, were also identified. Antimicrobial resistance rates differed among the groups, indicating the need for accurate identification for appropriate selection of treatment options.

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