Detection of the Smqrn quinolone protection gene and its prevalence in clinical isolates of Stenotrophomonas maltophilia in China

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The aim of this study was to detect novel variants of the Stenotrophomonas maltophilia Smqrn gene family and analyse the prevalence of Smqrn genes in clinical isolates of S. maltophilia in China. In total, 442 clinical isolates of S. maltophilia were collected from nine hospitals in four provinces in China. Antimicrobial susceptibility testing against six commonly used antibiotics was performed on these isolates. The sequences of the Smqrn genes amplified by PCR were aligned with those of known Smqrn genes in GenBank and an Smqrn database. The resistance rate against co-trimoxazole was highest at 48.6 %, followed by resistance rates against ceftazidime, chloramphenicol, ticarcillin/clavulanate and tigecycline at 28.7, 21.3, 19.0 and 16.1 %, respectively. The highest susceptibility was shown to levofloxacin, with a resistance rate of just 6.1 %. Smqrn genes were detected in 114 isolates, and comprised 11 previously identified genes and 20 new variants, bringing the total number of known Smqrn genes to 47. The 20 novel Smqrn genes were designated Smqrn28–47 and the encoded proteins showed only 1–12 amino acid differences among each other. The most common Smqrn genes in China were Smqrn8 and its variant Smqrn35 with prevalences of 17.5 % (20/114) and 13.2 % (15/114), respectively. Both the known and the novel Smqrn genes were discovered in both quinolone non-sensitive and sensitive isolates with similar frequency, suggesting that the Smqrn gene makes little contribution to quinolone resistance in this organism.

INTRODUCTION

Stenotrophomonas maltophilia is a non-fermentative Gram-negative bacillus that usually colonizes soil and water environments and causes opportunistic infections. It is an important pathogen in nosocomial infections.

Abbreviation: PMQR, plasmid-mediated quinolone resistance.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the Smqrn genes determined in this study are HQ874463, HQ874464 and HQ896258–HQ896575.

Drug resistance in S. maltophilia is an issue of concern, as S. maltophilia is inherently resistant to many antibiotics such as carbapenems and aminoglycosides. Resistance against antibiotics such as co-trimoxazole and ticarcillin/clavulanate, which are recommended for empirical treatment of S. maltophilia infections, has increased in the last few years (Looney et al., 2009). Until recently, quinolone antibiotics have been some of the few antibacterial agents that show relatively good activity against S. maltophilia. However, resistance to quinolones has now been reported (Krueger et al., 2001).
Quinolones with a broad spectrum of antibacterial activity have been widely used in clinical medicine and this has led to increasing resistance. Resistance to quinolones is mainly the result of chromosomally mediated mechanisms, including mutations in the targets of quinolones (DNA gyrase and topoisomerase IV) and decreased accumulation of quinolones (as a result of porin alteration or over-expression of efflux pump systems) (Martínez et al., 1998; Valdezate et al., 2002). It has also been found that quinolone resistance can be caused by plasmid-mediated quinolone resistance (PMQR) determinants, and the genes responsible for such resistance are named qnr (Martínez-Martínez et al., 1998). So far, various qnr genes, such as qnrA, -B, -C, -D and -S, have been found in various pathogens. However, no plasmid-mediated qnr genes have been reported for S. maltophilia. Instead, S. maltophilia contains a novel quinolone resistance gene named Smqnr, encoded by the chromosome, which confers lower-level resistance to quinolone antibiotics following expression in a heterologous host (Sánchez et al., 2008).

In this study, we collected 442 isolates of S. maltophilia from four provinces in PR China to detect novel variants of the Smqnr family and to analyse their prevalence in clinical isolates of S. maltophilia. The relationship between Smqnr genes and resistance of quinolones in S. maltophilia was also evaluated.

**METHODS**

**Bacterial strains.** A total of 442 non-duplicated clinical S. maltophilia isolates were collected from nine hospitals in PR China. Identification of S. maltophilia was performed using the VITEK system (bioMérieux).

**Antimicrobial susceptibility testing.** The MICs of levofloxacin, chloramphenicol, tetracycline, ticarcillin/clavulanate, co-trimoxazole and ceftazidime were determined by an agar dilution method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2010). Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as reference strains for susceptibility testing.

**PCR amplification of the Smqnr gene and PMQR determinants.** A loopful of bacteria was taken and suspended in 50 μl sterile TE buffer (pH 8.0) and boiled for 5 min. After centrifugation in a bench-top microfuge at 9600 g for 5 min, the supernatant was used as template for PCR. The primers used to amplify the full-length Smqnr gene were 5’-ACACGAACGGCTGGACTGC-3’ and 5’-TTCAACGACGTTGAGCTGT-3’ (Gordon & Wareham, 2010). The PCR process comprised denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min. For the resistant isolates, screening of the five PMQR determinants qnrA, qnrB, qnrC, qnrD and qnrS and identification of mutations in the gyrA and parE genes were carried out by PCR and sequence analysis. The primers used for the PMQR determinants and the PCR procedure have been described previously (Kim et al., 2009).

**Sequence analysis of Smqnr alleles and sequence alignment.** After purification, DNA sequencing of the PCR products was performed on an automated ABI PRISM 3730 sequencer (Applied Biosystems) with corresponding PCR primers. A 219-residue amino acid sequence of the translated proteins was determined using the DNAMAN 3.0 translation tool (http://www.lymon.com/). After comparison with Smqnr genes in GenBank and the Smqnr database (http://www.icsm.qmul.ac.uk/centres/immunologyandinfectiousdisease/Smqnr%20Web%20v2.htm), alleles were considered to be novel if one or more amino acid residues was changed in a number of alignments.

**Nucleotide accession numbers and phylogenetic tree.** The sequences of 20 novel Smqnr genes were determined using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and submitted to GenBank. A phylogenetic tree of the 20 novel Smqnr genes was constructed using DNAMAN 6.0 to analyse the relationship of each Smqnr gene. The tree was calculated using the mean percentage sequence identity.

**RESULTS**

**Antimicrobial susceptibility of S. maltophilia isolates**

Table 1 summarizes the resistance profiles, including the MIC50 and MIC90 values, the susceptibility rate and the resistance rate, of the 442 S. maltophilia isolates against six commonly used antibiotics. The resistance rate against co-trimoxazole was highest at 48.6 %, followed by resistance to ceftazidime, chloramphenicol and ticarcillin/clavulanate at rates of 28.7, 21.3 and 19.0 %, respectively. The rate of resistance to tigecycline was 16.1 %. The highest susceptibility was shown to levofloxacin, with a resistance rate of just 6.1 %.

**PMQR analysis**

The PMQR determinants qnrA, qnrB, qnrC, qnrD and qnrS were not detected in the resistant isolates. Although the mutations of the gyrA and parE genes at common sites

### Table 1. MICs of six common antibiotics for the 442 S. maltophilia isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC range (μg ml⁻¹)</th>
<th>MIC₅₀ (μg ml⁻¹)</th>
<th>MIC₉₀ (μg ml⁻¹)</th>
<th>Sensitivity rate (%)</th>
<th>Resistance rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tigecycline</td>
<td>1–64</td>
<td>4</td>
<td>16</td>
<td>51.8</td>
<td>16.1</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1–128</td>
<td>8</td>
<td>32</td>
<td>57.7</td>
<td>21.3</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.25–256</td>
<td>32</td>
<td>128</td>
<td>34.2</td>
<td>28.7</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>0.25–256</td>
<td>2</td>
<td>8</td>
<td>51.4</td>
<td>48.6</td>
</tr>
<tr>
<td>Ticarcillin/clavulanate</td>
<td>0.5–128</td>
<td>8</td>
<td>64</td>
<td>72.2</td>
<td>19.0</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.0625–64</td>
<td>0.5</td>
<td>4</td>
<td>86.9</td>
<td>6.1</td>
</tr>
</tbody>
</table>

**Figure 1.** Phylogenetic tree of the 20 novel Smqnr genes. The tree was calculated using the mean percentage sequence identity.
were found in half of the drug-resistant strains, some previous publications have indicated that mutations at these sites are not related to the drug resistance of *S. maltophilia* (Martínez *et al.*, 1998; Valdezate *et al.*, 2002).

**Detection of the Smqnr gene in *S. maltophilia* isolates**

Smqnr genes were detected in 114 of the 442 isolates (25.8%), and comprised 11 previously identified Smqnr subtypes and 20 novel Smqnr subtypes, which we designated Smqnr28–Smqnr47. The GenBank accession numbers of the sequences of the newly identified Smqnr subtypes are listed in Table 2. The various Smqnr genes identified were distributed among the isolates as follows: Smqnr4 (n=7), Smqnr6 (n=2), Smqnr8 (n=20), Smqnr9 (n=1), Smqnr10 (n=1), Smqnr11 (n=1), Smqnr12 (n=5), Smqnr13 (n=6), Smqnr15 (n=5), Smqnr17 (n=3), Smqnr20 (n=1), Smqnr28 (n=1), Smqnr29 (n=8), Smqnr30 (n=1), Smqnr31 (n=4), Smqnr32 (n=2), Smqnr33 (n=1), Smqnr34 (n=3), Smqnr35 (n=15), Smqnr36 (n=1), Smqnr37 (n=1), Smqnr38 (n=1), Smqnr39 (n=1), Smqnr40 (n=1), Smqnr41 (n=7), Smqnr42 (n=1), Smqnr43 (n=1), Smqnr44 (n=6), Smqnr45 (n=5), Smqnr46 (n=1) and Smqnr47 (n=1). The most common Smqnr subtypes were Smqnr8 and its variant Smqnr35 with rates of 17.5% (20/114) and 13.2% (15/114), respectively.

As shown in Fig. 1, among the *S. maltophilia* isolates, the Smqnr gene was detected in 42.3% of the resistant isolates (MIC ≥8 µg ml⁻¹) and 22.0–34.4% of the intermediately resistant isolates (MIC=1–4 µg ml⁻¹), respectively. The rates of detection of Smqnr from the sensitive (MIC=0.25 µg ml⁻¹) and highly sensitive (MIC <0.25 µg ml⁻¹) isolates were 23.9 and 37.8%, respectively. There was no clear tendency for a higher detection rate among resistant than among sensitive isolates.

To understand the distribution of the newly identified subtypes among the isolates with different resistance levels, we analysed the percentage of new subtypes compared with that of the known subtypes at different MIC values. We found that, among the 114 Smqnr-positive isolates, more than 45.5 and 35.1% were detected in susceptible isolates, whilst only 4.4 and 5.3% were detected in resistant isolates for the novel subtypes and known subtypes, respectively, indicating that Smqnr genes are more common in quinolone-sensitive than in quinolone-resistant isolates.

There was no significant difference between the known subtypes and the newly identified subtypes in terms of this distribution.

**Comparison of proteins encoded by the Smqnr genes and phylogenetic tree**

We compared the 20 novel Smqnr peptide sequences, which had one or more amino acids altered out of 219 residues in the translated protein, with the known Smqnr1–27 subtypes in GenBank (http://www.icms.qmul.ac.uk/centres/immunologyandinfectiousdisease/Smqnr%20Web%20v2.htm). Only between 1 and 12 amino acid residue differences were found. The highest number of changes was observed for the protein encoded by subtype Smqnr43, which had 12 amino acid residue differences, whilst the protein encoded by subtype Smqnr46 showed only a single residue alteration. The relative distances of the novel and known subtypes is shown in the phylogenetic tree in Fig. 2. Five major clusters were found. Some of the known subtypes such as Smqnr8–11 were concentrated in one of the clusters. The novel subtypes were distributed among the remaining four clusters with one of the known subtypes in each cluster.

**DISCUSSION**

Unlike in other bacteria, the mechanism of quinolone resistance in *S. maltophilia* is not due to mutation of topoisomerase genes. Rather, overexpression of the multi-drug efflux pump SmDEF can lead to high-level quinolone resistance in *S. maltophilia*, whilst low-level quinolone resistance is mediated by a qnr determinant encoded on the chromosome (Cavaco *et al.*, 2009; Valdezate *et al.*, 2005). A new chromosome-carried quinolone resistance gene in *S. maltophilia*, Smqnr, was first reported in Japan in 2008. It has been confirmed that the Smqnr gene is capable of decreasing quinolone and fluoroquinolone susceptibilities, similar to other *qnr* determinants (Shimizu *et al.*, 2008). Later, a publication from Spain demonstrated the role of the...
**Smqnr** gene in intrinsic bacterial resistance to quinolones using a gene knockout method (Sánchez & Martínez, 2010).

In this study, we identified 114 of 442 clinical *S. maltophilia* isolates containing **Smqnr** genes. Sequence analysis revealed that these isolates carried 20 novel **Smqnr** subtype genes, bringing the total number of **Smqnr** genes in *S. maltophilia* to at least 47. During the preparation of this manuscript, we found that two other **Smqnr** sequences had been submitted to the **Smqnr** database. We found that these two sequences were also different from our newly identified 20 **Smqnr** subtype genes. Sequence alignment and comparison indicated that our 20 novel **Smqnr** subtype genes were all variants of discovered genes, with 1–12 amino acid residue alterations in the encoded proteins. For example, **Smqnr**28–30 were variants of **Smqnr**2 with identities of 96–99 %, **Smqnr**31–34, **Smqnr**40 and **Smqnr**47 were variants of **Smqnr**4 with identities of 94–99 %, and **Smqnr**35–38 and **Smqnr**41–45 were variants of **Smqnr**6–11 with identities of 92–99 %. The **Smqnr**1–11 genes were initially detected in Japan and have 2–6 amino acid residue differences in the encoded proteins compared with the qnrB gene (Shimizu et al., 2008). The **Smqnr**12–18 genes prevail mainly in the UK and have different amino acid residue alterations in the encoded proteins from the **Smqnr**1–11 genes. The **Smqnr**19–27 genes were discovered in Spain and have higher sequence identity with **Smqnr**12–18. Likewise, the 20 novel **Smqnr** genes discovered in our study are all variants of the **Smqnr**1–11 genes from Japan. Thus, we believe that **Smqnr** genes in Asian counties and European countries are different. Therefore, identification and designation of **Smqnr** alleles will be important for future epidemiological investigations.

The detection rates of **Smqnr** genes among isolates with a high quinolone resistance (MICs of 4 and ≥8 µg ml⁻¹) were relatively high at 34.4 and 42.3 %, respectively (Fig. 1). However, the resistance rate was similar at 37.8 % for sensitive isolates. As so many **Smqnr** genes were detected in sensitive isolates, we believe that there is little correlation between **Smqnr** genes and direct resistance to quinolones in
S. maltophilia clinical isolates, and therefore the Smqnr gene may exist inherently in S. maltophilia.

Our finding that the Smqnr gene appears to contribute little to quinolone resistance, at least to high-level resistance, in S. maltophilia is in line with other studies. High variability in the Smqnr gene also supports this point. Nevertheless, Smqnr may provide an additional effect on top of other factors such as mutation in the DNA gyrase to elevate the resistance level once it is introduced into other organisms. Gordon & Wareham (2010) observed that mobilization of Smqnr from S. maltophilia to members of the Enterobacteriaceae could increase MICs against quinolones. Therefore, S. maltophilia may serve as a reservoir for the dissemination of quinolone resistance elements to members of the Enterobacteriaceae and other organisms.

In summary, this study has described new chromosome-carried quinolone resistance Smqnr genes in S. maltophilia and has provided data on the prevalence of Smqnr in China. However, further studies on the mechanisms and regulation of the intrinsic resistance of Smqnr genes are necessary in the future.

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REFERENCES


