

Prevalence of *Smqnr* and plasmid-mediated quinolone resistance determinants in clinical isolates of *Stenotrophomonas maltophilia* from Japan: novel variants of *Smqnr*

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Abstract

Stenotrophomonas maltophilia is an important pathogen in healthcare-associated infections. *S. maltophilia* may contain *Smqnr*, a quinolone resistance gene encoding the pentapeptide repeat protein, which confers low-level quinolone resistance upon expression in a heterologous host. We investigated the prevalence of *Smqnr* and plasmid-mediated quinolone resistance (PMQR) determinants in *S. maltophilia* isolates from Japan. A total of 181 consecutive and nonduplicate clinical isolates of *S. maltophilia* were collected from four areas of Japan. The antimicrobial susceptibility profiles for these strains were determined. PCR was conducted for *Smqnr* and PMQR genes, including *qnrA*, *qnrB*, *qnrC*, *qnrS*, *aac(6′)-Ib* and *qepA*. PCR products for *Smqnr* and *aac(6′)-Ib* were sequenced. For the *S. maltophilia* isolates containing *Smqnr*, pulsed-field gel electrophoresis (PFGE) was performed using XbaI. Resistance rates to ceftazidime, levofloxacin, trimethoprim–sulfamethoxazole, chloramphenicol and minocycline were 67.4%, 6.1%, 17.7%, 8.8% and 0%, respectively. The minimum inhibitory concentration required to inhibit the growth of 50% and 90% of organisms were 0.5 and 2 mg/L for moxifloxacin but 1 and 4 mg/L for levofloxacin, respectively. *Smqnr* was detected in 104 of the 181 *S. maltophilia* isolates (57.5%), and the most frequent was *Smqnr6*, followed by *Smqnr8* and *Smqnr11*. Eleven novel variants from *Smqnr48* to *Smqnr58* were detected. The 24 *Smqnr*-containing *S. maltophilia* isolates were typed by PFGE and divided into 21 unique types. Nine *S. maltophilia* isolates (5.0%) carried *aac(6′)-Ib-cr*. No *qnr* or *qepA* genes were detected. This study describes a high prevalence of *Smqnr* and novel variants of *Smqnr* among *S. maltophilia* from Japan. Continuous antimicrobial surveillance and further molecular epidemiological studies on quinolone resistance in *S. maltophilia* are needed.

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Introduction

In recent years, hospital infections and outbreaks due to glucose nonfermenting Gram-negative bacilli such as the multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have become a major concern worldwide. In hospital environments, *Stenotrophomonas maltophilia* has been isolated from tap water, nebulizers, ventilator circuits, contaminated disinfectants and other locations, and it is an important

opportunistic pathogen causing healthcare-associated infections [1]. Infections due to *S. maltophilia* are increasing as a result of developments in advanced medical treatments such as organ transplantation, chemotherapy, immunosuppressant drugs, increased numbers of compromised hosts and the increased use of broad-spectrum antibiotics. *S. maltophilia* causes various infections, including respiratory tract infections (pneumonia and acute exacerbations of chronic obstructive pulmonary disease); bacteremia; and infections of urinary tract, skin and soft tissues, bones and joints and eye [2]. Nosocomial infections have high mortality, which has been reported to range 12.5% to 41% due to bacteremia, 40% to 50% due to pneumonia and 39% due to endocarditis [1,3]. *S. maltophilia* infections are difficult to treat because the bacterium may exhibit high-level intrinsic resistance to many antimicrobial agents. Trimethoprim–sulfamethoxazole, ticarcillin/clavulanate, and quinolones are used for treatment of *S. maltophilia* infections, but resistance to these antibiotics has been increasingly described [3]. Few studies on the antimicrobial susceptibility of *S. maltophilia* have been reported in Japan.

Quinolone resistance is mainly caused by chromosomal mutations of the quinolone resistance–determining regions in DNA gyrase and DNA topoisomerase IV, as well as porin alterations or overexpression of efflux pump systems, resulting in decreased quinolone accumulation [4–6]. However, a plasmid-mediated horizontally transferable quinolone resistance gene encoding a pentapeptide repeat protein (*qnr*) was discovered in 1998 [7]. Since then, other plasmid-mediated quinolone resistance (PMQR) determinants, including *aac(6′)-Ib-cr*, encoding a variant aminoglycoside acetyltransferase, and *qepA*, encoding an efflux pump, have been reported worldwide in clinical isolates of Enterobacteriaceae [5,6]. Studies of PMQR determinants in Enterobacteriaceae have been increasing, though PMQR determinants in *S. maltophilia* remain unclear.

The main mechanism of high-level quinolone resistance in *S. maltophilia* is thought to involve overexpression of efflux pumps, in particular SmeDEF, and low permeability of the outer membrane [8]. Quinolone resistance in *S. maltophilia* can also occur as a result of mutations of DNA gyrase and topoisomerase genes [2]. *Smqnr* have been recently identified in *S. maltophilia* as a novel chromosomal quinolone resistance gene encoding the pentapeptide repeat protein SmQnr. Similar to PMQR determinants in Enterobacteriaceae, *Smqnr* confers low-level quinolone resistance in *Escherichia coli* and may transmit quinolone resistance genes to other bacteria [9–11]. There are few studies on the frequency of *Smqnr* and PMQR genes in *S. maltophilia*. In this study, we investigated the antimicrobial susceptibility of clinical isolates of *S. maltophilia* and the prevalence of *Smqnr* and PMQR determinants in *S. maltophilia* isolates from Japan.

Materials and Methods

Bacterial isolates

A total of 181 consecutive and nonduplicate clinical isolates of *S. maltophilia* were collected between November 2009 and March 2010 from four areas of Japan, including 43 isolates from Hokkaido, 49 from Tokyo, 43 from Osaka and 46 from Fukuoka. *S. maltophilia* were identified using the Microscan Walkaway System (Siemens Healthcare Diagnostics, Tokyo, Japan) and the Vitek-2 System (Sysmex-bioMérieux, Tokyo, Japan). Isolates included 148 from sputum (81.8%), 13 from nasal and throat swabs (7.2%), eight from urine (4.4%) and 12 from other specimens (6.6%). There were no isolates obtained from blood cultures in this study. The isolates included 160 (88.4%) from hospitalized patients and 21 (11.6%) from community patients. Institutional review board approval was not required for this study according to the research guidelines of the Japanese government (<http://www.niph.go.jp/wadai/ekigakurinri/guidelines.pdf>).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of nine antibiotics, including piperacillin (range, 0.06–128 mg/L), ceftazidime (0.06–128 mg/L), imipenem (0.06–128 mg/L), minocycline (0.06–128 mg/L), amikacin (0.06–128 mg/L), levofloxacin (0.06–128 mg/L), moxifloxacin (0.06–128 mg/L), trimethoprim–sulfamethoxazole (1/19–8/152 mg/L) and chloramphenicol (2–128 mg/L) were determined by the broth microdilution method. Clinical and Laboratory Standards Institute (CLSI) interpretive criteria were used for each antibacterial agent [12]. Quality control was performed using *E. coli* American Type Culture Collection (ATCC) 25922 and *E. coli* ATCC 35218.

PCR and sequencing of *Smqnr* and PMQR genes

The primers used to amplify an 811 bp fragment containing the entire *Smqnr* coding sequence were as follows: 5′-ACACA-GAACGGCTGGACTGC-3′ and 5′-TTCAACGACGTG-GAGCTGT-3′ [9,11]. Screening of *S. maltophilia* isolates for *aac(6′)-Ib* and PMQR genes, including *qnrA*, *qnrB*, *qnrC*, *qnrS* and *qepA*, was conducted by multiplex PCR amplification, as described previously [13]. PCR products for *Smqnr* and *aac(6′)-Ib* were sequenced using ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 3730xl Analyzer (Applied Biosystems). Nucleotide and amino acid sequences were analysed with BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), Expasy translate tool (<http://web.expasy.org/translate/>) and CLUSTALW2.1 (<http://www.ddbj.nig.ac.jp/searches-j.html>). If one or more amino acid differed compared to the previously reported *Smqnr* sequences in

GenBank and/or published articles, *Smqnr* alleles were considered to be novel and were named numerically according to *qnr* nomenclature [14].

Pulsed-field gel electrophoresis

For the 24 *S. maltophilia* isolates containing *Smqnr* variants which were detected from four Japanese areas, pulsed-field gel electrophoresis (PFGE) was performed using XbaI (Takara Bio Inc., Otsu, Japan). Electrophoresis was performed on a 1% PFGE agarose gel with a CHEF-DR III system (Bio-Rad Laboratories, Richmond, CA, USA). Electrophoretic patterns were analysed with GelCompar II version 3.0 (Applied Maths, Kortrijk, Belgium). Similarity between two tracks was calculated using the coefficient of Jaccard and band positions. Cluster analysis and dendrogram generation were performed by the unweighted pair group method using arithmetic averages. Isolates with similarity >85% were considered to be within a cluster [15].

Statistical analysis

Statistical significance was evaluated by two-sided chi-square test or Fisher's test, according to the sample size. A *p* value of <0.05 was considered significant.

Results

Antimicrobial susceptibility of *S. maltophilia* isolates

MICs and susceptibility profile of *S. maltophilia* for the tested antimicrobial agents are shown in Table 1. Resistance rates to ceftazidime, levofloxacin, trimethoprim–sulfamethoxazole and chloramphenicol were 67.4%, 6.1%, 17.7% and 8.8%, respectively. All isolates were susceptible to minocycline. The MIC required to inhibit the growth of 50% of organisms (MIC₅₀) of piperacillin, imipenem and amikacin was 128 mg/L, >128 mg/L and 128 mg/L, respectively. The MIC₅₀ and MIC required to inhibit the growth of 90% of organisms (MIC₉₀) of moxifloxacin

(0.5 and 2 mg/L, respectively) were lower than those of levofloxacin (1 and 4 mg/L, respectively). Fig. 1 shows a comparison of susceptibility to levofloxacin and moxifloxacin among *S. maltophilia* isolates with or without *Smqnr* genes. Eight (7.7%) and three (2.9%) of the 104 *Smqnr*-positive isolates were resistant to levofloxacin and moxifloxacin using the resistance breakpoint of MICs ≥8 mg/L, while three (3.9%) and three (3.9%) of the 77 *Smqnr*-negative isolates were resistant to levofloxacin and moxifloxacin, respectively (*p* >0.05) for both levofloxacin and moxifloxacin between *Smqnr*-positive and -negative isolates). However, 41 (39.4%) and 20 (19.2%) of the 104 *Smqnr*-positive isolates were resistant to levofloxacin and moxifloxacin using the resistance breakpoint of MICs ≥2 mg/L, while 17 (22.1%) and seven (9.1%) of the 77 *Smqnr*-negative isolates were resistant to levofloxacin and moxifloxacin, respectively (*p* 0.0134 for levofloxacin and *p* >0.05 for moxifloxacin between *Smqnr*-positive and -negative isolates).

Prevalence of *Smqnr* and PMQR genes in *S. maltophilia*

The distribution of *Smqnr* and PMQR genes for *S. maltophilia* is summarized in Table 2. *Smqnr* was detected in Japan at high frequencies in clinical isolates of *S. maltophilia* (57.5%; 104/181 isolates). The *Smqnr* frequency by region was 69.8% (30/43) in Hokkaido, 53.1% (26/49) in Tokyo, 46.5% (20/43) in Osaka and 60.9% (28/46) in Fukuoka. The most frequent was *Smqnr6* (27.9%, 29/104), followed by *Smqnr8* (19.2%, 20/104) and *Smqnr11* (13.5%, 14/104). PMQR determinants were detected in nine *S. maltophilia* isolates (5.0%). No *S. maltophilia* isolates carried *qnrA*, *qnrB*, *qnrC*, *qnrS* or *qepA*. Nine (5.0%) were positive for *aac(6')-Ib*, and all of them possessed the -*cr* variant.

PFGE typing of *Smqnr*-containing *S. maltophilia* isolates

Dendrogram and PFGE of XbaI-digested genomic DNA from 24 *S. maltophilia* with *Smqnr* are shown in Fig. 2, including 11 isolates with *Smqnr6* and 13 isolates with *Smqnr11*. The 24 *Smqnr*-containing *S. maltophilia* isolates were divided into 21 unique PFGE types. Of the 11 *Smqnr6*-containing *S. maltophilia*

TABLE 1. Minimum inhibitory concentrations (MICs) and susceptibility profiles of *Stenotrophomonas maltophilia* for the tested antimicrobial agents

Antibiotic	MIC range (mg/L)	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)	Resistance rate (%)
Piperacillin	0.06–128	128	>128	
Ceftazidime	0.06–128	64	128	67.4
Imipenem	0.06–128	>128	>128	
Minocycline	0.06–128	0.25	1	0
Amikacin	0.06–128	128	>128	
Levofloxacin	0.06–128	1	4	6.1
Moxifloxacin	0.06–128	0.5	2	
Trimethoprim–sulfamethoxazole	1/19–8/152	1/19	8/152	17.7
Chloramphenicol	2–128	8	16	8.8

MIC₅₀, minimum inhibitory concentration required to inhibit the growth of 50% of organisms; MIC₉₀, minimum inhibitory concentration required to inhibit the growth of 90% of organisms.

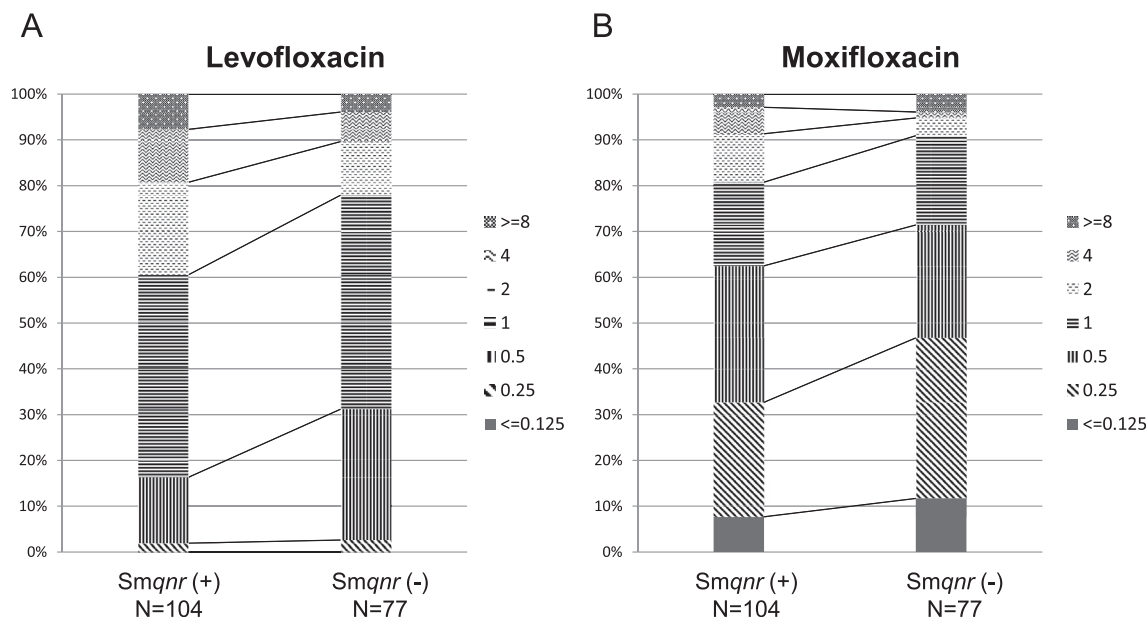


FIG. 1. Comparison of levofloxacin (A) and moxifloxacin (B) susceptibility among *Stenotrophomonas maltophilia* isolates with or without *Smqnr* genes. Susceptible, ≤ 2 mg/L; intermediate, 4 mg/L; resistant, ≥ 8 mg/L.

isolates, five isolates were novel, while two isolates from Osaka and Fukuoka, Tokyo and Osaka, or Fukuoka only clustered together (SM1, SM5 or SM6). All 13 isolates possessing *Smqnr11* were unique.

TABLE 2. Distribution of *Smqnr* and plasmid-mediated quinolone resistance (PMQR) genes in 181 clinical isolates of *Stenotrophomonas maltophilia*

Gene	n	%
<i>Smqnr</i>		
<i>Smqnr2</i>	5	2.8
<i>Smqnr4</i>	2	1.1
<i>Smqnr6</i>	29	16.0
<i>Smqnr8</i>	20	11.0
<i>Smqnr9</i>	3	1.7
<i>Smqnr11</i>	14	7.7
<i>Smqnr18</i>	1	0.6
<i>Smqnr35</i>	4	2.2
<i>Smqnr37</i>	5	2.8
<i>Smqnr41</i>	4	2.2
<i>Smqnr48</i>	1	0.6
<i>Smqnr49</i>	2	1.1
<i>Smqnr50</i>	1	0.6
<i>Smqnr51</i>	1	0.6
<i>Smqnr52</i>	6	3.3
<i>Smqnr53</i>	1	0.6
<i>Smqnr54</i>	1	0.6
<i>Smqnr55</i>	1	0.6
<i>Smqnr56</i>	1	0.6
<i>Smqnr57</i>	1	0.6
<i>Smqnr58</i>	1	0.6
Total	104	57.5
PMQR genes		
<i>qnrA</i>	0	0
<i>qnrB</i>	0	0
<i>qnrC</i>	0	0
<i>qnrS</i>	0	0
<i>qepA</i>	0	0
<i>aac(6)-Ib-cr</i>	9	5.0
Total	9	5.0

Novel variants of *Smqnr* and comparison with previous variants

In this study, 11 novel variants of *Smqnr* were discovered, and the following nucleotide accession numbers were assigned in the EMBL/GenBank/DDBJ database: *Smqnr48*, AB852568; *Smqnr49*, AB852569; *Smqnr50*, AB852570; *Smqnr51*, AB852571; *Smqnr52*, AB852572; *Smqnr53*, AB852573; *Smqnr54*, AB905279; *Smqnr55*, AB905280; *Smqnr56*, AB905281; *Smqnr57*, AB905282; and *Smqnr58*, AB905283. The novel variants were compared to those reported previously: *Smqnr1–Smqnr11* (accession numbers AB430839–AB430843, AB430845–AB430850); *Smqnr12–Smqnr18* (accession numbers FJ596752–FJ596758); *Smqnr19–Smqnr27* (accession numbers EU681371–EU681373, EU681375, EU681378, EU681381–EU681382, EU681384–EU681385); *Smqnr28–Smqnr47* (accession numbers HQ874463–HQ874464, HQ896258–HQ896275). A phylogenetic tree showing the relationship from *Smqnr1* to *Smqnr58* is shown in Fig. 3. Differences in amino acid sequences between novel variants in this study and previous variants are as follows: *SmQnr48* (55S → T, 194T → A of *SmQnr36*), *SmQnr49* (18D → G of *SmQnr30*), *SmQnr50* (119A → V of *SmQnr35*), *SmQnr51* (20T → H of *SmQnr9*), *SmQnr52* (72R → H, 203V → M of *SmQnr11*), *SmQnr53* (67K → E, 146W → G of *SmQnr37*), *SmQnr54* (119A → E, 169V → I of *SmQnr11*), *SmQnr55* (82S → N, 115G → W, 194A → T of *SmQnr38*), *SmQnr56* (6S → F, 57T → A, 82S → N of *SmQnr39*), *SmQnr57* (105S → N, 169V → I of *SmQnr11*), and *SmQnr58* (6S → F, 57T → A, 82S → N, 205Q → X of *SmQnr39*).

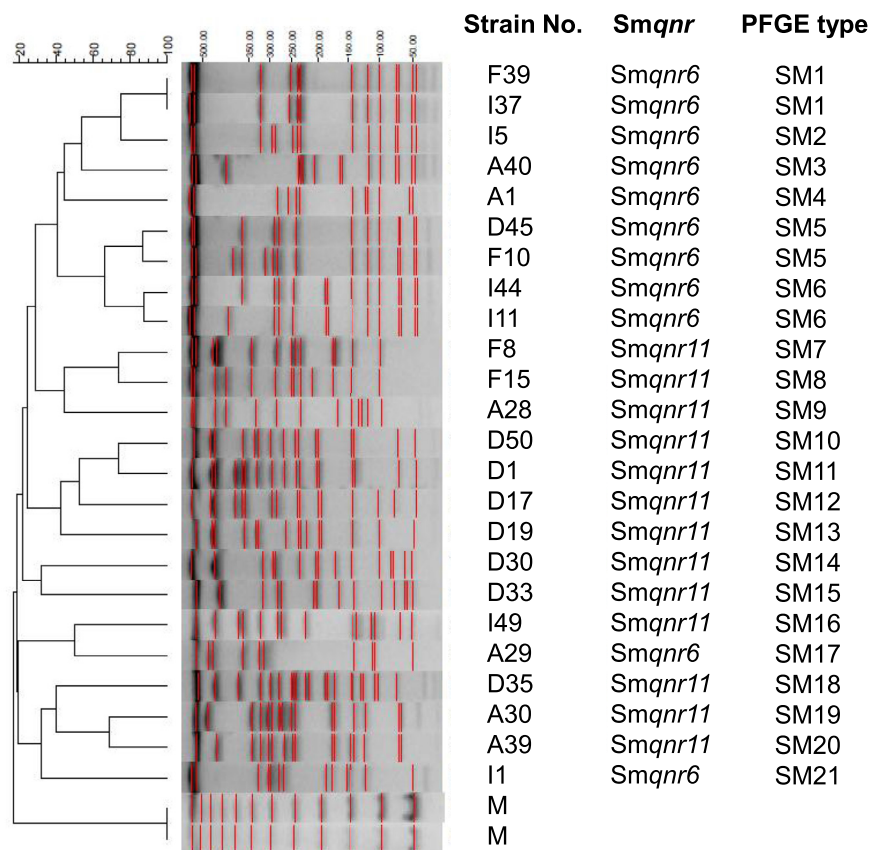


FIG. 2. Dendrogram and pulsed-field gel electrophoresis of XbaI-digested genomic DNA from 24 Smqnr-containing *Stenotrophomonas maltophilia* isolates. A, Hokkaido; D, Tokyo; F, Osaka; I, Fukuoka; M, marker.

Discussion

Global infectious disease surveillance indicates that resistance rates for trimethoprim–sulfamethoxazole, ticarcillin–clavulanic acid, ciprofloxacin, levofloxacin and minocycline in *S. maltophilia* isolates are less than 4.7%, 16.1%, 40%, 6.5% and 5%, respectively [16]. However, large differences in the susceptibility of *S. maltophilia* were seen, depending on the country and/or region. In our study, resistance to trimethoprim–sulfamethoxazole was approximately 18% in Japan, while it has been reported to be 8% in the Asia-Pacific region, 10% in Europe, 15% in Turkey, 25% in Taiwan and 27% in Spain [3]. The resistance rate to levofloxacin in this study was 6.1%, which was the same as seen in a previous study in China [17]. Although the percentage of strains resistant to quinolones ranged from 13–96% for ciprofloxacin, 3–11% for levofloxacin, 0–46% for gatifloxacin, 6% for moxifloxacin, 56% for norfloxacin and 5% for ofloxacin [3], the levofloxacin resistance rate in this study showed a higher tendency in a MIC scale than to moxifloxacin. Furthermore, our resistance rate using a MIC of ≥ 2 mg/L for levofloxacin among Smqnr-positive isolates was significantly higher than that in Smqnr-negative isolates; however, there was

no significant difference in levofloxacin resistance rate using a MIC of ≥ 8 mg/L (the same as the CLSI criteria) between isolates with or without Smqnr, although the number of sample strains tested in this study was small. Little association was seen between Smqnr presence and high-level quinolone resistance in *S. maltophilia*, though Smqnr can provide low-level quinolone resistance, as described in previous studies [9–11,17]. The role of Smqnr remains unclear, but the reduced susceptibility may be associated with the selection of mutants having higher resistance, as described in PMQR determinants [7].

In this study, we identified 21 different PFGE types among the 24 *S. maltophilia* Smqnr-containing isolates in Japan. These results suggest that *S. maltophilia* Smqnr-containing isolates in Japan are not a single clone but rather are multiple clones. Superinfection with *S. maltophilia* is common, with a reported frequency of 20–40% [3,18], and *S. maltophilia* may be a reservoir for the dissemination of quinolone resistance to other bacteria. Thus, it is necessary to monitor Smqnr genes in Enterobacteriaceae and other organisms as well as in *S. maltophilia*.

Smqnr, a chromosome-carried quinolone resistance gene in *S. maltophilia*, was first reported by Shimizu and colleagues in 2008 [9]. There have been several experimental and epidemiologic studies regarding the mechanism and distribution of

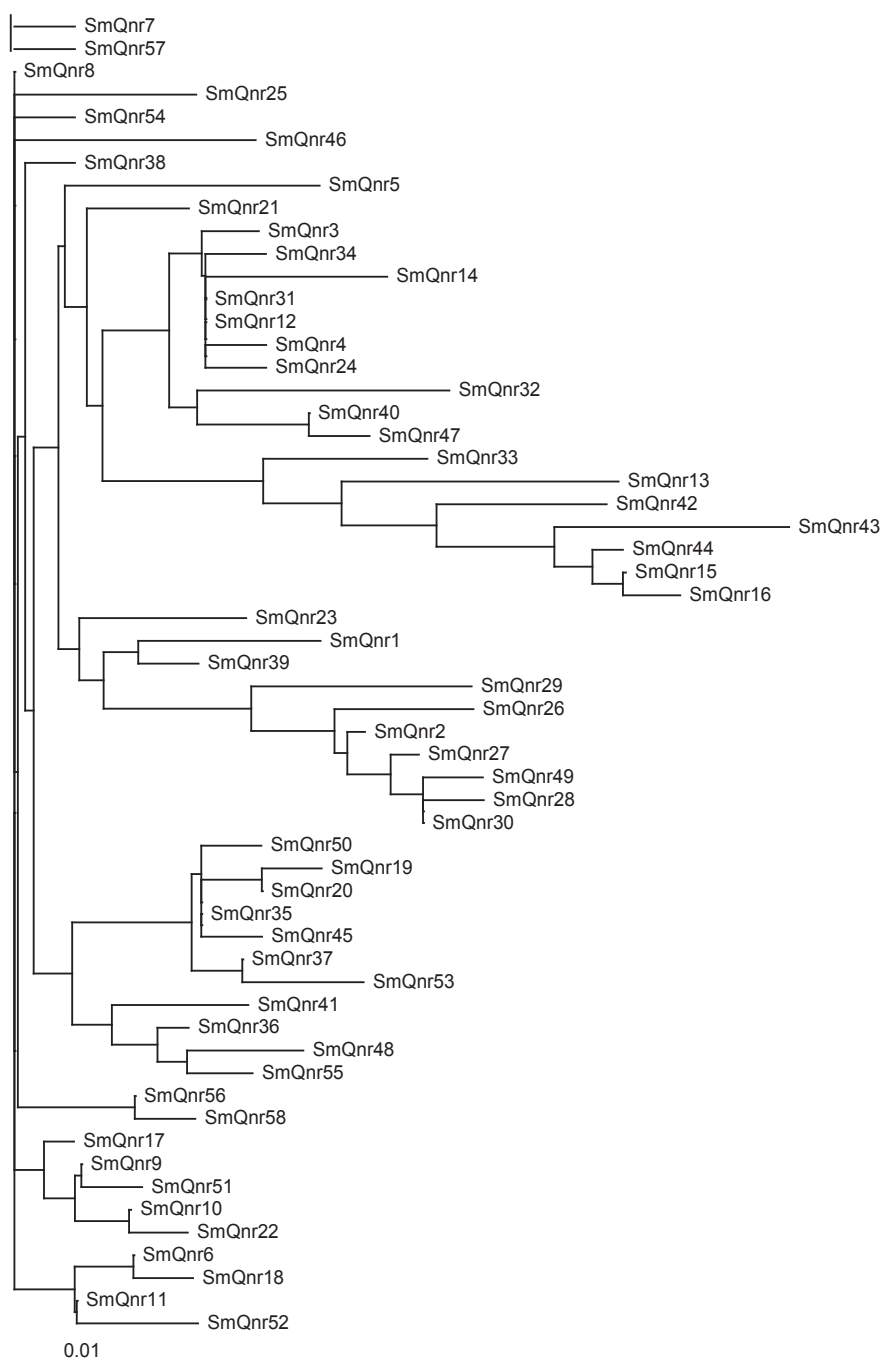


FIG. 3. Phylogenetic analysis of the relationship of SmQnr1–58. The phylogram was provided by the neighbor-joining method using ClustalW2.1.

Smqnr in clinical isolates of *S. maltophilia* [9–11,17,19,20]. SmQnr1–11 proteins with 2–6 amino acid alterations were initially identified in Japan [9], and subsequent SmQnr variants were described as follows: SmQnr12–18 in UK [11], SmQnr19–27 in Spain [10], SmQnr28–47 in China [17] and SmQnr48–58 in Japan (this study). All 11 novel *Smqnr* variants in our study were derived from *Smqnr* genes reported previously from Japan and China. Our study suggests a high prevalence of *Smqnr* in *S. maltophilia* from Japan (Table 2), and there

were regional differences of 47–70% in *Smqnr* frequency. The most common *Smqnr* gene in our study was *Smqnr6*, followed by *Smqnr8* and *Smqnr11*, while *Smqnr8* and *Smqnr35* were most common in China. Further molecular epidemiological studies investigating the prevalence of *Smqnr* variants in other countries are needed.

Unlike Enterobacteriaceae, PMQR determinants in *S. maltophilia* have not been fully clarified. A previous study in China showed that PMQR determinants, including *qnrA*, *qnrB*,

qnrC, *qnrD* and *qnrS*, were not detected in *S. maltophilia* [17]. In this investigation, we report the detection of *aac(6′)-Ib-cr* in *S. maltophilia*, but no *qnr* or *qepA* genes were seen. The *aac(6′)-Ib-cr* gene, which confers resistance to both quinolones and aminoglycosides, has often been detected in CTX-M-producing Enterobacteriaceae [6,21] and is a concern for facilitating further multidrug resistance in *S. maltophilia*.

In conclusion, our study revealed a high prevalence of *Smqnr* among *S. maltophilia* from Japan and described novel variants of *Smqnr*. We also investigated the prevalence of PMQR determinants and demonstrated the presence of *aac(6′)-Ib-cr* genes in clinical isolates of *S. maltophilia*. Continuous antimicrobial surveillance for *S. maltophilia* and further investigations into quinolone resistance mechanisms in *S. maltophilia* are required.

Conflict of interest

None declared.

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