Antimicrobial susceptibility and mechanisms of high-level macrolide resistance in clinical isolates of Moraxella nonliquefaciens

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We investigated antimicrobial susceptibility and the molecular mechanism involved in conferring high-level macrolide resistance in 47 clinical isolates of Moraxella nonliquefaciens from Japan. Antimicrobial susceptibility was determined using Etest and agar dilution methods. Thirty-two erythromycin-non-susceptible strains were evaluated for the possibility of clonal spreading, using PFGE. To analyse the mechanism related to macrolide resistance, mutations in the 23S rRNA gene and the ribosomal proteins, and the presence of methylase genes were investigated by PCR and sequencing. The efflux system was examined using appropriate inhibitors. Penicillin, ampicillin, amoxicillin, cefixime, levofloxacin and antimicrobials containing β-lactamase inhibitors showed strong activity against 47 M. nonliquefaciens isolates. Thirty-two (68.1 %) of the 47 isolates showed high-level MICs to macrolides (MIC > 128 mg l⁻¹) and shared the A2058T mutation in the 23S rRNA gene. The geometric mean MIC to macrolides of A2058T-mutated strains was significantly higher than that of WT strains (P < 0.0001). Thirty-two isolates with high-level macrolide MICs clustered into 30 patterns on the basis of the PFGE dendrogram, indicating that the macrolide-resistant strains were not clonal. In contrast, no common mutations of the ribosomal proteins or methylase genes, or overproduction of the efflux system were observed in A2058T-mutated strains. Moreover, of the 47 M. nonliquefaciens strains, 43 (91.5 %) were bro-1 and 4 (8.5 %) were bro-2 positive. Our results suggest that most M. nonliquefaciens clinical isolates show high-level macrolide resistance conferred by the A2058T mutation in the 23S rRNA gene. This study represents the first characterization of M. nonliquefaciens.

INTRODUCTION

Moraxella nonliquefaciens, a Gram-negative coccobacillus, is part of the normal microbiota of the human respiratory tract (Vaneechoutte et al., 2012). Previous reports have suggested that M. nonliquefaciens causes various infectious diseases, such as endocarditis, endophthalmitis and pneumonia (Davis et al., 2004; Laukeland et al., 2002; Rafiq et al., 2011), and it has been reported to show susceptibility to various antimicrobials, including macrolides (Davis et al., 2004; Laukeland et al., 2002; Vaneechoutte et al., 2012).

Macrolides act by binding to the 23S rRNA gene and prevent elongation of the peptide chain (Leclercq & Courvalin, 1998). Currently, clarithromycin and azithromycin are frequently used for the treatment of respiratory tract infections (Grover et al., 2012; Higashi & Fukuhara, 2009; Kawai et al., 2012). Mechanisms of resistance against these antimicrobials, including in Moraxella catarrhalis, which is among the Moraxella species most frequently isolated from clinical samples, are known to involve active efflux of...
antimicrobials and modification of the ribosomal target by enzymes such as rRNA methylase, or mutations in domain V of the 23S rRNA gene (Chironna et al., 2011; Chisholm et al., 2010; Li et al., 2011; Saito et al., 2012; Tait-Kamradt et al., 2000; Versalovic et al., 1997). Another mechanism, involving mutations in the ribosomal proteins L4 and L22 encoded by rplD and rplV, respectively, has been reported to underlie resistance in some clinical isolates (Berisio et al., 2006; Tait-Kamradt et al., 2000).

There is, however, a need for more data from clinical isolates to assess the risk of emergence of antimicrobial-resistant M. nonliquefaciens strains. Therefore, we investigated the antimicrobial susceptibility of 47 clinical isolates of M. nonliquefaciens and characterized the molecular mechanism related to high-level resistance to macrolides in these strains. We demonstrated for the first time that a point mutation in the 23S rRNA gene confers high-level resistance to macrolides in M. nonliquefaciens.

**METHODS**

**Bacterial strains and culture conditions.** A total of 47 M. nonliquefaciens clinical isolates collected at four university hospitals in Tokyo, Japan, from October 2008 to July 2009, and a type strain, M. nonliquefaciens ATCC 17953, were examined in this study. The clinical isolates were obtained from nasopharyngeal specimens of patients with respiratory tract infections. They were identified using the VITEK 2 system (SYSMEX; bioMérieux) and 16S rRNA gene sequencing using primers 10F and 800R (Table 1). M. nonliquefaciens strains were cultured at 37 °C on tryptone soya agar (TSA) plates containing 5 % sheep blood (Nippon Becton Dickinson) and brain heart infusion (BHI) agar plates (Oxoid) in an atmosphere of 95 % air and 5 % CO2. Cultures were maintained in 10 % skimmed milk and stored at −80 °C until required for subsequent experiments.

**Antimicrobial susceptibility testing.** The antimicrobial susceptibility of M. nonliquefaciens strains was determined using the Etest method (AB BIODISK) on TSA containing 5 % sheep blood, according to the manufacturer’s recommendations. The following antibiotics were tested by Etest: penicillin, ampicillin, amoxicillin, ampicillin-sulbactam, amoxicillin-clavulanic acid, cefotaxime and levofloxacin. Antimicrobial susceptibility to erythromycin (Wako Pure Chemical), clarithromycin (Taisho Pharmaceutical), azithromycin (Tokyo Chemical Industry) and josamycin (Astellas Pharma) was determined by the agar dilution method, using BHI agar. MICs of antimicrobials were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria for M. catarrhalis (CLSI, 2010). *Staphylococcus aureus* ATCC 29213 was used for quality control.

**PFGE.** Genomic DNA of 32 erythromycin-non-susceptible M. nonliquefaciens strains was prepared in agarose plugs treated with proteinase K (Takara Bio). The DNA was digested with 25 U SpeI (New England Biolabs) and electrophoresis was performed using a CHEF DRII system (Bio-Rad). Fingerprinting II software (Bio-Rad) was used to analyse the DNA restriction patterns and determine their similarity, based on calculation of the Dice similarity coefficient and using the unweighted pair group method with arithmetic mean algorithm.

**DNA sequencing of macrolide-resistance targets and detection of methylase and bro ß-lactamase genes.** Bacterial genomic DNA was extracted by boiling. The 23S rRNA gene, rplD and rplV were amplified by PCR using the relevant primer sets (Table 1), as follows:

**Table 1. Primers used in this study**
3 min of initial denaturation at 95°C, 30 cycles each consisting of 30 s at 95°C, 30 s at 53°C and 1 min at 72°C and 3 min of final extension at 72°C. PCR amplions were sequenced on an ABI PRISM 3100 genetic analyser (Applied Biosystems). DNA sequences were compared with the M. nonliquefaciens ATCC 17953 sequence (GenBank accession no. AB745464 for 23S rRNA, AB745465 for rplD and AB745466 for rplV) using BLAST.

The presence of the methylase genes erm(A), erm(B), erm(C) and erm(F) was screened for using the relevant primer sets (Table 1) as described previously (Chung et al., 1999; Nakaminami et al., 2008; Roberts et al., 1999; Saito et al., 2012; Sutcliffe et al., 1996). S. aureus, Streptococcus pneumoniae and Bacteroides vulgatus were used as positive controls for erm(A) and erm(C), erm(B) and erm(F), respectively.

PCR amplification of bop was performed as described previously (Khan et al., 2010).

Analysis of the efflux system. To investigate the role of efflux systems in resistance to macrolides, efflux pump activity was assessed by the addition of two different inhibitors, phenylalanine arginine β-naphthylamide (PAβN; Sigma-Aldrich) and carbonyl cyanide m-chlorophenylhydrazone (CCCP; Nacalai Tesque) to the growth medium at the final concentration of 10 and 0.1 mg l⁻¹, respectively. A greater than twofold decrease in MICs with and without inhibitors for each antibiotic was considered to indicate a significant change. The effects of the relevant inhibitors were determined at least twice.

Statistical analysis. A Mann–Whitney U test was used to determine statistical differences in MICs. In all analyses, P<0.05 was considered significant.

RESULTS

Antimicrobial susceptibility testing

The results of antimicrobial susceptibility testing of 47 clinical isolates to 12 antibiotics are shown in Table 2. The MIC of antibiotics for an M. liquefaciens type strain ATCC 17953 were as follows: penicillin, 0.040 mg l⁻¹; ampicillin, ≤0.016 mg l⁻¹; amoxicillin, ≤0.016 mg l⁻¹; ampicillin–sulbactam, ≤0.016 mg l⁻¹; amoxicillin–clavulanic acid, ≤0.016 mg l⁻¹; cefixime, ≤0.016 mg l⁻¹; levofloxacin, 0.064 mg l⁻¹; erythromycin, ≤0.125 mg l⁻¹; clarithromycin, ≤0.125 mg l⁻¹; azithromycin, ≤0.125 mg l⁻¹; josamycin, 1 mg l⁻¹.

The MIC₉₀ values of antibiotics, excluding macrolides, for the isolates in this study were: penicillin, 4 mg l⁻¹; ampicillin, 0.25 mg l⁻¹; amoxicillin, 0.5 mg l⁻¹; cefixime, 0.25 mg l⁻¹; levofloxacin, 0.064 mg l⁻¹. The MIC₉₀ values of ampicillin and amoxicillin containing a β-lactamase-inhibitor were 0.125 and 0.125 mg l⁻¹, respectively. These antibiotics showed strong activity against M. nonliquefaciens.

In contrast, macrolides were less active against M. nonliquefaciens than the other seven tested antibiotics: the MIC₉₀ values of erythromycin, clarithromycin, azithromycin and josamycin were ≥512, 512, 512 and 256 mg l⁻¹, respectively. Based on the CLSI breakpoints for M. catarrhalis, the non-susceptible rates of amoxicillin–clavulanic acid (˃4 mg l⁻¹), levofloxacin (˃2 mg l⁻¹), erythromycin (˃2 mg l⁻¹), clarithromycin (˃1 mg l⁻¹) and azithromycin (˃0.125 mg l⁻¹) were 0% (0/47), 0% (0/47), 68.1% (32/47), 68.1% (32/47), and 68.1% (32/47), respectively.

PFGE

To investigate the possibility of clonal spreading of macrolide-resistant M. nonliquefaciens strains, 32 erythromycin-non-susceptible strains were evaluated by PFGE. The resulting dendrogram showed 30 PFGE patterns (Fig. 1). Strains in which a similar band pattern was detected (Mn8 and Mn10, as well as Mn13 and Mn19) had not been isolated from the same hospital.

Analysis of macrolide-resistance targets

In comparison with the 23S rRNA gene sequence (nt 1964–2430; Eschericia coli numbering, GenBank accession no.
V00331) of *M. nonliquefaciens* ATCC 17953 (GenBank accession no. AB818473), 18 different patterns of 23S rRNA gene sequences were identified in the 47 *M. nonliquefaciens* clinical isolates (GenBank accession nos AB818474–AB818489). Although a large number of mutations were observed in the 32 erythromycin-non-susceptible and 15 erythromycin-susceptible strains, mutations including A1986C, G1987A, A2058T, A2105G, T2113C, T2132A, A2150T, T2151C, G2154A, C2160T and T2184C, and an insertion at nt 2212 (*E. coli* numbering) were detected only in the erythromycin-non-susceptible strains. However, a single mutation, A2058T, was present only in erythromycin-non-susceptible strains and was found in all of these strains. The geometric mean MIC for erythromycin, clarithromycin, azithromycin and josamycin of 15 erythromycin-susceptible strains with the WT sequence at nt 2058 was significantly lower than those of 32 erythromycin-non-susceptible strains bearing the A2058T mutation \( (P<0.0001; \text{Table 3}) \).

Furthermore, five different amino acid sequence patterns in L4 and two in L22 were found among the 47 *M. nonliquefaciens* (data not shown). No sequence patterns present only in erythromycin-nonsusceptible strains were observed.

To investigate the presence of methylase genes, a PCR detection assay was performed. We did not detect *erm(A), erm(B), erm(C)* or *erm(F)* in any of the 47 clinical isolates.

**Analysis of bro β-lactamase genes**

Among the 47 *M. nonliquefaciens* clinical isolates, 43 (91.5%) were positive for *bro-1* and 4 (8.5%) for *bro-2*. The results of DNA sequencing of *bro-1* (165 bp) and *bro-2* (144 bp) showed 100% similarity to those of *M. catarrhalis* (GenBank accession nos Z54180 and Z54181, respectively).

**Analysis of the efflux system**

MICs of erythromycin for the 47 *M. nonliquefaciens* isolates were evaluated in the absence or presence of efflux pump inhibitors. In the presence of PAβN, the MICs of 61.7% (29/47) isolates remained unchanged, 25.5% (12/47) were decreased twofold and 8.5% (4/47) were decreased fourfold (three erythromycin-non-susceptible strains: \( \geq 512 \) to \( 256 \) mg l\(^{-1}\); one erythromycin-susceptible strain: \( 0.5 \) to \( 0.125 \) mg l\(^{-1}\)) compared with the MICs in the absence of PAβN; 6.4% (3/47) did not grow. On the other hand, in the presence of CCCP, the MICs of 95.7% (45/47) remained similar to those observed in the absence of CCCP, while those of 6.4% (3/47) were decreased twofold.

**DISCUSSION**

To date, although there are few case reports of *M. nonliquefaciens*-associated infections, and *M. nonliquefaciens* has not yet been characterized in detail, including its antimicrobial susceptibility. In general, *Moraxella* species are susceptible to penicillin and its derivatives, cephalosporins, tetracyclines, quinolones, aminoglycosides, and macrolides (Vaneechoutte et al., 2012). Furthermore, previous studies have reported that *M. nonliquefaciens* is susceptible to penicillin G, ampicillin, cepfazidime, tetracycline, ciprofloxacin, gentamicin, erythromycin and imipenem (Davis et al., 2004; Laukeland et al., 2002). In this study, most *M. nonliquefaciens* demonstrated low penicillin MICs and showed extremely low MICs for ampicillin, amoxicillin, ampicillin-sulbactam, amoxicillin-clavulanic acid, cefixime and levofloxacin, as reported in previous studies, while 68.1% (32/47) of clinical isolates demonstrated high-level MICs for 14-, 15- and 16-membered macrolides (e.g. erythromycin MIC >256 mg l\(^{-1}\)). Therefore, although the present study has certain
Table 3. MICs of macrolides in WT and A2058T mutant strains of *M. nonliquefaciens*

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>23S rRNA sequence*</th>
<th>n</th>
<th>MIC (mg l⁻¹)</th>
<th>P value†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Range</td>
<td>MIC₉₀</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>WT</td>
<td>15</td>
<td>≤0.125–1</td>
<td>≤0.125</td>
</tr>
<tr>
<td></td>
<td>A2058T</td>
<td>32</td>
<td>512–&gt;512</td>
<td>512</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>WT</td>
<td>15</td>
<td>≤0.125–0.5</td>
<td>≤0.125</td>
</tr>
<tr>
<td></td>
<td>A2058T</td>
<td>32</td>
<td>128–&gt;512</td>
<td>512</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>WT</td>
<td>15</td>
<td>≤0.125</td>
<td>≤0.125</td>
</tr>
<tr>
<td></td>
<td>A2058T</td>
<td>32</td>
<td>64–&gt;512</td>
<td>256</td>
</tr>
<tr>
<td>Josamycin</td>
<td>WT</td>
<td>15</td>
<td>0.5–4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>A2058T</td>
<td>32</td>
<td>128–256</td>
<td>256</td>
</tr>
</tbody>
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*E. coli numbering (GenBank accession no. V00331).
†Statistical significance determined by a Mann–Whitney U test.

limitations in that sample numbers were small and medical records of patients were not examined, our results indicate that most *M. nonliquefaciens* clinical isolates in Japan may have high-level resistance to macrolides, which are frequently used for treatment of respiratory tract infections such as pneumonia caused by *Mycoplasma pneumoniae* (Grover et al., 2012; Higashi & Fukuhara, 2009; Kawai et al., 2012). Furthermore, 32 erlythromycin-non-susceptible strains showed 30 banding pattern types by PFGE analysis, indicating that various clones with high-level resistance to macrolides are spreading throughout this country.

Recently, macrolide- and lincosamide-resistant *M. catarrhalis* strains have been reported in Japan (Saito et al., 2012). Saito et al. (2012) demonstrated that the A2058T mutation in the 23S rRNA gene (*E. coli* numbering) confers high-level resistance to macrolides and lincosamides in *M. catarrhalis*. Furthermore, it has been described that a single mutation at position A2058T or A2059 in the 23S rRNA gene confers high-level resistance to macrolides and/or lincosamides in a variety of bacteria, including *S. pneumoniae*, *Mycoplastermum smegmatis*, *Neisseria gonorrhoeae*, *Helicobacter pylori* and *M. pneumoniae* (Chironna et al., 2011; Chisholm et al., 2010; Li et al., 2011; Tait-Kamradt et al., 2000; Versalovic et al., 1997). In the present study, although 32 erythromycin-non-susceptible strains harboured various sequencing profiles in the 23S rRNA gene, a single A2058T mutation was common to all erythromycin-non-susceptible strains. Furthermore, the geometric mean MIC for macrolides in A2058T-mutated strains was significantly higher than that of WT strains (P<0.0001). In contrast, erlythromycin-non-susceptible strains did not share common amino acid sequences in L4 and L22, and erm genes were absent. Moreover, an active efflux system was not only observed in three erythromycin-non-susceptible strains, but also in one erythromycin-susceptible strain. Therefore, although overexpression of efflux systems in addition to the A2058T mutation in the 23S rRNA gene may contribute synergistically to the acquisition of resistance to macrolides, our findings suggest the possibility that the presence of the A2058T mutation is required to acquire high-level macrolide resistance in *M. nonliquefaciens*, as described previously for *M. catarrhalis* (Liu et al., 2012; Saito et al., 2012). However, as we have not investigated the role of the A2058T mutation in the 23S rRNA alleles of *M. nonliquefaciens* to the same extent as in our previous *M. catarrhalis* study (Saito et al., 2012), we propose to perform further studies to analyse the role of the A2058T mutation in more detail.

It is already known that *M. nonliquefaciens* produces BRO β-lactamase (Eliasson et al., 1992). In this study, all 47 *M. nonliquefaciens* clinical isolates carried bro β-lactamase genes, and the frequency of bro-1- and bro-2-positive isolates was 91.5 and 8.5 %, respectively. These observations are similar to previous findings of *M. catarrhalis*, which is closely related to *M. nonliquefaciens*, in that more than 90 % of the global clinical isolates are BRO β-lactamase producers, of which bro-1-positive isolates make up a large majority (Khan et al., 2010; Murphy & Parmeswaran, 2009).

The present study demonstrates that *M. nonliquefaciens* strains with high-level macrolide resistance have already spread through clinical settings in Japan, but it is not clear whether these results are related to the use of macrolides in the treatment of infectious diseases, including respiratory tract infections. Moreover, high-level macrolide-resistant *M. catarrhalis* clinical isolates have already been reported in Japan and China (Flamm et al., 2012; Liu et al., 2012; Saito et al., 2012). To ensure rapid detection of the emergence and prevention of further dissemination of high-level macrolide-resistant phenotypes in *Moraxella* species, surveillance of antimicrobial susceptibility to macrolides should be continued.

In conclusion, we demonstrate that 68.1 % of *M. nonliquefaciens* clinical isolates from Japan showed high-level macrolide resistance and provide the first evidence that a single A2058T mutation in the 23S rRNA gene confers this
high level of macrolide resistance. To our knowledge, this is the first report investigating the role of mutations in the 23S rRNA gene in *M. nonliquefaciens*. As the emergence and spread of high-level macrolide-resistant *Moraxella* species threatens effective treatment of these infections, continued surveillance for these strains should be undertaken.

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