Rapid assay of A2058T-mutated 23S rRNA allelic profiles associated with high-level macrolide resistance in *Moraxella catarrhalis*

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We report on a restriction fragment-length polymorphism (HpyCH4III) assay for profile analysis of 23S rRNA gene A2058T-mutated alleles associated with high-level macrolide resistance in *Moraxella catarrhalis*. Our assay results were supported by DNA sequencing analysis, allowed for simultaneous testing of many strains, and produced results from pure-cultured colonies within 4 h.

INTRODUCTION

*Moraxella catarrhalis*, an important human respiratory tract pathogen, can cause various infectious diseases, including otitis media (OM) and lower respiratory tract infections with chronic obstructive pulmonary disease (COPD) (Coco et al., 2010; Seemungal et al., 2008). 14- and 15-membered macrolides are commonly used to treat OM and exacerbations of COPD (Coco et al., 2010; Seemungal et al., 2008).

Although the resistance to macrolides and/or lincosamides in *M. catarrhalis* is a rare phenotypic characteristic, high-level macrolide-resistant *M. catarrhalis* strains have been reported in Japan (Iwata et al., 2015; Saito et al., 2012) and China (Liu et al., 2012). It is well documented that a mutation of the 23S rRNA gene at position 2058 or 2059 (*Escherichia coli* numbering) is associated with decreased susceptibility to macrolides and/or lincosamides in various organisms such as *Helicobacter pylori* (Debets-Ossenkopp et al., 1998), *Neisseria gonorrhoeae* (Ng et al., 2002) and *Streptococcus pneumoniae* (Tait-Kamradt et al., 2000). We have also shown that the presence of three or more A2058T-mutated alleles in the 23S rRNA gene is associated with the acquisition of high-level resistance to macrolides in *M. catarrhalis* (Saito et al., 2012).

Since the emergence of macrolide-resistant strains threatens effective treatment of *M. catarrhalis* infections, their rapid detection is important. However, current methods for detection of the A2058T mutation can take up to 2 days at the earliest to complete from pure-cultured colonies. We here report on a rapid assay developed to analyse profiles of A2058T mutations in each 23S rRNA allele using the naturally occurring restriction enzyme HpyCH4III cleavage site.

METHODS

Bacterial strains and culture conditions. The erythromycin-susceptible *M. catarrhalis* reference strain ATCC 49143, the clinical isolate Mc19 and their erythromycin-resistant transformants (49143TF48 and 49143TF53, MIC = 4 μg·ml⁻¹; 49143TF25 and 19TF24, MIC = 512 μg·ml⁻¹), whose 23S rRNA allele sequences were previously confirmed (Saito et al., 2012), were included in this study. Furthermore, eight erythromycin-susceptible and 13 erythromycin-resistant strains from a previous study (Kasai et al., 2015) were also examined. They were cultured on Mueller–Hinton (MH) broth and MH agar (Oxoid).

PCR-RFLP assay. PCR amplification of alleles 1, 3, and 4 in the 23S rRNA gene, and DNA sequencing were performed as previously reported (Saito et al., 2012). Allele 2 of the 23S rRNA gene was amplified using the primer Mc23S-allele2-2 (5'-CCAGATTACACG-AAGAACATCAA-3'), using the same PCR conditions as applied for the other alleles. Restriction enzyme digestion with HpyCH4III (New England Biolabs Japan) was performed in a 20 μl mixture containing 1 μl of the PCR product of each 23S rRNA allele, 0.2 μl (1 unit) of *HpyCH4III* and 2 μl 10 × digestion buffer supplied by the manufacturer. Another restriction enzyme, DraI (New England Biolabs Japan), was added to digest the PCR product of only allele 2 of the 23S rRNA gene. The digested samples were then separated by
RESULTS AND DISCUSSION

When allele-specific PCR products without an A2058T mutation in the 23S rRNA gene are digested with HpyCH4III, the digested DNA fragments in 23S rRNA alleles 1, 3, and 4 should theoretically produce six, six and seven fragments, respectively (including a fragment of 379 bp), according to the M. catarrhalis BBH18 genome sequence (GenBank accession number CP002005) (Fig. 1a). Conversely, because the PCR products containing A2058T mutations naturally create an HpyCH4III cleavage site, as shown in Fig. 1a, the 379 bp fragment disappears and is replaced by 135 bp and 244 bp fragments.

The HpyCH4III-digested DNA fragments in allele 2 of 23S rRNA should theoretically produce seven fragments, including 355 bp and 379 bp fragments. Since the 355 bp fragment cannot be distinguished from the 379 bp fragment by agarose gel electrophoresis, we used DraI in addition to HpyCH4III to digest it. This process allowed for clearer analysis of the presence or absence of the 379 bp fragment in this allele.

In this study, the digestion patterns of each 23S rRNA allele could be sufficiently confirmed in strains ATCC 49143, Mc19 and their four transformants that were used in our previous study (Fig. 1b, c). Furthermore, these results were concordant with those of the DNA sequencing analysis conducted in eight erythromycin-susceptible and 12 erythromycin-resistant strains. Additionally, our assay allowed for simultaneous testing of many strains and produced results within 4 h from pure-cultured colonies. However, although no A2058T mutation was observed in allele 2 of the highly erythromycin-resistant strain NIH28 (MIC=512 μg ml⁻¹), we could not detect the expected 379 bp fragment because this allele contains an HpyCH4III cleavage site. In a previous study, we indicated that transformants with two mutated 23S rRNA alleles in alleles 3 and 4 showed low-level resistance to macrolides, and that transformants with A2058T mutations in alleles 1, 3

Fig. 1. RFLP assay using HpyCH4III in M. catarrhalis strains. (a) Schematic representation of the assay taking advantage of a naturally occurring restriction enzyme (HpyCH4III) cleavage site. The digested fragment of the WT 23S rRNA allele is shown above, and its A2058T-mutated 23S rRNA allele is shown below. Position 2058 (E. coli numbering) in the 23S rRNA gene is indicated by bold type and underlined. Note that the DNA fragment size in the schematic is not to scale. (b) Profiles of 23S rRNA allele-specific PCR products. Lanes 1, 2, 3 and 4 are 23S rRNA alleles 1, 2, 3 and 4 (erythromycin-resistant M. catarrhalis strain NSH1), respectively. Lane MW, 1 kb DNA ladder molecular-mass standard. (c) Digestion profiles of 23S rRNA allele-specific PCR products with HpyCH4III or HpyCH4III and DraI. Lanes 1, 2, 3 and 4 are 23S rRNA alleles 1, 2, 3 and 4 without an A2058T mutation (erythromycin-susceptible M. catarrhalis ATCC 49143), respectively, and lanes 5, 6, 7 and 8 are 23S rRNA alleles 1, 2, 3 and 4 with the A2058T mutation (erythromycin-resistant M. catarrhalis strain NSH1), respectively. Lane MW, 100 bp DNA ladder molecular-mass standard.
and 4 showed high-level resistance to macrolides (Saito et al., 2012). Our results therefore suggest that this assay is useful as a rapid screening tool for the profile analysis of A2058T mutations of the 23S rRNA gene in highly macrolide-resistant *M. catarrhalis* isolates, although a large-scale evaluation might be needed for validation of allele 2 of the 23S rRNA gene.

Rapid detection of the 23S rRNA mutation could have a major impact on patients infected by *M. catarrhalis* with decreased susceptibilities to macrolides and lincosamides in terms of timely administration of appropriate antibiotic treatment. We plan to use this assay in further epidemiological studies of macrolide-resistant *M. catarrhalis* clinical isolates in the future.

**ACKNOWLEDGEMENTS**


**REFERENCES**


