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Isolation and Characterizations of Clarithromycin-Resistant *Mycobacterium avium* Clinical Isolates

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Mycobacterium avium is an important intracellular pathogen, particularly in AIDS patients. It also shows the second frequency among nontuberculous mycobacteria infections in Korea. Point mutations of domain V region of the 23S rRNA gene has been known to confer clarithromycin resistance to *M. avium*. In order to isolate the clarithromycin-resistant strains from clinical isolates of *M. avium* and characterize them, we isolated the clarithromycin-resistant strains from clinical isolates of *M. avium* using reverse hybridization assay (RHA) and broth microdilution test (BMT). Three clarithromycin-resistant isolates with high level of MICs were found from 274 clinical isolates by BMT. Two of three resistant strains were also found by RHA, which revealed point mutations in the domain V region of the 23S rRNA. We report here clarithromycin-resistant clinical isolates of *M. avium* with the different characteristics from those of the resistant strains reported from earlier studies. J. Clin. Lab. Anal. 25:33–36, 2011.

Key words: *Mycobacterium avium*; clarithromycin; resistance; point mutation

INTRODUCTION

Mycobacterium avium is an important intracellular pathogen, particularly in AIDS patients (1). It also shows the second frequency among nontuberculous mycobacteria (NTM) infections in Korea. Macrolides, such as clarithromycin and azithromycin, are important agents for the treatment of a variety of mycobacterial infections, including *M. avium* complex (MAC) infection (2). However, resistance to these antibiotics is known to emerge in patients receiving macrolide therapy (3–5).

The predominant resistance mechanism is posttranscription methylation of an adenine residue within the domain V region of the 23S rRNA by rRNA methylase gene, *erm*. This mechanism of macrolide resistance has been studied, including *M. tuberculosis*, *M. smegmatis*, *M. fortuitum*, and other NTMs (6–9). Another mechanism has been reported that point mutations in the domain V region of the 23S rRNA occur in many bacteria (10). Nash et al. characterized the point mutations in the 23S rRNA genes of macrolide-resistant *M. avium* isolated from patients with disseminated disease (4). Also, mutation in ribosomal proteins, changes in membrane permeability, and the active drug efflux pump are known to be related with the resistance (11,12).

Until recently, the relationship between the mutation and resistance of clarithromycin-resistant clinical isolates in Korea was not evaluated. In this study, we tried to isolate clarithromycin-resistant strains from clinical isolates of *M. avium* using reverse hybridization assay (RHA) and broth microdilution test (BMT), and represented the characteristics of the resistant strains isolated by using both methods.

MATERIALS AND METHODS

Mycobacteria

M. avium ATCC 700898 and ATCC 25291 were used in this study as reference strains. Clinical isolates of *M. avium* were obtained from Korean Institute of Tuberculosis, Seoul, Korea. We selected 274 clinical isolates, which were identified as *M. avium* with PCR-restriction fragment length polymorphism analysis and had good growth condition on the Lowenstein–Jensen medium.

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Preparation of Clarithromycin Solution

Clarithromycin (The United States Pharmacopeial Convention, Inc., Rockville, MD) was dissolved in methanol (the final concentration, 50%/v) and then was immediately diluted with sterile 0.1 M phosphate buffer (pH 6.8). A stock solution of 1,024 μg/ml clarithromycin was prepared.

Media

The media used for the culture of *M. avium* were Middlebrook 7H10 agar and Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; BBL, Becton Dickinson).

Drug Susceptibility Testing

Susceptibility testing to clarithromycin was performed by broth microdilution in Middlebrook 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase (512~0.25 μg/ml, pH 6.8), according to the guidelines of the Clinical and Labortory Standards Institute (13). Clinically significant resistance of clarithromycin was determined as a MIC of >32 μg/ml at pH 6.8.

PCR

The mycobacterial DNA was isolated by heating method. Primer synthesis included biotinylated-reverse primer (Table 1) and PCR for amplifying the domain V of 23S rRNA gene were performed according to the previously described protocols (4).

Reverse Hybridization Assay

Capture probes corresponding to the mutation site of the domain V of 23S rRNA gene were synthesized with a 3′-amino group to facilitate covalent linkage to Biodyne C membranes (Pall Corporation, East Hills, NY). The method for membrane preparation was performed according to the previously described protocols (14). PCR fragments were added to four volumes of hybridization buffer (HB; 6 × SSPE/0.1% SDS), boiled for 10 min at 100°C, and cooled in ice. After membrane strips were incubated with prewarmed HB (55°C) for 10 min, HB was discarded. Sample HB mixture were added and incubated with the strips, in a shaking water bath at 50°C for 60 min. After discarding the mixture solution and washing the strips twice with prewarmed washing buffer (WB; 4 × SSPE/0.1% SDS) at 62°C for 15 min, the 1/2,000-diluted streptavidin–alkaline phosphatase conjugate (Roche Diagnostics, Mannheim, Germany) in WB was added and incubated in a shaking water bath at 42°C for 40 min. After discarding the conjugate solution, strips were washed twice with TBS at room temperature for 10 min. For colorimetric detection, diluted NBT/BCIP stock solution (Roche Diagnostics, Mannheim, Germany) in staining buffer (0.1 M Tris-buffer pH 9.5/0.05 M $MgCl_2$, 0.1 M NaCl) was added and when the band appeared, the detection was stopped with distilled water.

TABLE 1. Primers and Probes for Detection of Mutation in 23S rRNA Gene

Primers and probes	Sequence (5′→3′)	Remark
Primers		
23S forward primer	aatggcgtaacgacttctcaactgt	
23S reverse primer	gcactagaggttcgtccgtccc	Biotin labeling at 5′ end
Probes		Amine linking at 3′ end
Wild type	ggacgaaaagaccccggg	
A2058G	ggacga**g**aagaccccggg	
A2058C	ggacga**c**aagaccccggg	
A2058T	ggacga**t**aagaccccggg	
A2059G	ggacgaa**g**agaccccggg	
A2059C	ggacgaa**c**agaccccggg	
A2059T	ggacgaa**t**agaccccggg	

Sequencing

The PCR products were sequenced by using 23S forward primer (Table 1) and sequencing was performed on 3,730 × DNA analyzer (Applied Biosystems, Foster city, CA).

RESULTS

Three clarithromycin resistant isolates were found from 274 clinical isolates by BMT. Two of three resistant strains were also found by RHA. Of the three resistant isolates, point mutations in domain V of the 23S rRNA gene were observed in two strains which were isolated by RHA. One isolate, MA23, showed the transition of A to T at position 2058 (*Escherichia coli* equivalent base number) with MIC 256 μg/ml and the other, MA223, showed the transition of A to C at position 2059 with MIC 256 μg/ml. However, a strain which was isolated only by BMT, MA251, had no mutation in the domain V region of the 23S rRNA gene and MIC was 128 μg/ml. In MICs of the reference strains and all susceptible clinical isolates, MICs of reference strains were 2 μg/ml and those of all susceptible clinical isolates were ≤ 2 μg/ml (Table 2).

DISCUSSION

M. avium is the important pathogen in patients infected with HIV (1). Although the incidence of tuberculosis is decreasing, the incidence of NTM infection is increasing

TABLE 2. Nucleotide Sequence at the Position 2,058 and 2,059 in 23S rRNA Gene and MICs for Clarithromycin

Strains	Positions (*E. coli* equivalent base number)		Clarithromycin MIC (μg/ml)
	2058	2059	
Reference strains			
M. avium ATCC 25291	A	A	2
M. avium ATCC 700898	A	A	2
Clinical susceptible isolates	A	A	≤2
Clinical resistant isolates			
MA23	**T**	A	256
MA223	A	**C**	256
MA251	A	A	128

in Korea. Because the macrolides has been effective agent against MAC infection (2), the problems of the macrolide resistance have occurred (3–5). Many reports have shown the results about drug susceptibility testing and the mechanisms for macrolide resistance (6–10).

We developed the method for RHA in order to detect the clarithromycin resistant strains with point mutations and tried to isolate the resistant strains from *M. avium* clinical isolates. RHA is a rapid and inexpensive method to easily detect resistant strains that have point mutations. Reports from other countries have shown point mutations in the 23S rRNA gene conferring clarithromycin resistance. We confirmed the usefulness of this method by comparing the results of RHA with the sequencing analyses in the domain V of the 23S rRNA gene from all tested *M. abscessus* clinical isolates (15). In this study, we isolated two resistant strains from the total 274 clinical isolates by RHA, and the sequencing analyses for point mutations in 23S rRNA gene showed the different transitional patterns with high level of MIC (256 μg/ml). Especially, isolate MA23 showed the transition of A to T at position 2058 with high level of MIC (256 μg/ml). Nash et al. reported that they isolated the resistant strain which showed the transition of A to T at position 2058 with a low level of MIC (32 μg/ml) from patients after 10 months of therapy (4). Also, Jamal et al. reported that they isolated the resistant strain which showed the transition of A to T at position 2058 with low level of MIC (50 μg/ml) from clinical isolates (16). It has been suggested that MIC values of mutants from clinical isolates or in vitro generated mutants with the transition of A to T were lower than those of mutants with the transition of A to G or C; therefore, transition of A to G or C at position 2058 or 2059 conferred high-level resistance because of the difference of binding affinity between antibiotics and 23S rRNA (4,16). However, isolate MA23 in this study showed very high level of MIC, unlike MICs of the previously reported resistant strains with the transition of A→T.

Many reports had tested in vitro-generated mutants or the resistant strains after treatment, because resistance is inducible and their MICs showed high level of resistance (4,16,17). Most of all tested isolates in this study were thought to be primary strains because they were requested Korean Institute of Tuberculosis for the identification of NTM from private hospitals or institutes. Therefore, it is likely that the resistant strains were comprised of low proportion (3/274, 1.1%) in clinical isolates. We confirmed that isolate MA223 is the resistant strain from patient who had received clarithromycin therapy and isolate MA251 is from patient who had not received any. But, isolate MA23 was not able to confirm whether it is from that patient who had contact with clarithromycin or other macrolides previously concerned with *M. avium* infection or not.

In case of the resistant strains without mutations, it has been reported that the clinical resistant isolates without mutations showed low level of MICs (50 μg/ml) (16), but isolate MA251, a resistant strain without mutations from patient who had not received macrolide therapy, showed high level of MIC (128 μg/ml), the same as Nash et al. who reported an isolate from patient with the treatment (4). We did not investigate other resistance mechanisms except for 23S rRNA gene mutation. So, this result is suggested further investigation about the alternative mechanisms (for example, ATP-binding cassette transport systems or efflux mechanism) of macrolide resistance.

In conclusion, we confirmed the nonreported characteristics for the clarithromycin resistance in clinical *M. avium*, owing to the facts that the existence of *M. avium* clinical isolate which do not have correlation between MIC level and base selection (A→T) for substitution in the clarithromycin resistance and an high-level resistant isolate without point mutation in 23S rRNA gene from a patient with no treatment history of macrolide.

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