Journal of Antimicrobial Chemotherapy (2002) **49**, 679–681

JAC

Comparison of phenotypic and genotypic methods for the detection of clarithromycin resistance in *Mycobacterium avium*

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The MICs of clarithromycin for 10 clinical isolates of *Mycobacterium avium* were determined using three methods: Bactec 460-TB, broth microdilution and Etest. The results were compared with the presence of resistance mutations in the 23S rRNA gene. Isolates were obtained from five AIDS patients who were treated with clarithromycin. Five isolates were recovered before and five during treatment. MICs were reproducible and comparable between the three methods. They were ≤4 mg/L for pre-treatment isolates and ≥128 mg/L for strains recovered during treatment. An MIC ≥ 128 mg/L was associated with the presence of mutations in the 23S rRNA gene that were absent in the isolates exhibiting MIC ≤ 4 mg/L.

Introduction

Mycobacterium avium is an important pathogen, particularly in immunocompromised patients.[1] Owing to the HIV pandemic, there has been a considerable increase in the frequency of *M. avium* infections in recent years.[2] Macrolides such as clarithromycin constitute the cornerstone of treatment for, and prophylaxis against, these infections. However, resistance to these antibiotics is known to emerge in patients receiving macrolide therapy.[1] In the clinical mycobacteriology laboratory the detection of resistance is restricted by the fact that the methods available are expensive, inadequately standardized and of low reproducibility. As a result, generally recognized breakpoints have not yet been published.[3]

The aim of this study was to assess the phenotypic reproducibility and comparability of three quantitative susceptibility testing methods for the detection of clarithromycin resistance in *M. avium* isolates and to compare these with the detection of resistance mutations within the 23S rRNA gene.

Materials and methods

Ten clinical isolates of *M. avium* recovered from five AIDS patients were studied. From each patient, one isolate was obtained before the start of anti-mycobacterial therapy, and one follow-up isolate was recovered during the treatment with a clarithromycin-containing drug regimen. Two strain pairs had been described earlier and were included for reference.[4]

MICs were determined in triplicate. The methods used were Bactec 460-TB (Becton Dickinson, Franklin Lakes, NJ, USA), broth microdilution assay and Etest (AB Biodisk, Solna, Sweden).[5–7]

Clarithromycin was kindly provided by the manufacturer (Abbott Laboratories, Cham, Switzerland). A stock solution of 2048 mg/L clarithromycin dissolved in methanol was prepared according to the manufacturer's instructions.

Serial two-fold dilutions of the stock solution were prepared with 0.1 mol/L phosphate buffer pH 6.5. Dilutions ranged from 256 to 0.25 mg/L (Bactec) and from 512 to 0.125 mg/L (broth microdilution). The Etest strips cover a clarithromycin gradient from 256 to 0.016 mg/L.

Bactec 460-TB

The test strains were inoculated into a Bactec 12B vial and incubated until they attained a growth index (GI) of 999. The strains were then diluted 1:100 in sterile distilled water. Bacterial density was specified as 10^4–10^5 cfu/mL. A control vial and the vials containing clarithromycin were each inoculated with 0.1 mL of this bacterial suspension diluted 1:100. The MIC was defined as the lowest drug concentra-

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tion that inhibited >99% of the bacterial population within 8 days of culture, as described by Heifets.[5]

Broth microdilution assay

Each strain was inoculated in 4 mL of 7H9SF broth (Difco Laboratories, Detroit, MI, USA), and incubated at 35°C for 7 days. On the day before the MIC test was performed, this culture was diluted in 1:20 fresh 7H9SF broth, and incubated overnight at 35°C. The following day this diluted culture was mixed by tilting it 10 times, and then diluted 1:50 in 7H9SF broth. This produced a concentration of $\sim 10^6$ cfu/mL. A clarithromycin-containing microtitre plate was inoculated with 100 μL of the 1:50 diluted bacterial suspension. The microtitre plates were sealed in a plastic bag and incubated aerobically at 35°C. Readings were made after 2, 3, 4, 6 and 7 days of incubation. The MIC was taken to be the lowest antibiotic concentration at which no growth could be determined by visual inspection.[6]

Etest

Mueller–Hinton agar plates enriched with 10% OADC (Difco Laboratories) were used for the Etest. Each of the plates was inoculated with 100 μL of the same bacterial suspension (1:50 dilution) employed for the broth microdilution assay according to the manufacturer's instructions. After pre-incubation for 18 h at 35°C in 5% CO_2, the Etest strips were applied. The plates were then sealed in a plastic bag (permeable to CO_2), and incubated at 35°C in 5% CO_2. The Etest strips used gave rise to a gradient representing a range of 0.016–256 mg/L of clarithromycin. After a 7 day incubation period, the MIC was read according to the manufacturer's instructions.[7]

In all three phenotypic methods *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used for quality control purposes.[5,6]

Detection of resistance mutations within the 23S rRNA gene

In order to define mutations conferring resistance to macrolides, the partial sequence of the 23S rRNA gene was determined in all isolates. For this purpose mycobacterial DNA was extracted as described previously.[8] The sequencing of the 23S rRNA gene was performed according to the protocol described by Kirschner & Böttger.[9]

Results

The reproducibility of the phenotypic methods was good. In the radiometric method and the broth microdilution assay, 29/29 replicates were within ±1 dilution step from the modal MIC; in the Etest 56/59 replicates were within ±1 dilution step and 3/59 were within ±2 dilution steps (Etest dilution step).

All strains recovered prior to anti-mycobacterial therapy had a wild-type genotype, i.e. no mutation was detected within the 23S rRNA gene, and the respective MICs were ⩽4 mg/L by either the radiometric method (range 0.5–4 mg/L), the broth microdilution assay (range 1–4 mg/L) or the Etest (range 0.094–4 mg/L). The follow-up isolates recovered during treatment had one of the following mutations within the 23S rRNA gene: 2058 A→C/G (n = 3) or 2059 A→C (n = 2). The respective MICs were ⩾128 mg/L by all three phenotypic methods (Table).

One strain, 271659, was considered to be resistant by all three phenotypic assays. However, by initial DNA sequencing, the isolate was the wild-type, i.e. no mutation was detected. The isolate was subcultured in a quantitative manner on Middlebrook 7H11 agar (Difco Laboratories), with or without 64 mg/L clarithromycin. Only 0.1% of the bacterial population were resistant to clarithromycin. In the case of the other resistant strains, values between 3.5% and 66% were found. Sequence analysis of the clarithromycin-resistant sub-population showed an A→G base substitution at position 2058 in the 23S rRNA gene.

Discussion

M. avium is the cause of one of the most common opportunistic bacterial infections in patients infected with HIV. The emergence of resistance during clarithromycin therapy and/or prophylaxis poses a therapeutic problem. Ideally, treatment should be given on the basis of the *in vitro* determination of resistance. This requires a rapid and standardized method. Nash & Inderlied[10] assumed a breakpoint for the clarithromycin resistance of *M. avium* of ⩾32 mg/L. Lebrun *et al.*[7] used a breakpoint of ⩾64 mg/L. In the present work all strains found to carry a point mutation associated with clarithromycin resistance had an MIC ⩾ 128 mg/L as demonstrated by the three different methods. With the exception of one single isolate, an MIC of ⩽4 mg/L was identified in all wild-type isolates investigated. This exception exhibited an MIC ⩾ 128 mg/L, although initially no mutation was detected in the 23S rRNA gene. In the case of this strain, growth was delayed in the Bactec method and in microdilution. Furthermore, two populations were apparent in the Etest. This strain had a critical proportion of only 0.1%. Nash & Inderlied[10] have shown that *Taq* DNA polymerase-based sequencing can produce false-negative results if the critical proportion is <20%. Therefore, the initial discrepancy could be due to the relative insensitivity of DNA polymerase-based sequencing in the presence of a very low critical proportion.

The present work has demonstrated that mutation-associated resistance to clarithromycin in *M. avium* may be detected with each of the three phenotypic methods evaluated. Thus, additional considerations such as resources, in terms of personnel and facilities, as well as turnaround time

Table. Results of sequencing the 23S rRNA gene, and the phenotypic determination of MIC

Strain pairs	Strain	MIC (mg/L)			23S rRNA mutation
		Bactec	microdilution	Etest	
A	171428	2	4	1.5–3	wild type
	256979	>256	>256	>256	2059→C
B	191045	1	2–4	0.5–0.75	wild type
	271659	128	256	>256	2058→G
C	228753	2	2–4	1.5–3	wild type
	280141	>256	⩾256	>256	2059→C
D[a]	W6165	2–4	2–4	1.5–2	wild type
	W14482	>256	>256	>256	2058→C
E[a]	W4697	0.5	1–2	0.094–0.19	wild type
	H3291	>256	>256	>256	2058RC

[a]Heifets *et al.*[4]

and cost of a given assay will guide the choice of method for the detection of clarithromycin-resistant *M. avium*. While access to sequencing facilities may generally be limited, each of the three phenotypic assays evaluated can be introduced to any diagnostic mycobacteriology laboratory. In our hands, the turnaround times for the unambiguous identification of resistance were 7, 8 and 8 days, respectively, for the Etest, broth microdilution assay and radiometric method. The radiometric method proved to be the most expensive, costing an estimated €52 per test, as compared with €23 and €10 per test for the broth microdilution method and Etest, respectively.

On the basis of these considerations the implementation of Etest for the detection of mutation-associated resistance to clarithromycin in *M. avium* may prove to be a good choice for many diagnostic mycobacteriology laboratories, because of the ease of use and cost-effectiveness.

Acknowledgements

The authors gratefully acknowledge the technical support of Martina Scholkmann, Andrea Aebischer, Sabine Michel and Jasmine Portmann, and wish to thank W. John Looney for critical reading of the manuscript. This work was supported in part by an educational grant from the Division of Bacteriology of the Institute for Infectious Diseases of the University of Bern.

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Received 25 July 2001; returned 8 October 2001; revised 23 November 2001; accepted 17 December 2001