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Resazurin Microtiter Assay for Clarithromycin Susceptibility Testing of Clinical Isolates of *Mycobacterium abscessus* Group

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Background: Mycobacterium abscessus group has heterogeneous susceptibility pattern among species. The species is most common cause of nosocomial infections. Macrolides minimum Inhibitory concentration (MIC) determination is essential for the treatment. *Methods:* Thirty-six strains were randomly selected for performing Resazurin Microtiter Assay (REMA) for clarithromycin testing in comparison to MIC test according to Clinical and Laboratory Standards Institute (2011) recommendation. REMA has been used for detection of drug resistance in *M. tuberculosis.* Extended incubation was performed to detect induced resistance. *Results:* Thirty microliters of resazurin (0.01%) was added after visually taking MIC reading. Resistance was observed in 11.1% of *M. bolletti* and 4.8% of *M. abscessus* strains; and induced resistance was detected in 77.8% and 95.2% of *M. bolletti and M. abscessus strains*, respectively. All strains of *M. massiliense* were susceptible. The samples presented same MIC value both by visual reading and through resazurin. *Conclusion:* The present study showed 100% concordance between both readings, with REMA providing easier to read and report results benefit. This change in reading can also reflect on the MIC determination and report, improving the test. J. Clin. Lab. Anal. **30**:751–755, 2016. © 2016 Wiley Periodicals, Inc.

Key words: clarithromycin; *Mycobacterium abscessus*; resazurin microtiter assay; susceptibility testing

INTRODUCTION

Mycobacterium abscessus group is one of the "rapidly growing mycobacteria" (RGM) groups most frequently isolated from pulmonary infections. The group comprises very closely related species, but with a controversial species classification. As a matter of fact, they are named *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, the latter encompassed the old classification of *M. massiliense* and *M. bolletii* (1). This species is also associated with nosocomial infections (2–9), because of the environmental factor and often opportunistic nature of this species. These organisms are very resistant *in vitro* to most chemotherapeutic agents, leading to treatment failure (10–12). Clarithromycin and azithromycin are the main drugs recommended by the American Thoracic Society/Infectious Diseases Society of America, as part of a treatment regimen of multiple drugs (13). However, induction of the *erm*(41) gene, responsible for inducible macrolide resistance, is greater after exposure to clarithromycin compared to azithromycin (14).

It is necessary to perform drug susceptibility testing to choose the appropriate treatment (10, 15) as *M. abscessus* group isolates have variable susceptibility to clarithromycin. In the present study, the old classification is maintained because of the different susceptibility features of the group. The Clinical and Laboratory Standards

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Institute (CLSI) recommends the Minimum Inhibitory Concentration (MIC) determination for clinically significant isolates, and for those recovered from immunocompromised patients such as HIV-positive patients and people with malignances (13, 16). For correct MIC reading, results interpretation requires an experienced professional who understands the different characteristics among the mycobacteria species. The use of dyes, such as resazurin, can make the visual reading easier and more accurate. Resazurin is an oxidation-reduction indicator used for the evaluation of cell proliferation and bacterial growth (17). Resazurin Microtiter Assay (REMA) was recommended in 2009 by the World Health Organization for testing susceptibility in *M. tuberculosis* (18). Ramis et al. (19) for the first time described the use of resazurin as a good alternative method for MIC determination in *Mycobacterium* species, but the detection of inducible resistance by REMA was not verified. In order to fulfill this issue, we aimed to evaluate the performance of resazurin in a determining routine MIC of clarithromycin on *M. abscessus* group strains from a reference laboratory in Brazil.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

During January 2010 to December 2011, we selected all *M. abscessus* group isolates sent to the Tuberculosis and Mycobacteriosis Branch at Adolfo Lutz Institute, Brazil, a reference laboratory. The 21 *M. abscessus*, nine *M. bolletii*, and six *M. massiliense* strains were originally identified by PRA-*hsp*65 method (20) and *rpo*B gene sequencing (21). The strains were cultivated on Lowenstein Jensen and transferred to grow for 3 days on Mueller Hinton cation adjusted broth (MHC) before clarithromycin testing.

Chemicals

Clarithromycin (Sigma-Aldrich) was prepared in dimethyl sulphoxide to obtain 10 μg/ml stock solutions and stored at –20ºC. Resazurin (Sigma-Aldrich) was prepared at 0.01% concentration in sterile distilled water.

Clarithromycin Susceptibility Testing With Resazurin

The MIC test was performed according to CLSI recommendation and with extended incubation for 14 days to detect *M. abscessus* induced resistance. The same operator performed tests and readings, but readings were performed without previously known sample identification (blind reading). Briefly, 100 μl MHC was dispensed in each well of a sterile 96-well plate, and serial twofold dilutions of clarithromycin were prepared directly on the plate by adding 100 μl of the working solution of drug to achieve the final concentration. The clarithromycin concentration range used was 0.5–64 μg/ml. The inoculum was prepared from the MHC growth, adjusted to a McFarland tube number 0.5. The suspension was diluted 1:100 and 100 μl was added to each well. Control wells were prepared with medium culture only (negative control) and bacterial suspension only (positive control). The plates were covered, sealed in plastic bags, and incubated for 14 days at 37ºC in the normal atmosphere. Visual readings were taken on days 3, 5, 7, 10, and 14. After the final visual reading, 30 μl of 0.01% resazurin was added to each well and reincubated overnight. A change in color from blue (oxidized state) to pink (reduced) indicated bacterial growth and MIC was defined as the lowest drug concentration that prevented the color change. If positive control maintains blue color, the plate was discharged. *Staphylococcus aureus* ATCC 29213 was used as control of the susceptibility test, according to CLSI recommendation.

Isolates were considered susceptible when the MICs were less than or equal to 2 μg/ml and resistant when MIC has higher values. Isolates with intermediate MICs were also considered resistant. Susceptible isolates at day 3 and resistant after day 5 were considered induced resistant.

Statistical Analysis

Sensitivity, specificity, and confidence intervals were calculated using Epi Info version 6.0.

Ethics

The present study was approved by the Research Ethical Committee of IAL (CEPIAL process n. 032/2011).

RESULTS

Thirty-six strains of *M. abscessus* group had their clarithromycin susceptibility evaluated (Table 1). Twenty *M. abscessus* strains presented induced resistance and one presented resistance since the third day. Seven *M. bolletii* strains presented induced resistance, one was susceptible, and one resistant. All *M. massiliense* strains were susceptible to clarithromycin.

We observed that all samples (100%) presented the same MIC value by visual reading and after the use of resazurin (Table 1). The reading of the standard strain used as a positive control had no change in the MIC value obtained in visual readings and through resazurin. CLSI recommends that the MICs of this strain, for clarithromycin test, should range between 0.12 and 0.5 μg/ml after 3 days of

TABLE 1. Evaluation of Clarithromycin Susceptibility Test, According CLSI Protocol, Through the Use of Resazurin, Comparing the Minimum Inhibitory Concentration (MIC) Values Read Visually (Days 3 and 14) With the MIC Values Obtained After Using the Resazurin Dye After 14 Days of Incubation

			MIC		
Species	Samples	Type of susceptibility	Day 3 (μg/ml)	Day 14 (μg/ml)	Resazurin (μg/ml)
M. abscessus	1	IR	≤0.5	>64	>64
M. abscessus	2	IR	≤0.5	>64	>64
M. abscessus	3	R	4	>64	>64
M. abscessus	4	IR	≤0.5	>64	>64
M. abscessus	5	IR	≤0.5	>64	>64
M. abscessus	6	IR	≤0.5	>64	>64
M. abscessus	7	IR	1	>64	>64
M. abscessus	8	IR	≤0.5	>64	>64
M. abscessus	9	IR	≤0.5	>64	>64
M. abscessus	10	IR	≤0.5	>64	>64
M. abscessus	11	IR	≤0.5	>64	>64
M. abscessus	12	IR	≤0.5	>64	>64
M. abscessus	13	IR	≤0.5	>64	>64
M. abscessus	14	IR	≤0.5	>64	>64
M. abscessus	15	IR	≤0.5	>64	>64
M. abscessus	16	IR	≤0.5	>64	>64
M. abscessus	17	IR	≤0.5	8	8
M. abscessus	18	IR	≤0.5	8	8
M. abscessus	19	IR	≤0.5	32	32
M. abscessus	20	IR	≤0.5	32	32
M. abscessus	21	IR	≤0.5	32	32
M. bolletii	22	IR	≤0.5	>64	>64
M. bolletii	23	S	≤0.5	≤0.5	≤0.5
M. bolletii	24	IR	≤0.5	>64	>64
M. bolletii	25	R	4	>64	>64
M. bolletii	26	IR	≤0.5	>64	>64
M. bolletii	27	IR	1	>64	>64
M. bolletii	28	IR	≤0.5	>64	>64
M. bolletii	29	IR	≤0.5	>64	>64
M. bolletii	30	IR	≤0.5	>64	>64
M. massiliense	31	S	≤0.5	≤0.5	≤0.5
M. massiliense	32	S	≤0.5	≤0.5	≤0.5
M. massiliense	33	S	≤0.5	≤0.5	≤0.5
M. massiliense	34	S	≤0.5	≤0.5	≤0.5
M. massiliense	35	S	≤0.5	≤0.5	≤0.5
M. massiliense	36	S	≤0.5	4	4
S. aureus	PC	–	≤0.5	≤0.5	≤0.5

S, susceptible; IR, inducible resistant; R, resistant; PC, positive control.

incubation, which occurred in the initial test of the control strain (0.5 μg/ml).

Sensitivity and specificity were 100.0% for resazurin reading (95% confidence interval 85.9–100.0% for sensitivity and 51.7–100.0% for specificity), when compared to visual reading.

DISCUSSION

The present study evidenced that use of resazurin does not affect the MIC values, but makes it more evident to read. Besides, we verified high prevalence of clarithromycin-induced resistance in *M. abscessus* group. The infections caused by *M. abscessus* are very difficult to be treated, mainly due to the acquired resistance of those organisms (22). Clarithromycin is a very important drug in the treatment of these infections; however, several countries have reported the occurrence of induced resistance. Therefore, it is necessary to conduct a clarithromycin susceptibility testing so that a physician can choose the best regimen for the patient (8, 9, 22–24)

In this study, MIC was performed according to the CLSI recommendation and with extended incubation to identify a possible induced resistance. This type of

resistance can solely be detected when there is an extended incubation period, as it is expressed in the presence of the drug. We observed that most *M. abscessus* and *M. bolletii* isolates presented induced resistance and that all *M. massiliense* isolates were susceptible to clarithromycin. These results are in agreement with those found in studies by Nash et al., Bastian et al., Maurer et al., Shallom et al., and Lee et al., all of these studies used the same methodology for the detection of induced resistance. The susceptibility test with clarithromycin extended for 14 days incubation allowed to identify the true susceptibility profile of the isolates, since 78.5% of isolates would be considered susceptible to clarithromycin, were resistant to this drug. However, visual reading can be difficult to estimate. Sometimes, a slight precipitate related to the inoculum may be confounded with a true growth, which only an experienced analyst could differentiate.

For an accurate reading, the use of resazurin was established in the present study. Previous studies have shown the use of REMA against *M. tuberculosis*, and concluded that this technique has high specificity and sensitivity, in addition to being simple, practical and with no requirement of sophisticated equipment, enabling deployment in laboratories with limited resources (25–27).

The evaluation of the use of resazurin showed a concordance of 100% between the visual and resazurin readings, presenting high specificity and sensitivity. Using resazurin in the susceptibility test for *M. tuberculosis*, Martin et al. (28) verified the robustness of the REMA with visual reading. Castilho et al. (29) tested the use of resazurin to observe MIC of linezolid and ciprofloxacin for RGM and observed good agreement with standard test.

The visual reading may not be accurate, especially when bacterial growth is very thin, making it difficult to read visually. In addition, previous validation of resazurin in MIC determination alerts for the importance of clarithromycin induced resistance in RGM (19), which is the aim of the present study. Based on this test, we verified the accuracy of resazurin reading in clarithromycin susceptibility testing, which minimizes possible reading errors enabling an accurate reading of the susceptibility testing. It also opens opportunities for the development of automated reading protocols due to the fluorescence issued.

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CONFLICT OF INTEREST

None.

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