ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2001, p. 1934–1936 Vol. 45, No. 6
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.6.1934–1936.2001

Kanamycin Susceptibility Testing of *Mycobacterium tuberculosis* Using Mycobacterium Growth Indicator Tube and a Colorimetric Method

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Received 3 November 2000/Returned for modification 18 December 2000/Accepted 16 March 2001

Two novel systems were evaluated for performing indirect kanamycin susceptibility tests on 72 strains of *Mycobacterium tuberculosis*. The microplate Alamar blue colorimetric method (breakpoint, 2.5 μg/ml) and the Mycobacterium Growth Indicator Tube (MGIT) system (breakpoint, 5.0 μg/ml) both produced 98.6% agreement when compared with the conventional proportion method performed on 7H10 agar using 5.0 μg of kanamycin/ml. Both systems provided results within an average of 1 week.

Multidrug-resistant tuberculosis (MDRTB), defined by isolates of *Mycobacterium tuberculosis* resistant to isoniazid and rifampin, is a significant public health problem in several countries, particularly those that comprised the former USSR (3, 6, 7, 15). Laboratories supporting TB control programs in populations of these countries with endemic MDRTB are increasingly required to provide rapid, reliable drug susceptibility testing (DST) not only for the first-line drugs (i.e., isoniazid, rifampin, ethambutol, and streptomycin) but also for second-line agents (e.g., kanamycin and the quinolones) (3, 14). Unfortunately, the protocols for performing second-line DST are not standardized and the recommended critical concentrations for the various media are often based on scattered small-scale studies (1, 10). Collaborative efforts have already attempted to optimize the methods for performing second-line DST on solid media and by the radiometric BACTEC method (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) (18). This study continues this process by evaluating the microplate Alamar blue (MAB) colorimetric method (8, 16, 21) and the Mycobacterium Growth Indicator Tube (MGIT) system (4, 17, 19) for performing kanamycin susceptibility tests.

A panel of *M. tuberculosis* strains was compiled comprising 68 clinical isolates sent to the World Health Organization (WHO) Supranational Reference Laboratory in Antwerp, Belgium, from Azerbaijan (n = 42), Siberia, Russia (n = 16), Georgia (n = 8), and Kazakhstan (n = 2) and four reference strains, ATCC 35826, ATCC 35827, ATCC 35828, and ATCC 35830 (all from American Type Culture Collection, Manassas, Va.), with known monoresistance to cycloserine, kanamycin, pyrazinamide, and ethionamide, respectively. The clinical isolates had been characterized as kanamycin resistant or susceptible by using the conventional proportion method (5), Middlebrook 7H10 agar, and recommended parameters (e.g., a critical proportion of 1% and a critical concentration of 5.0 μg/ml) (11, 13, 18). The panel was maintained on Löwenstein-Jensen medium and freshly subcultured in 7H9-S broth (Middlebrook 7H9 supplemented with 0.1% Casitone, 0.5% glycerol, and oleic acid, albumin, dextrose and catalase [OADC; Becton Dickinson Microbiology Systems]) prior to evaluation. A stock solution of kanamycin (1 mg/ml) was prepared from chemically pure powder (Roche, Brussels, Belgium), filter sterilized, and kept in aliquots at −20°C until use.

For the MAB procedure, 0.1 ml of 7H9-S broth containing serial dilutions of kanamycin (to provide final drug concentrations of 0.5 to 20 μg/ml) was dispensed into the wells of a 96-well flat-bottom microtiter plate (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J.). Inocula were prepared from 5- to 7-day-old 7H9-S broth cultures, the turbidity was adjusted to a 0.5 McFarland standard, and 0.1 ml of a 1:5 dilution was added to the test wells. A growth control well containing no antibiotic and a sterile control well were also prepared for each specimen. The plate was covered, sealed in a polyethylene bag, and incubated at 37°C in normal atmosphere. After 6 or 7 days, 20 μl of the Alamar blue indicator (10× sterile solution) and 20 μl of 10% Tween 20 were added separately to all wells and the plate was reincubated overnight. Mycobacterial growth was indicated by a blue-to-pink color change. The MIC was defined as the lowest concentration of kanamycin that prevented a color change.

For the MGIT DST, an inoculum was prepared as described for the MAB assay or by vortexing a 1- to 3-day-old positive MGIT tube for 10 s and pipetting 1 ml of the medium into 4 ml of sterile saline (1:5 dilution) (19). Aliquots (0.5 ml) of this inoculum were added to two MGIT tubes that had been supplemented with 0.5 ml of OADC; one tube also contained kanamycin at a final concentration of 5.0 μg/ml. The tubes were tightly capped and incubated in normal atmosphere at 37°C. Starting on day 3 after inoculation, the tubes were examined daily using a 365-nm UV transilluminator as previously described (4, 17, 19). An isolate was considered susceptible if the drug-containing tube did not fluoresce within 2 days of the drug-free tube; conversely, if the drug-containing tube fluoresced before or within 2 days of the drug-free tube, the strain was defined as resistant. By including a series of tubes containing dilutions of kanamycin between 1 and 20 μg/ml, the MGIT system was also used to perform kanamycin MIC determinations in the initial stages of the study and for specimens producing discordant results. The MIC was defined as the lowest

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TABLE 1. Distribution of MICs of kanamycin by MAB assay for panel of 72 *M. tuberculosis* strains[a]

Result by proportion method	No. of strains for which the MIC of kanamycin (μg/ml) was:					
	≤0.5	1.0	2.5	5.0	10	≥20
Susceptible (n = 37)	15	13	8	1		
Resistant (n = 35)				2	9	24

[a] In this study, a breakpoint of 2.5 μg/ml provided the best performance characteristics for performing kanamycin susceptibility tests using the MAB method: sensitivity, 100% (95% CI, 90.0 to 100%); specificity, 97.3% (95% CI, 85.8 to 99.9%); predictive value for resistance, 97.2% (95% CI, 85.5 to 99.9%); predictive value for susceptibility, 100% (95% CI, 90.3 to 100%); accuracy, 98.6% (95% CI, 92.5 to 100%).

concentration of kanamycin that prevented the drug-containing tube from fluorescing within 2 days of the drug-free tube.

The MAB and MGIT assays were performed in parallel, and the results read visually by one observer (I.B.). In both assays, the tests were compared with growth and sterile controls; only those tests with color development or fluorescence equivalent to that of the growth control were considered positive. For strains producing results discordant with the proportion method, the discordant assay was repeated, as was the proportion method test on 7H10 and 7H11 Middlebrook agar (10, 12, 18).

Previous studies have reported that wild-type kanamycin-susceptible *M. tuberculosis* strains require MICs of 3 μg/ml or less when tested on 7H11 agar or in BACTEC 7H12 broth (1, 10). The MAB method using 7H9-S broth yielded similar results (Table 1). Thirty-six of 37 kanamycin-susceptible strains required MICs of 2.5 μg/ml or less while all 35 kanamycin-resistant strains required MICs of 5.0 μg/ml or greater. For these 72 *M. tuberculosis* strains, the greatest concordance (98.6%) between the MAB and conventional proportion methods was achieved when a breakpoint concentration of 2.5 μg/ml was used in the MAB assay.

As an initial step in evaluating kanamycin DST by MGIT, this study used the MGIT system to determine the MICs for eight kanamycin-susceptible strains (five clinical isolates and three ATCC strains) and four kanamycin-resistant strains (three clinical isolates and one ATCC strain). All eight susceptible strains required MICs of 2.5 μg/ml or less, while the MICs for the four resistant strains were greater than 20 μg/ml. A critical concentration of 5 μg/ml was therefore chosen as the breakpoint for evaluating kanamycin DST by MGIT. This breakpoint was also considered justifiable because 5 μg/ml is the recommended critical concentration for kanamycin susceptibility testing in several media (e.g., BACTEC 7H12 broth) (11, 13, 18).

Using this breakpoint concentration, the initial kanamycin DST by MGIT produced three discordant results compared with the conventional proportion method when testing the panel of 72 *M. tuberculosis* isolates. One strain (98-1825) was confirmed as "false resistant" by MGIT, requiring a MIC of ≤2.5 μg/ml by MAB but a MIC of >5.0 μg/ml by MGIT on repeated occasions; supplemental testing by the conventional proportion method on 7H10 and 7H11 agars verified that this isolate was truly kanamycin susceptible. However, two other isolates that were repeatedly "susceptible" by MGIT were classified as "resistant" by the original proportion method tests, but supplemental investigations on 7H10 and 7H11 agars found that these two strains were kanamycin susceptible. Sequencing of the 16S rRNA gene supported the final classification of these two initially discordant strains as kanamycin susceptible (data not shown), finding no mutations commonly associated with kanamycin resistance (i.e., at positions 1400, 1401, and 1483) (20). The ultimate concordance of the MGIT system with the proportion method was therefore 98.6%. The other performance characteristics of the MGIT system were as follows: sensitivity, 100% (95% confidence interval [CI], 90.0 to 100%); specificity, 97.3% (95% CI, 85.8 to 99.9%); predictive value for resistance, 97.2% (95% CI, 85.5 to 99.9%); predictive value for susceptibility, 100% (95% CI, 90.3 to 100%). The mean turnaround time for these indirect kanamycin DSTs by MGIT was 5.1 days (range, 3 to 14).

MDRTB strains often demonstrate resistance to other first-line drugs and occasionally to second-line agents, such as kanamycin. For example, studies of MDRTB strains from two prisons in areas of the former USSR have reported that 97.1% are streptomycin resistant (6) and 15.5% are kanamycin resistant (3). Considering the widespread and uncontrolled use of kanamycin in areas of the former USSR (7), this high rate of resistance is not surprising but is alarming. A quinolone and a cheap injectable agent, such as kanamycin, form the basis of effective treatment regimens for MDRTB (2, 12), but kanamycin resistance produces cross-resistance with amikacin (1), leaving the expensive polypeptide capreomycin as the only effective injectable agent (2, 3, 12).

Laboratories supporting TB services in areas of the former USSR and other areas where MDRTB is endemic must therefore be able to provide prompt, reliable DSTs for kanamycin. Protocols have been described for performing kanamycin DST by the conventional proportion method on solid media and by BACTEC (1, 11, 13, 18). However, the conventional method requires 3 to 6 weeks for completion while BACTEC, which involves the use of radioisotopes and machinery, is a rapid but inappropriate technology for low-resource countries. In contrast, the manual MGIT system uses a fluorescence quenching-based oxygen sensor and can be read visually with a simple Wood's lamp. Previous studies from high-income countries have validated the system for first-line DST (4, 17, 19). The system is robust, safe, and simple and has been easily implemented in a TB laboratory in a Siberian prison hospital (9). This study has now demonstrated that the MGIT system can provide accurate indirect kanamycin susceptibility results within an average of 5.1 days. In fact, cost is the only factor prohibiting widespread application of the MGIT system.

The MAB method is a cheaper alternative that can also provide indirect DST results within 1 week without the need for expensive machinery. Studies in high- and low-income countries have validated the MAB method for first-line DST (8, 16, 21). Using the microplate format and selected critical concentrations, the reagent costs for performing susceptibility testing for first-line drugs and kanamycin could be less than $1 per isolate. However, the MAB method requires a greater level of technical expertise to provide reliable results. Furthermore, the microplates do not have a tight seal and may represent a biohazard unless handled carefully in the laboratory.

In summary, this study has demonstrated that the MGIT and MAB systems can provide indirect kanamycin DST results

within an average of 1 week and with 98.6% agreement with the conventional proportion method. While further studies with larger isolate collections are required to confirm the optimal breakpoint concentrations, these two systems appear to be appropriate techniques for performing rapid indirect kanamycin susceptibility tests in low-resource settings where MDRTB is endemic, where such tests are increasingly required.

These studies were funded by the Damien Foundation Belgium and the "Belgische Bond Tegen de Tuberculose." BD Biosciences-Europe provided the MGIT media. I.B. was supported by a Neil Hamilton Fairley Fellowship (987069) from Australia's National Health and Medical Research Council.

We thank our colleagues in the International Committee of the Red Cross, Médecins sans Frontières, and the Ministry of Justice in Siberia for their cooperation.

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