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Selection of Strains for Quality Assessment of the Disk Induction Method for Detection of Inducible Clindamycin Resistance in Staphylococci: a CLSI Collaborative Study

Adrian M. Zelazny,[1] Mary Jane Ferraro,[2] Anita Glennen,[3] Janet F. Hindler,[4] Linda M. Mann,[5] Susan Munro,[6] Patrick R. Murray,[1] L. Barth Reller,[7] Fred C. Tenover,[8] and James H. Jorgensen[9]*

Microbiology Service, Department of Laboratory Medicine, Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland 20892[1]; Microbiology Laboratory, Clinical Microbiology Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114[2]; Acute Disease Epidemiology, Minnesota Department of Health, Minneapolis, Minnesota 55414[3]; Department of Pathology and Laboratory Medicine, University of California at Los Angeles Medical Center, Los Angeles, California 90025[4]; Clinical Microbiology Laboratory, Baylor University Medical Center, Dallas, Texas 75246[5]; Clinical Microbiology Laboratory, Stanford University Medical School, Stanford, California 94305[6]; Clinical Microbiology Laboratory, Duke University Medical Center, Durham, North Carolina 27710[7]; Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[8]; and Department of Pathology, University of Texas Health Science Center, San Antonio, Texas 78229[9]

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A nine-laboratory collaborative study was conducted to select positive and negative quality assessment control strains for the detection of inducible clindamycin resistance in staphylococci. Four strains of *Staphylococcus aureus* were tested as unknowns on 10 different days in each laboratory using the recently recommended CLSI (formerly NCCLS) disk diffusion method and the inoculum purity control method. Strains contained either macrolide-lincosamide-streptogramin B (MLS_B) resistance genes encoded by *erm*(A) or *erm*(C) or a macrolide resistance efflux pump encoded by *msr*(A). Based upon the results of this study, strain UT 32 (now designated ATCC strain BAA-977) containing *erm*(A) is recommended as the positive control organism for inducible clindamycin resistance. Strain UT 25 (now designated ATCC BAA-976), which harbors the efflux pump encoded by *msr*(A), is recommended as the negative control organism.

Macrolide (e.g., erythromycin), lincosamide (e.g., clindamycin), and streptogramin (e.g., quinupristin-dalfopristin) antimicrobial agents (collectively MLS agents) are widely used in the treatment of staphylococcal infections. Macrolide resistance may be due to an active efflux mechanism encoded by *msr*(A) (conferring resistance to macrolides and type B streptogramins but not to clindamycin) or to a ribosomal target modification that affects the activities of macrolides, lincosamides, and type B streptogramins (MLS_B resistance). MLS_B resistance in staphylococci is usually encoded by *erm*(A) or *erm*(C) (2, 4, 5) and can be either constitutive or inducible (MLS_Bi). While constitutive resistance to clindamycin can be detected by standard susceptibility testing methods, inducible clindamycin resistance is not detected by standard broth- or agar-based susceptibility test methods (8, 10). Thus, standard susceptibility tests cannot reliably differentiate *msr*(A) resistance (clindamycin susceptible) and MLS_Bi resistance (inducibly clindamycin resistant).

Fiebelkorn et al. recently described a practical disk diffusion method for the detection of MLS_Bi strains (D-zone test) (3). It involves placing standard erythromycin and clindamycin disks in adjacent positions from 15 to 26 mm apart on a Mueller-Hinton agar plate when performing the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) disk diffusion test. Inducible resistance to clindamycin is manifested by a flattening or blunting of the clindamycin zone of inhibition adjacent to the erythromycin disk, giving a D shape to the zone of inhibited growth. The same authors have also shown that the D-zone test can be performed by placing erythromycin and clindamycin disks 15 mm from edge to edge in the heavy-inoculum area of standard blood agar plates used for purity checks with automated or broth-based susceptibility test systems (6). There is increasing interest in assessing the incidence of inducible clindamycin resistance in hospitals and the community (3, 9, 10). However, there are no standardized quality assessment strains for training and competency assessment purposes for the D-zone test.

The purpose of this multicenter study was to evaluate and select positive and negative control strains for the detection of inducible clindamycin resistance in *Staphylococcus* spp.

MATERIALS AND METHODS

Study sites. The study was conducted during November 2003 in nine laboratories: Baylor University Medical Center, the Centers for Disease Control and Prevention, Duke University Medical Center, Massachusetts General Hospital, Minnesota Department of Health Laboratory, National Institutes of Health Clinical Center, Stanford University Medical Center, University of California at Los Angeles Medical Center, and University of Texas Health Science Center at

* Corresponding author. Mailing address: Department of Pathology, The University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229. Phone: (210) 567-4088. Fax: (210) 567-2367. E-mail: jorgensen@uthscsa.edu.

TABLE 1. Summary of the D-zone test results

Center	% of correct results[a]											
	NCCLS disk diffusion								Purity-15			
	UT 25		UT 32		UT 751		UT 944		UT 25	UT 32	UT 751	UT 944
	15	26	15	26	15	26	15	26				
1	80	80	100	100	100	100	100	90	90	100	100	100
2	100	100	100	100	100	100	100	100	100	100	100	100
3	100	100	100	100	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100	100	100	100	100
6	100	100	100	100	100	100	100	100	100	100	100	100
7	100	100	100	100	100	100	100	100	100	100	100	100
8	100	100	100	60	100	60	100	60	100	100	100	100
9	100	100	100	100	100	100	100	100	100	100	100	100

[a] Expressed as percent correct results of tests for NCCLS-15, NCCLS-26, and the purity control method (purity-15).

San Antonio. Four of the laboratories used a commercial broth microdilution instrument (MicroScan; Dade-Behring, Inc., West Sacramento, CA) or the VITEK or VITEK 2 instrument (bioMerieux, Inc., Durham, NC). Three of the laboratories routinely employed disk diffusion testing of staphylococci, and two of the laboratories employed more than one of the above methods for their testing.

Organisms. Four strains of *Staphylococcus aureus* were evaluated: one clindamycin-susceptible strain with *msr*(A) (labeled UT 25) and three strains with inducible clindamycin resistance, including one strain with *erm*(A) (UT 32) and two strains with *erm*(C) (UT 751 and UT 944). Stability of the resistance traits was assessed in two of the participating laboratories (Centers for Disease Control and Prevention and University of Texas Health Science Center at San Antonio). This involved daily subcultures and performance of the D-zone test on each strain for 33 days.

Study design. Each of the four strains was tested on 10 different days by three methods: the standard CLSI disk diffusion method with the 15-μg erythromycin and 2-μg clindamycin disks placed at 15 mm (designated NCCLS-15) and 26 mm (NCCLS-26) apart on Mueller-Hinton agar (7) and the inoculum purity control method by placing the erythromycin and clindamycin disks on standard sheep blood agar plates 15 mm apart (purity-15) (6). For the inoculum purity control method, a standard 0.5 McFarland inoculum suspension or a further dilution (e.g., 1:10 or 1:20 in saline according to the standard protocol for the broth-based susceptibility testing method used by the participating laboratory) was used as the inoculum source. The two disks were placed in the initial inoculum area of expected confluent growth. Following 16 to 18 h of incubation at 35°C in ambient air, all plates were examined for evidence of inducible clindamycin resistance (flattening of the clindamycin zone adjacent to the erythromycin disk, or a "D-zone"). Any flattening of the clindamycin zone was considered a positive test reaction.

RESULTS AND DISCUSSION

The results for the four test strains of *S. aureus* were recorded for the nine participating laboratories. The expected results were negative D-zone tests for strain UT 25 and positive D-zone tests for strains UT 32, UT 751, and UT 944. A summary of the results expressed as the percentage of correct results/total number of tests is shown in Table 1. Seven of the nine participating laboratories observed the expected results with all tests performed with the four test strains. One laboratory (center 8) erroneously interpreted the NCCLS-26 results during the first 4 days of testing but then did obtain the expected results on the final 6 days of testing after a review of the results identified the errors. The errors involved failure to note a flattening of the inner zone around the clindamycin disk when the strain had the appearance of a double zone of inhibition. When the zone was examined carefully for any flattening in subsequent tests, all results were interpreted as positive. Three laboratories observed the expected results with all tests performed with the NCCLS-15 method but recorded that the results with two or more organisms were subtle when the NCCLS-26 method was used. One laboratory (center 1) observed erroneous results with strain UT 25, with 2 of 10 results using both the NCCLS-15 method and the NCCLS-26 method and 1 of 10 of the purity-15 tests; and with strain UT 944, with 1 of 10 results using the NCCLS-26 method. Center 1 also noted an inadequate inoculum density for the purity plate method when attempting to use a commercial direct inoculum wand (BBL Prompt System; Becton-Dickinson, Sparks, MD) to streak the purity plates prior to disk placement.

This study has assessed the potential utility of four strains for the purposes of training and competency assessment of laboratory staff in the performance of macrolide disk induction testing for detection of inducible clindamycin resistance in staphylococci. In fact, the results of this study illustrate the importance of using such strains when testing is first initiated. Those laboratories with prior experience in D-zone testing generally obtained very consistent results in accordance with the expected findings with each candidate strain. In contrast, some laboratories had difficulty in recognizing the more subtle D-zones when the disks were placed 26 mm apart on Mueller-Hinton agar. The 26-mm disk separation was proposed initially (3) because it provided the convenience of simply placing the disks in adjacent positions of a standard disk dispenser. Disk placement at a distance of 15 mm provides a more obvious indication of a positive D-zone but requires hand placement of the disks. None of the labs had difficulty recognizing the D-zones obtained when the disks were placed 15 mm apart on either Mueller-Hinton or sheep blood agar plates. However, this study illustrated the importance of using either the 0.5 McFarland suspension directly or only a modest dilution of that suspension: i.e., no more than a 1:20 dilution in order to avoid potential false-negative results. The BBL Prompt System wand should not be used to inoculate purity plates for D-zone determinations.

The stability of clindamycin resistance in the three strains with inducible resistance was assessed in two of the participating laboratories. A total of 32 tests were performed over a 33-day period. Stable resistance was observed for all tests with UT 32 [*erm*(A)]. Inducible clindamycin resistance was ob-

served for the first 16 tests with UT 751 [*erm*(C)]; however, this strain lost the inducible resistance and became clindamycin susceptible in subsequent tests. Inducible clindamycin resistance was observed with all tests with strain UT 944 [*erm*(C)], although the zone of inhibition between erythromycin and clindamycin was less distinct than with UT 32. For these reasons, UT 32 was selected as the best positive control strain for D-zone quality assessment testing. Following the study, UT 32 was deposited with the American Type Culture Collection (ATCC), and has received the catalogue number BAA-977. Strain UT 25 (now ATCC BAA-976) that contains *msr*(A) performed acceptably in this study and can be recommended as a D-zone negative control strain.

Failure to identify inducible clindamycin resistance may lead to clinical failure when clindamycin is used therapeutically (1, 10). On the other hand, the broad assumption of clindamycin resistance based on erythromycin resistance may prevent the use of clindamycin in cases where it would be effective therapy. Since the occurrence of inducible resistance to clindamycin varies widely by hospital and geographic region (9), it is important to perform the D-zone test when staphylococci appear by routine tests to be erythromycin resistant and clindamycin susceptible. ATCC BAA-977 and ATCC BAA-976 have been accepted by the CLSI as the positive and negative control strains, respectively, for D-zone quality assessment purposes.

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