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Influence of Disk Separation Distance on Accuracy of the Disk Approximation Test for Detection of Inducible Clindamycin Resistance in *Staphylococcus* spp.▽

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We undertook this study to assess the accuracy of the clindamycin-erythromycin disk approximation test (D-test) for detection of inducible clindamycin resistance in *Staphylococcus* spp. One hundred sixty-three *Staphylococcus aureus* and 68 coagulase-negative *Staphylococcus* (CoNS) spp. which were erythromycin nonsusceptible but clindamycin susceptible were tested using the D-test performed at both 15-mm and 22-mm disk separations and compared with genotyping as the "gold standard." The rate of inducible clindamycin resistance was 96.3% for *S. aureus* and 33.8% for CoNS spp. The sensitivities of the D-tests performed at 15 mm and 22 mm were 100% and 87.7%, respectively, and specificities were 100% for both. The use of 22-mm disk separation for the D-test to detect inducible clindamycin resistance results in an unacceptably high very major error rate (12.3%). All isolates with false-negative results harbored the *ermA* gene, and the majority were methicillin-resistant *Staphylococcus aureus*. False-negative results were associated with smaller clindamycin zone sizes and double-edged zones. We recommend using a disk separation distance of ≤15 mm. There is wide geographic variation in the rates of inducible clindamycin resistance, and each laboratory should determine the local rate before deciding whether to either perform the D-test routinely or else report that all erythromycin-resistant *S. aureus* isolates are also clindamycin resistant.

Acquired staphylococcal resistance to lincosamides such as clindamycin is largely mediated by ribosomal methylases encoded by one of several *erm* genes. These enzymes methylate the bacterial ribosome at the binding site for macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics, thus inhibiting antibiotic activity. Such resistance may be inducible ($iMLS_B$) or constitutive. Induction occurs in the presence of erythromycin but not clindamycin (16). However, if clindamycin is used for treatment of an isolate with $iMLS_B$ resistance, selection for a mutation in the macrolide-responsive promoter region upstream of the *erm* gene may occur, leading to constitutive clindamycin resistance and treatment failure (5, 16–18, 23, 32, 35). It is thus recommended by most experts that clindamycin therapy be avoided for *Staphylococcus* sp. isolates that display $iMLS_B$ resistance, despite a low clindamycin MIC (5, 16, 27, 32).

Macrolide resistance due to active efflux encoded by the *msrA* gene is also found in *Staphylococcus* spp. It results in resistance to macrolides and streptogramin B antibiotics, but not lincosamides (MS phenotype), and clindamycin is active against such isolates (16). The prevalence of this type of resistance shows great geographical variation but is generally less common than $iMLS_B$ resistance (9, 20, 29).

It is important for laboratories to distinguish between MS and $iMLS_B$ resistance before reporting an erythromycin-nonsusceptible *Staphylococcus* sp. isolate as clindamycin susceptible. However, the two are indistinguishable when routine broth microdilution (BMD) testing is used. They can be distinguished by the erythromycin-clindamycin disk approximation test or D-test. When an organism expressing $iMLS_B$ resistance is tested according to Clinical and Laboratory Standards Institute (CLSI) methods with a 15-μg erythromycin disk placed close to a 2-μg clindamycin disk, the zone of inhibition around the clindamycin disk is flattened to form a "D" shape (positive D-test), whereas in the MS phenotype, the clindamycin zone remains circular (7). It is not clear from the literature what distance between antibiotics disks is ideal for the D-test. A false-negative D-test will result in reporting an isolate as clindamycin susceptible when it should be reported as resistant (very major error), while a false-positive test will result in reporting an isolate as resistant when it should be reported as susceptible (major error).

The need to test each *Staphylococcus* sp. isolate for inducible clindamycin resistance depends on the local prevalence of the two phenotypes. If MS resistance is uncommon, many laboratories will not perform the D-test but simply report all erythromycin-resistant isolates as clindamycin resistant (31). There are few published data on the prevalence of the two phenotypes in Australia, but what information is available suggests the $iMLS_B$ phenotype predominates (25; S. Aurangabadkar, V. Sintchenko, and D. Rankin, Abstr. Natl. Conf. Aust. Soc. Microbiol., abstr. PP02.2, 2004).

The aims of this study were (i) to determine the accuracy of the D-test when disks were placed 15 mm apart manually or 22 mm apart using an automated dispenser and (ii) to establish

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TABLE 1. Primers used in multiplex PCRs, and corresponding oligonucleotide probes used in the reverse line blot assay

Gene	Primer: sequence	Probe: sequence
ermA	ermA/TRSb: 35690 TCA GGA AAA GGA CAT TTT ACC 35671 ermAAb: 35320 ATA TAG TGG TGG TAC TTT TTT GAG C35344	ermAAp: 35647 TCG ACT CAT TTT GAC TAG CTC TT 35669 ermASp: 35387 GAG CTT TGG GTT TAC TAT TAA TGG 35364
ermB	ermBSb: 739 GGT AAA GGG CAT TTA ACG AC 758 ermBAb: 1232 CGA TAT TCT CGA TTG ACC C 1214	ermBAp: 787 TTA CCT GTT TAC TTA TTT TAG CCA G 763 ermBSP: 1147 CTT ACC CGC CAT ACC ACA 1164
ermC	ermCsb: 655 CTTGTTGATCACGATAATTTCC 676 ermCab: 838 TAGCAAACCCGTATTCCACG 819	ermCAp: 700 GCA ATA TAT CCT TGT TTA AAA CTT GG 675 ermCSp: 756 CATAAGTACGGATATAATACGCA 778
ermTR	ermA/TRSb: 35690 TCA GGA AAA GGA CAT TTT ACC 35671 ermTRAb: 699 AAA ATA TGC TCG TGG CAC 682	ermTRAp: 398 CCT TCA TCA ATC TCT ATA GCA TTC 375 ermTRSp: 641 TGC TGT TAA TGG TGG AAA TG 660
msrA	msrASb: 2895 TCCAATCATTGCACAAAATC 2914 msrAAb: 3058 CAATTCCCTCTATTTGGTGGT 3038	msrAAp: 2938 TTT GAC TTC CTT TAA CCA ATG TTA G 2914 msrASp: 3006 TGT AGG TAA GAC AAC TTT ACT TGA AGC 3032
nuc	nucSb: 511 GCGATTGATGGTGATACGGTT 531 nucAb: 789 AGCCAAGCCTTGACGAACTAAAGC 766	nucAp: 558 CATTGGTTGACCTTTGTACATTAA 535 nucSp: 745 GATGGAAAAATGGTAAACGAAG 766
mecA	mecASb: 1190 TCC AGA TTA CAA CTT CAC CAG G 1211 mecAAb: 1357 CAT TTA CCA CTT CAT ATC TTG TAA CG 1332	mecAAp: 1236 TGCTGTTAATATTTTTTGAGTTGAAC 1211 mecASp: 1309 GAT AAA TCT TGG GGT GGT TAC AAC 1332

the prevalence of the two mechanisms of resistance in isolates obtained in our laboratory.

MATERIALS AND METHODS

Collection of isolates. The Centre for Infectious Diseases and Microbiology provides microbiology laboratory services to hospitals, general practitioners, and other health facilities in metropolitan Sydney, Australia, as well as regional and rural New South Wales. Between 24 July and 5 November 2005, all significant isolates (i.e., those for which antibiotic susceptibility testing was performed) of *Staphylococcus* spp. which were erythromycin resistant (MIC, ≥8 mg/liter) or intermediate (MIC, 1 to 4 mg/liter) and clindamycin susceptible (MIC ≤ 0.5 mg/liter) by BMD were collected. Duplicate isolates from the same patient were excluded. Identification and BMD were performed using the Phoenix Automated Microbiology System (Becton Dickinson, North Ryde, New South Wales, Australia). Tube coagulase testing was also performed on each isolate using an established method (37). All phenotypic and genotypic testing was performed on-site at the Centre for Infectious Diseases and Microbiology.

Phenotypic testing. Disk approximation testing was performed twice for each isolate according to the CLSI method using 2-μg clindamycin disks and 15-μg erythromycin disks placed on inoculated Mueller-Hinton agar (2). For the first test, disks were placed 15 mm apart edge to edge manually and, for the second, 22 mm apart using a six-disk disk dispenser (Bio-Rad, Hercules, California). A single 0.5 McFarland suspension in normal saline was used to inoculate both plates. Part of this suspension was also inoculated onto blood agar to ensure purity. Plates were incubated at 35°C in atmospheric conditions for 18 h. To minimize bias, plates containing the 15-mm D-test were identified by the accession number of the specimen only and placed in numerical order prior to reading while plates containing the 22-mm D-test were identified by an alphanumeric code and placed in alphabetical order. The D-tests were read independently by three observers using reflected light. Tests showing flattening of the clindamycin zone adjacent to the erythromycin disk were classified as D-test positive, while those with a circular zone were classified as D-test negative. The result recorded by two or more was taken as correct. Clindamycin zone sizes were recorded for all D-test-negative isolates as well as the last 84 consecutive isolates collected. Testing was performed in weekly batches, and *Staphylococcus aureus* ATCC BAA-976 (negative) and ATCC BAA-977 (positive) were included with each batch for quality control.

Genetic testing. (i) DNA extraction. Single colonies from the susceptibility purity plate were used for extraction of bacterial DNA. Colonies were placed in 100 μl of digestion buffer (10 mM Tris-HCl [pH 8.0], 0.45% Triton X-100, 0.45% Tween 20), heated to 100°C for 10 min, frozen at −20°C, and then thawed and centrifuged. The supernatant containing DNA was then stored at −20°C until required for PCR.

(ii) PCR. Genes encoding MLS_B resistance (*ermA*, *ermB*, *ermC*, and *ermTR*) and MS resistance (*msrA*) as well as *mecA* (for methicillin resistance) and *nuc* (specific for *S. aureus*) were amplified in multiplex reactions using the primers indicated in Table 1. Reaction mixtures consisted of 5 μl of template, 12.5 pmol of each primer, 2.5 mM of each deoxynucleoside triphosphate, 2.5 μl 10× PCR buffer, 3 mM additional $MgCl_2$ (final concentration, 4.5 mM), 0.5 U QIAGEN HotStart *Taq* polymerase, and water to make a final volume of 25 μl. Cycling conditions were 95°C for 15 min and then 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 10 min. A reverse line blot assay was performed as described elsewhere (38). Two oligonucleotide probes for each amplified gene were used (Table 1). A test was regarded as positive only if both probes for the gene were positive. If no erythromycin resistance gene was detected, repeat PCR using single oligonucleotide primer pairs was carried out and amplification products were detected using agarose gel electrophoresis.

Data analysis and statistics. Data were analyzed using Microsoft Access 2000 and statistical calculations performed using Microsoft Excel 2000, Statistical Program for the Social Sciences (SPSS) for Windows, Confidence Interval Analysis for Windows (available at http://www.medschool.soton.ac.uk/cia), and Program for Reliability Assessment with Multiple Coders (PRAM). The Student *t* test, Mann-Whitney test, chi-squared test, and Cohen's kappa test were used where appropriate. The very major error rate was defined as the percentage of isolates carrying one or more *erm* genes that were incorrectly identified as having the MS phenotype by D-test (12).

RESULTS

Isolate collection. During the study period, 1,012 *S. aureus* (632 methicillin susceptible [MSSA] and 380 methicillin resis-

TABLE 2. Interobserver agreement for the D-test[a]

Result[b]	15-mm disk separation			22-mm disk separation		
	No. (%)	No. of:		No. (%)	No of:	
		False negatives	False positives		False negatives	False positives
+++	180 (71.7)	NA[c]	0	150 (64.9)	NA	0
++−	0	NA	0	8 (3.4)	NA	0
−−+	0	0	NA	25 (10.8)	10	NA
−−−	51 (22.1)	0	NA	48 (20.8)	12	NA
Total	231	0	0	231	22	0

[a] Genotyping was considered the gold standard.

[b] +++, all observers read test as positive; ++−, positive reading for two observers, negative reading for one observer (considered a positive result); −−+, negative reading for two observers, positive reading for one observer (considered a negative result); −−−, all observers read test as negative.

[c] NA, not applicable.

TABLE 3. Genotype frequency among MSSA, MRSA, and CoNS isolates, and D-test results for each genotype

Genotype	No. (%) of isolates with genotype			No. of isolates with indicated D-test result			
	MSSA	MRSA	CoNS	15 mm		22 mm	
				Negative	Positive	Negative	Positive
ermA	42 (68.9)	46 (45.1)	2 (2.9)	0	90	22	68
ermA msrA	1 (1.6)	0	1 (1.5)	0	2	0	2
ermC	14 (23.0)	53 (52.0)	20 (29.4)	0	87	0	87
msrA	3 (4.9)	3 (2.9)	45 (66.2)	51	0	51	0
No gene amplified	1 (1.6)	0	0	0	1	0	1
Total	61	102	68	51	180	73	158

tant [MRSA]) and 139 coagulase-negative staphylococcus (CoNS) isolates were identified and tested for antibiotic susceptibility, excluding duplicate isolates from the same patient. Of these, 60 MSSA (9.5%), 103 MRSA (27.0%), and 68 CoNS (48.9%) isolates were erythromycin nonsusceptible but clindamycin susceptible by BMD and so were included in the study. All study isolates were erythromycin resistant (MIC $\geq$ 8 mg/liter) except one MSSA isolate which had intermediate susceptibility.

D-test. Using 15-mm edge-to-edge disk separation, 180 of the 231 isolates were recorded as D-test positive, with 100% consensus between the three observers (kappa value of 1; Table 2). Using 22-mm disk separation, there was discordance between the three observers for 33 of 231 tests (14.3%; kappa value of 0.77; Table 2). All 51 of the isolates that were D-test negative at 15 mm were also D-test negative at 22 mm. However, of the 180 isolates that were D-test positive at 15 mm, 22 were said to be D-test negative at 22 mm (20 *S. aureus* isolates and 2 CoNS isolates). Control strains were read correctly on each occasion by all three observers at both distances.

Genotyping. The frequency of the various genotypes among the study isolates is shown in Table 3. No macrolide resistance gene was detectable in 1 of the 231 study isolates (0.4%), a MSSA isolate that showed $iMLS_B$ resistance by D-test. This is similar to the rate described in other studies (6, 19, 30). Possible explanations include mutations in the primer binding site and the presence of rare resistance mechanisms (21, 26, 30, 36).

Using the presence of at least one *erm* gene as the "gold standard" for detection of inducible resistance and the presence of *msrA* in the absence of an *erm* gene as the negative gold standard, the 15-mm D-test had a 100% sensitivity (95% confidence interval [CI], 98.0% to 100%) and specificity (95% CI, 93.0% to 100%) while the 22-mm D-test had a sensitivity of 87.7% (95% CI, 82.9% to 92.5%) and specificity of 100% (95% CI, 93% to 100%). The very major error rate using 22 mm was 12.3% and was higher for MRSA isolates (18.2%) than for MSSA (3.5%) or CoNS (8.7%) isolates.

The median zone diameter for false-negative tests was 21 mm (range, 20 to 26 mm) versus 27 mm (range, 20 to 31 mm) for true negatives ($P < 0.0001$), indicating that false-negative results occur when the edge of the clindamycin zone is too far from the erythromycin disk for induction to occur. Likewise, *ermA*-containing isolates which produced false-negative results were more likely to have a smaller zone diameter than *ermA*-containing isolates which produced true-positive results (median diameter, 21 mm [range, 20 to 26 mm] versus 27 mm [range 20 to 37 mm]; $P < 0.0001$). For the sample of consecutive isolates, there were no significant differences in clindamycin zone sizes between the iMLSB and MS phenotypes or between the *ermA* and *ermC* genotypes.

A "double zone" around the clindamycin disk was present in 9 of 22 (40.9%) false-negative D-tests at 22 mm compared with 3 of 51 (5.9%) true-negative tests ($P = 0.0002$). An example of the double zone and small zone size associated with false-negative tests is given in Fig. 1.

Only *ermA*-containing isolates were read as false negative at 22 mm (22 of 92, 23.9%), while all 87 *ermC*-containing isolates were read correctly ($P < 0.0001$). Eighteen of the 22 (81.8%) false-negative isolates were MRSA isolates, while 2 were MSSA and 2 were CoNS.

DISCUSSION

This study confirmed a high frequency (96.3%) of $iMLS_B$ resistance among *S. aureus* isolates with an erythromycin-nonsusceptible, clindamycin-susceptible phenotype in an Austra-

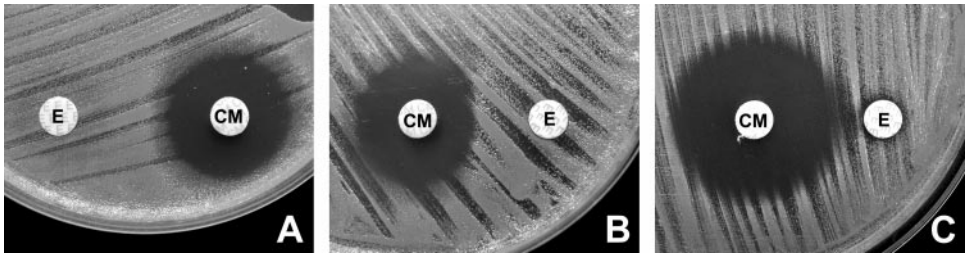

FIG. 1. A. False-negative D-tests were more likely at 22 mm when the clindamycin zone diameter was smaller and showed a double zone of inhibition. B. The same isolate tested at 15 mm. C. D-test-negative isolate tested at 15 mm. E, erythromycin disk; CM, clindamycin disk.

lian laboratory. This high rate was observed in both MSSA and MRSA isolates, from community- and hospital-acquired infections, and from metropolitan and rural areas (data not shown). The frequency of $iMLS_B$ resistance in such isolates shows marked geographic variability, ranging from 7 to 100% in published studies (1, 4, 7–11, 14, 15, 18, 20, 22, 24, 25, 28, 29, 31–33). There are fewer published data for CoNS, but generally $iMLS_B$ resistance in erythromycin-nonsusceptible but clindamycin-susceptible CoNS isolates is less common than in *S. aureus*. Rates in published studies range from 28 to 84% (4, 7, 8, 11, 14, 28, 31).

It is important for laboratories to be aware of the local prevalence of $iMLS_B$ isolates so they can choose whether to perform the D-test routinely or whether to report all erythromycin-resistant *Staphylococcus* sp. isolates as also being clindamycin resistant. This prevalence may change over time with the emergence of strains with different sensitivity patterns, so periodic surveys should be performed if testing is not routine (1).

Currently in our laboratory, approximately 28 isolates of *S. aureus* would need to be tested to detect one for which clindamycin susceptibility could be reported. As a result, we perform the D-test only in special circumstances. Such circumstances include instances where clindamycin therapy is most likely to be useful, such as bone and joint infections and infections due to nonmultiresistant MRSA, particularly where the patient is known to have hypersensitivity to beta-lactams.

A number of authors suggest that a range of distances up to 28 mm may be used for performance of the D-test, and the CLSI states that edge-to-edge distances of 15 to 26 mm may be used (3). The greater distance is more convenient since automated disk dispensers generally place disks 20 to 26 mm apart. We chose to compare the minimum distance recommended by the CLSI (15 mm) to the distance produced by automated dispensers used in our laboratory (22 mm) since there is conflicting opinion about the accuracy of the D-test when distances above 20 mm are used (7, 13, 34). One study which validated a distance of 26 mm found that all but one of 59 *Staphylococcus* sp. isolates with $iMLS_B$ resistance were positive by D-test using 26 mm and that all were positive using 20 mm and 15 mm (7). The low rate of very major errors in that study may relate to the nature of resistance determinants in local strains: only 8 of 28 $iMLS_B$ *S. aureus* isolates genotyped in the study harbored *ermA*, and the remainder harbored *ermC*, which we found to be less associated with false-negative D-test results. Furthermore, since all three distances were compared on a single plate, the possibility of observer bias cannot be excluded.

The present study has found that the D-test using a 22-mm distance is inaccurate, with a sensitivity of only 87.7% when compared with genotyping, and a very major error rate of 12.3% (18.2% for MRSA isolates). Moreover, the results are more difficult to read, with a significant level of disagreement between observers, which was not apparent when the shorter separation distance was used. We recommend that a distance of $\leq$15 mm should be used for disk approximation testing.

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