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Performance of an Agar Dilution Method and a Vitek 2 Card for Detection of Inducible Clindamycin Resistance in *Staphylococcus* spp.[▽]

Christian Lavallée,[1,2]* Danielle Rouleau,[2,3] Christiane Gaudreau,[2,4] Michel Roger,[2,3] Catherine Tsimiklis,[2] Marie-Claude Locas,[1] Simon Gagnon,[3] Jocelyn Delorme,[1,2] and Annie-Claude Labbé[1,2]

Département de Microbiologie, Hôpital Maisonneuve-Rosemont, 5415 Boulevard de l'Assomption, Montréal, Québec, Canada[1]; Département de Microbiologie et d'Immunologie, Faculté de Médecine, Université de Montréal,[2] and Hôpital Notre-Dame du Centre Hospitalier Universitaire de Montréal,[3] 1560 Rue Sherbrooke Est, Montréal, Québec, Canada; and Hôpital Saint-Luc du Centre Hospitalier Universitaire de Montréal, 1058 Rue Saint-Denis, Montréal, Québec, Canada[4]

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The D-zone test detects inducible clindamycin resistance in *Staphylococcus* spp. Two other methods not described by the Clinical and Laboratory Standards Institute (CLSI) are available to test for this resistance mechanism: an agar dilution method and new Vitek 2 cards. This study evaluated the performance of both methods in detecting inducible clindamycin resistance. Nonduplicate clinical strains of *Staphylococcus* spp. (111 *Staphylococcus aureus* and 52 coagulase-negative staphylococcus strains), intermediate or resistant to erythromycin but susceptible to clindamycin, were obtained from three hospitals in Montreal, Quebec, Canada. Molecular analysis to detect resistance genes was conducted on all strains. A Mueller-Hinton agar containing 1 mg of erythromycin and 0.5 mg of clindamycin/liter was used for the dilution method, and two inocula were tested: 10^4 and 10^5 CFU per spot. Plates were read at 24 and 48 h. The Vitek 2 AST-P580 card was used according to the manufacturer's recommendations. The results were compared to those of the D-zone test. The D-zone test was positive in 134 of 163 (82%) strains. With the 10^4 CFU inoculum, the sensitivities were 84 and 99% at 24 and 48 h, respectively. The 10^5 CFU inoculum increased the sensitivities at 24 and 48 h to 91 and 100%, respectively. The specificity was 100% for the 10^4 CFU inoculum at 24 h and 97% for the other combinations. The sensitivity and specificity for the Vitek 2 card were 93 and 100%, respectively. The performance of both the agar dilution method and the Vitek 2 card was good, but these methods were not as sensitive as the D-zone test at 24 h.

Inducible clindamycin resistance is caused by a ribosomal methylase encoded by *erm* genes; it results in resistance to macrolides, lincosamides, and streptogramin B antibiotics (MLS_B phenotype). This phenotype can be either inducible ($iMLS_B$) or constitutively expressed ($cMLS_B$) (10, 11, 19). The inducible phenotype is expressed only in the presence of macrolides, but not lincosamides (10). *In vitro*, isolates harboring the $iMLS_B$ phenotype appear resistant to macrolides but susceptible to clindamycin. Treatment failures with the use of clindamycin have been described with $iMLS_B$ phenotype and raise concern about the selection of a $cMLS_B$ phenotype, especially in infections that are deep seated or with a large bacterial burden (11).

The Clinical and Laboratory Standards Institute (CLSI) recommends testing of erythromycin-resistant and clindamycin-susceptible isolates of *Staphylococcus* spp. by either D-zone test or broth microdilution to detect inducible clindamycin resistance (3). In some clinical microbiology laboratories, this increases the turnaround time by 24 h since isolates that meet the CLSI criteria have to be further tested by the D-zone test. One way to circumvent this problem would be to report all macrolide-resistant *Staphylococcus* spp. resistant to lincosamides but, because the prevalence of the $iMLS_B$ phenotype varies between different geographic locations and populations (1, 2, 6, 7, 14, 15, 18, 20, 22), it would prevent the use of clindamycin for patients that would likely respond to clindamycin therapy (20). Another option is to test all isolates of *Staphylococcus* spp. for inducible clindamycin resistance by performing the D-zone test on purity plates used with automated systems (9) but, for laboratories with a high workload, this can be time-consuming.

Two other methods not described in CLSI's M100 document allow inducible clindamycin resistance to be detected while conducting the other antimicrobial susceptibility tests. Fernandes et al. (5) recently described an agar dilution method to detect this resistance mechanism. A Mueller-Hinton agar with 3.3% defibrinated horse blood containing 0.5 mg of clindamycin and 1 mg of erythromycin/liter resulted in a sensitivity and specificity of 100%. The automated system Vitek 2 (bioMérieux, Marcy l'Étoile, France) now offers a panel that detects inducible clindamycin resistance directly.

The objectives of the present study were to evaluate the performance of the agar dilution method, using Mueller-Hinton agar unsupplemented with horse blood, and a Vitek 2 card

* Corresponding author. Mailing address: Hôpital Maisonneuve-Rosemont, 5415 Assomption, Montreal, Quebec H1T 2M4, Canada. Phone: (514) 252-3400, ext. 2693. Fax: (514) 252-3898. E-mail: lavallee_c@hotmail.com.

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TABLE 1. Characteristics of clinical isolates of *Staphylococcus* spp.

Identification	No. of isolates					
	D-zone test negative[a]	D-zone test positive				Total
		ermA positive	*ermC* positive	*ermA* and *ermC* positive	*ermA* and *ermC* negative	
MSSA	8	64	21	0	1	94
MRSA	6	11	0	0	0	17
CNS	15	1	35	1	0	52
Total	29	76	56	1	1	163

[a] All D-zone test-negative isolates were PCR negative for *ermA* and *ermC* genes.

in detecting inducible clindamycin resistance compared to the D-zone test.

(The results of the present study were presented in part at the 109th Annual Meeting of the American Society for Microbiology in Philadelphia, PA, May 2009 [poster C-010].)

MATERIALS AND METHODS

Collection and characterization of isolates. Consecutive, nonduplicate clinical isolates of *Staphylococcus* spp. resistant (MIC, ≥8 mg/liter) or intermediate (MIC, 1 to 4 mg/liter) to erythromycin but susceptible to clindamycin (MIC, ≤0.5 mg/liter) were identified and collected from two teaching hospitals in Montreal, Quebec, Canada (35 isolates from Hôpital Saint-Luc du Centre Hospitalier Universitaire de Montréal [CHUM] and 55 isolates from Hôpital Maisonneuve-Rosemont [HMR]), between May and July 2008. A collection of 73 isolates that met the same selection criteria from a third teaching hospital, but collected from August 2006 to March 2007 (Hôpital Notre-Dame du CHUM), was also included. Routine antimicrobial susceptibility testing was done by the Vitek 2 system in both CHUM hospitals and by agar dilution in HMR. The D-zone test was performed according to the CLSI recommendations using a disk separation distance of 15 mm (3).

Molecular analysis of all isolates was conducted. The presence of *ermA* or *ermC*, the two most common genes implicated in inducible clindamycin resistance in *Staphylococcus* spp., was detected by a multiplex PCR according to previously outlined methods (12).

Agar dilution. The induction agar was prepared with Mueller-Hinton agar (BBL, Sparks, MD) containing 0.5 mg of clindamycin (Nucro-Technics, Scarborough, Canada)/liter and 1 mg of erythromycin (Sigma-Aldrich, St. Louis, MO)/liter and poured in 100-mm plates to a depth of 5 mm. The decision not to supplement the agar with horse blood was made because all other agars used in the laboratory are unsupplemented with blood according to CLSI recommendations. Plates were stored at 4°C, and a new lot was produced each month. Plates with 0.5 mg of erythromycin/liter and plates without antibiotics were used as growth controls. Three to five colonies of an 18- to 24-h-old culture of *Staphylococcus* spp. were inoculated in Mueller-Hinton broth (BBL) and adjusted to a concentration equivalent to a 0.5 McFarland standard (~10^8 CFU/ml). A 500-μl volume of the suspension was placed in a 37-well seed block. A manual inoculum replicator was then used. Pins of 1 mm, delivering ~0.1 μl of suspension (~10^4 CFU) per spot, were selected. Because of the possible inoculum effect (5), a second induction agar plate was also inoculated with 3-mm pins that delivered ~1 μl (~10^5 CFU) per spot. Plates were incubated at 35°C and read at 24 and 48 h. Growth of more than one colony on the induction agar plate was considered a positive result for inducible clindamycin resistance.

Vitek 2. The Vitek 2 AST-P580 card (bioMérieux, Marcy l'Étoile, France) was used according to the manufacturer's recommendations. Briefly, three to five colonies of an 18- to 24-h-old culture of *Staphylococcus* spp. were inoculated in a 0.45% NaCl solution and adjusted to a concentration equivalent to a 0.5 to 0.63 McFarland standard. The solution was then loaded with the card in the Vitek 2 system. The incubation period was determined by the Vitek 2 system. Two wells are used to detect inducible clindamycin resistance in the Vitek 2 card: one with 0.5 mg of clindamycin/liter and another one with a combination of 0.25 and 0.5 mg of clindamycin and erythromycin/liter, respectively. Both the instrument and the Advanced Expert System (AES) results were considered.

TABLE 2. Performance of the agar dilution method in detecting inducible clindamycin resistance in *Staphylococcus* spp. compared to the D-zone test

Inoculum (CFU/spot) and time point	Agar dilution method result	No. of isolates with:		% (95% CI)	
		Positive D-zone test	Negative D-zone test	Sensitivity	Specificity
10^4					
24 h	Positive	113	0	84 (77–90)	100 (85–100)
	Negative	21	29		
48 h	Positive	133	1	99 (95–100)	97 (80–100)
	Negative	1	28		
10^5					
24 h	Positive	122	1	91 (85–95)	97 (80–100)
	Negative	12	28		
48 h	Positive	134	1	100 (97–100)	97 (80–100)
	Negative	0	28		

Quality control. Positive (ATCC BAA-977, *ermA*) and negative (ATCC BAA-976, efflux pump *msrA*) strains were used for the D-zone test, the agar dilution, and the Vitek 2 card. Because the PCR used was a multiplex PCR, two in-house isolates—one with an *ermC* gene and one with both *ermA* and *ermC*—were added to the ATCC BAA-976 and the ATCC BAA-977 strains with each run of PCR. These additional quality control strains made us confident that *ermC* could be detected in each run and that there was no competition between the two sets of primers.

Statistical analysis. Confidence intervals (CI) were calculated using Stata 10.0 (Stata Corp., College Station, TX).

RESULTS

A total of 163 erythromycin-resistant or -intermediate, but clindamycin-susceptible *Staphylococcus* clinical isolates were included in the present study (Table 1). The $iMLS_B$ phenotype was detected by the D-zone test in 134 (82%) isolates. Of the D-zone test-positive isolates, all but one isolate were positive for *ermA* and/or *ermC*. All D-zone test-negative isolates were PCR negative for *ermA* and *ermC* genes.

Results of inducible clindamycin resistance by agar dilution, for both inocula at both reading times, compared to the D-zone test, are shown in Table 2. At 24 h, increasing the inoculum from 10^4 to 10^5 CFU/spot allowed the detection of inducible clindamycin resistance in 9 more strains, raising the sensitivity from 84 to 91%. Only the 10^5 CFU/spot inoculum, read at 48 h, permitted the detection of inducible resistance in all strains. Only one *Staphylococcus* isolate that was negative for inducible clindamycin resistance by D-zone test and negative for *ermA* or *ermC* grew on the induction agar plates with the 10^4 CFU/spot inoculum at 48 h and with the 10^5 CFU/spot inoculum at both 24 and 48 h. When only methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) were considered, the sensitivity and specificity were similar (data not shown).

The performance of the Vitek 2 card is showed in Table 3. There was no difference between the results obtained from the instrument and the AES. Inducible clindamycin resistance was not detected in 10 strains (i.e., 5 MSSA and 5 coagulase-negative staphylococcus [CNS] strains) that were D-zone test positive and *ermA* or *ermC* positive by PCR (sensitivity of 93%). The specificity was 100%. When these strains were re-

TABLE 3. Performance of the Vitek 2 card in detecting inducible clindamycin resistance in *Staphylococcus* spp. compared to the D-zone test

Vitek 2 result	No. of isolates with:		% (95% CI)	
	Positive D-zone test	Negative D-zone test	Sensitivity	Specificity
Positive	124	0	93 (86–96)	100 (85–100)
Negative	10	29		

tested, 6 of the 10 were still negative with the Vitek 2 card the second time. Again, the results were similar when considering MSSA and MRSA separately (data not shown).

DISCUSSION

In the context of increasing prevalence of community-acquired MRSA, alternative drugs to treat skin and soft tissue infections are needed. Clindamycin appears to be an interesting option because of the availability of an oral formulation, good bioavailability, and distribution in skin and abscesses (4). Resistance to clindamycin is highly variable in different patient populations (15, 20) and, if this drug is to be used, rapid susceptibility testing for inducible clindamycin resistance must be available. In order to choose an appropriate method for each laboratory and patient populations, the performance has to be evaluated.

For the agar dilution method, our study confirmed the importance of the inoculum effect. Unfortunately, the two inocula tested were not 100% sensitive at 24 h. Fernandes et al. (5) mentioned that horse blood was added in their agar because the opaque red background facilitates the detection of scanty growth. The absence of horse blood in the agar might explain why, even in the hands of experienced technicians, some positive results were missed at 24 h. It is also possible that without horse blood supplementation, other antibiotic concentrations should be used to reach 100% sensitivity at 24 h (5). Laboratories that choose to use unsupplemented agar should be aware of this difference in sensitivity and should probably keep the plates for 48 h. This will affect turnaround time, but with our isolates, 91% of $iMLS_b$ phenotype strains would have been reported resistant at 24 h (24 h earlier than with the use of the D-zone test when it is done only after the susceptibilities to erythromycin and clindamycin are known). Moreover, a second reading at 48 h is less labor-intensive in a laboratory using agar dilution than performing the D-zone test on isolates that meet the screening criteria.

The Vitek 2 system was reported to be 98% sensitive in detecting inducible clindamycin resistance in a study that tested 62 strains of *Staphylococcus* spp. (16). In two other studies that evaluated the new cards, the sensitivity for inducible clindamycin resistance detection was 99% (8, 21). In the present study, the Vitek 2 card failed to detect inducible clindamycin resistance in 10 strains (negative predictive value of 74%; 95% CI = 58 to 87%). All of these isolates were from clinical specimens of different patients and were found in the three participating hospitals. Half were MSSA harboring the *ermA* gene and the others were CNS harboring the *ermC* genes so we know it is not a problem associated with one single clone that would be present in the three hospitals. Moreover, these results were reproducible in more than half of the isolates. There were no false-positive results for inducible clindamycin resistance with the Vitek 2 card, so positive results can be reported without further confirmation with D-zone test. Our findings indicate that the Vitek 2 card would allow 93% of isolates to be correctly reported as resistant concomitantly with the other antimicrobial susceptibility results. Laboratories that want to reach 100% sensitivity would still have to test erythromycin resistant/clindamycin susceptible isolates that showed negative results for inducible clindamycin resistance with the Vitek 2 card with the D-zone test.

Interestingly, one strain of MSSA in our study was D-zone test positive and also positive for inducible clindamycin resistance by the agar dilution method and by the Vitek 2 card but was negative for *ermA* or *ermC*. This strain was tested in another laboratory for the presence of *ermB* by PCR (13) and was negative for this gene. At least two other similar strains have been reported (5, 17). The reason why this strain has an $iMLS_B$ phenotype but is not harboring a *ermA*, *ermB*, or *ermC* gene is still unclear. *ermY* genes have been described in *Staphylococcus* spp. (19). It is also possible that mutations at the target sites of primers for *ermA*, *ermB*, and *ermC* could be responsible for this observation.

This is the first study comparing these two methods of detection of inducible clindamycin resistance to D-zone test. We raise the concern that the agar dilution method is probably not as sensitive as previously described at 24 h if not supplemented by horse blood. We also raise the concern that some strains of *Staphylococcus* spp. with the $iMSL_B$ are not detected with the Vitek 2 card. Although the clinical significance of the $iMSL_B$ phenotype is still uncertain, testing of inducible clindamycin is recommended. Our results will help clinical microbiologists to decide which technique to use depending on their local epidemiology and the techniques already in place in their institution.

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