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Accuracy of Sensititre ARIS™ 2X Microbroth Dilution Panels in Detecting Oxacillin Resistance in *Staphylococcus aureus* and Coagulase-negative Staphylococci

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ABSTRACT (revised)

Objectives: *Staphylococcus aureus* (SA) and coagulase negative staphylococci (CoNS) can be significant human pathogens. Oxacillin resistance in both groups of organisms has continued to expand and must be considered when treating staphylococcal infections. We prospectively evaluated Sensititre® microdilution panels incubated in the TREK Diagnostic Systems Automated Reading and Incubation System (ARIS® 2X) for accuracy in detecting oxacillin resistance in clinical isolates of SA and CoNS, with comparison to *mecA* PCR, and oxacillin-salt agar.

Methods: Susceptibility testing was completed on 674 clinical isolates of SA and 85 strains of CoNS using Sensititre panels (Part No. GPN2F). Sensititre panels were auto-inoculated and then placed in the ARIS 2X for incubation and autoreading. All panels were read by the instrument after 24 hours of incubation; MICs were determined and interpreted using Sensititre SWIN® software and CLSI standards. *S. aureus* strains were also tested on Mueller-Hinton agar supplemented with 4% NaCl and 6.0 mg of oxacillin. Real-time PCR for detection of the *mecA* gene was performed on all isolates.

Results: Oxacillin resistance was confirmed in 326 (48%) and 53 (62%) isolates of SA and CoNS respectively. Resistance was accurately detected with Sensititre panels, except in one strain of CoNS; this isolate was reproducibly PCR positive for *mecA*, and repeatedly yielded an MIC of <=0.25 µg/ml. For all 759 isolates, the MIC interpretation and the *mecA* PCR result were in agreement 98% of the time. False-negative *mecA* PCRs were noted in 11 instances involving 3 isolates of CoNS and 8 isolates of SA. As expected, oxacillin-salt agar was very reliable in detecting resistance in SA; however, four resistant strains, with MICs of 4.0 µg/ml were not identified; all had reproducible MICs of 4.0 µg/ml, and two of these isolates were *mecA* PCR positive.

Conclusions: Susceptibility testing remains an important function in clinical microbiology, and laboratories increasingly rely on commercial systems to standardize and expedite this process. The inability of automated susceptibility testing systems to accurately detect oxacillin resistance in staphylococci has been reported, necessitating supplemental testing. This study evaluated the precision of the ARIS 2X and Sensititre microbroth dilution panels in detecting oxacillin resistance. Sensititre panels proved to be quite reliable in detecting resistance, and compared favorably with *mecA* PCR and oxacillin agar.

INTRODUCTION

Human staphylococcal infections are very common in all patient populations, and involve a number of different species. *Staphylococcus aureus* (SA), arguably the most important gram positive pathogen, is often associated with benign infections such as folliculitis. However, severe and potentially life threatening conditions including cellulitis, deep abscess, pneumonia, sepsis, and endocarditis are also common. The broad spectrum of disease, coupled with great environmental adaptability and unique capacity to develop resistance to virtually all antibiotics continues to make SA an impressive pathogen.

Collectively the coagulase-negative staphylococci (CoNS) are ubiquitous colonizers of the skin and mucous membranes of all animals. While frequently recovered as contaminants in clinical cultures, these organisms are also associated with infections, often involving venous catheters and prosthetic devices. While infections caused by the CoNS are typically more indolent and less devastating, the high level of antibiotic resistance and production of biofilms can make CoNS infections a challenge to treat.

While surgical intervention and/or removal of indwelling devices or foreign bodies may be required, antibiotics continue to play an important role in managing staphylococcal infections. Clearly oxacillin, or a similar beta-lactam antibiotic remain the drugs of choice for treating susceptible strains of staphylococci; however, increasing resistance in both SA and CoNS to oxacillin continues, requiring the clinical laboratory to maintain methods that accurately and rapidly detect susceptibility or resistance. Detection of the *mecA* gene, or gene product are described by the CLSI as the most accurate methods for detecting oxacillin resistance (4). Assays for *mecA* gene detection (PCR) or PBP2a protein (latex agglutination) can be performed rapidly and complement phenotypic studies that provide a more extensive antibiogram for the isolate. Many clinical laboratories utilize commercial products to perform and report phenotypic susceptibility studies. Not all of the commercial systems can be relied on to accurately and consistently detect oxacillin resistance, unless supplemental testing is included. This is particularly true of "rapid" systems as 24 hours of incubation is necessary to accurately determine the oxacillin phenotype (6).

We evaluated the Automated Incubation and Reading System (ARIS 2X) and Sensititre microbroth dilution susceptibility panels, coupled with *mecA* real-time PCR and oxacillin-salt agar for the rapid and accurate detection of oxacillin resistance in both SA and CoNS (TREK Diagnostic Systems, Cleveland, OH, USA. Cepheid, Sunnyvale, CA, USA. Remel, Lenexa, KS, USA)

METHODS

Isolates and Initial Testing: Significant isolates of SA (n=674) and CoNS (n=85) recovered from clinical cultures using standard microbiology techniques were included in the study (6). All susceptibility and amplification testing was performed prospectively using protocols established in our laboratory. Briefly, these included direct *mecA* PCR testing of broth from positive blood culture bottles, followed by phenotypic susceptibility studies completed from growth on solid media. The *mecA* genotype was also determined on clinically significant isolates of SA and CoNS recovered from wound, body fluid, and LRT cultures. In both instances the *mecA* result was reported on the same day, followed with phenotypic susceptibility results in 24-48 hours.

Sensititre Panel Inoculation & Automated Reading: The ARIS 2X has been designed to incubate and then "auto-read" Sensititre microdilution susceptibility and identification panels. Each ARIS 2X unit can hold 64 different panels, tracking inventory with barcode information present on each microtiter tray. A temperature controlled, and timed incubation is maintained within the instrument, after which, robotics are used to transport plates to the reading unit. Hydrolysis of a fluorogenic substrate by the bacterial isolate is used to measure growth in each well and determine MIC endpoints. Data management software included with the ARIS 2X System was used to interpret MICs, generate a printed report, and to transmit data to the LIS. All MIC interpretations were based on CLSI guidelines (4).

Twenty-four hour growth was used for all phenotypic susceptibility studies and all susceptibilities were incubated a full 24 hours prior to reading and interpretation. A suspension equal to a 0.5 McFarland turbidity standard, verified by nephelometry, was prepared in 5.0 ml of sterile demineralized water, sampling isolated, but similar colonies. A precision pipette was used to transfer 10.0 µl of the 0.5 McFarland suspension to a tube containing 11.0 ml of cation-adjusted Mueller-Hinton (MH) broth. Using an Autoinoculator, the MH broth was used to simultaneously rehydrate and inoculate Sensititre panels, dosing each well with 50 µl of broth, final concentration $5.0 \times 10^4 - 5.0 \times 10^5$ cfu/ml. Colony counts were performed and recorded for each Sensititre plate by sampling the positive growth control well using a 0.001 µl calibrated loop and sub-culture on a blood agar plate; colonies were counted after 24 hours of incubation.

Oxacillin-Salt Agar Screening: Mueller-Hinton agar plates supplemented with 4% NaCl and 6 *ug* of oxacillin were used as recommended by the CLSI as a supplemental test to screen SA isolates for oxacillin resistance. Using 24 hour growth, a direct suspension equal to a 0.5 McFarland standard was prepared in 5.0 ml of sterile demineralized water. A dacron tipped swab was dipped into the suspension, excess liquid was expressed, and the swab was used to spot inoculate the oxacillin agar plate. Inoculated plates were incubated at 35°C in ambient air for 24 hours. Any growth on the plate was interpreted as resistant to oxacillin (3,4,6).

***mecA* PCR Assay**: An in-house real-time PCR amplification protocol employing analyte specific reagents (ASR) in a hydrolysis probe format for the detection of *mecA* gene sequences was performed on a SmartCycler II System (Cepheid, Sunnyvale, CA, USA). As mentioned previously, PCR was completed directly from a positive blood culture bottle, or by using growth from plated-culture medium. For positive blood culture bottles, 1.0 µl of broth was diluted in 1.0 ml of nuclease-free water. This suspension was vortexed and 5.0 µl was used as template in the PCR (1,2). With plated isolates, a suspension equal to a 0.5 McFarland Standard (1-2 10^8 cfu/ml) was prepared in 5.0 ml of sterile demineralized water. From this suspension,1.0 ml was transferred to a microfuge tube containing 1.0 ml of nuclease-free water. The sample was vortexed and similarly, 5.0 µl was used as template in the PCR.

RESULTS

Results for 759 staphylococcal isolates, 674 SA and 85 CoNS, were evaluated. For SA, oxacillin resistance was confirmed in 326 (48%) strains, with Sensititre MIC panels accurately detecting oxacillin resistance in all of these isolates. For seven different SA isolates; Sensititre panels yielded reproducible oxacillin MICs of 4.0 µg/ml (resistant based on CLSI standards). The *mecA* PCR was positive in all but two of these strains, validating the phenotypic resistant call obtained by Sensititre MIC testing. The remaining two SA with MICs of 4.0 µg/ml were repeatedly negative for *mecA* by PCR suggesting the possibility of hyper-beta lactamase producing strains. Only three of these seven isolates grew on oxacillin-salt agar, confirming an MIC in the 4-6 µg/ml range.

Eight SA isolates produced false-negative *mecA* PCR assays compared to phenotypic susceptibility studies on initial testing. Two of these strains yielded positive *mecA* PCR results, but CTs were >38 cycles; values that we do not attempt to interpret, and were therefore initially recorded as indeterminate. Repeat *mecA* PCR testing of the remaining six discordant samples yielded positive *mecA* PCRs for all isolates with CTs ranging from 33.3-36.6, an average of 3.4 cycles higher than the mean for samples tested with this assay, suggesting either low target copy due to sample preparation (e.g., Poisson distribution) or possibly because of a highly mixed or heterogenous population being present. While not uncommon, heterogeneous populations are efficiently selected for in phenotypic susceptibility systems due to added salt in the medium and decreased incubation temperatures. All six of these isolates grew on oxacillin-salt agar, further confirming the initial Sensititre MIC and interpretation.

Only one MRSA strain with an MIC of >8 µg/ml and positive by PCR for *mecA* failed to grow on oxacillin-salt agar. Overall there was 98% agreement between all three methods, with Sensititre panels being the only one that accurately detected all MRSA strains (Table 1).

Table 1. Accuracy of the three methods in detecting Oxacillin Resistant *S. aureus*

Oxacillin MIC	Sensititre*	mecA PCR Positive*	Oxacillin Agar Positive*
<0.25-2.0	350	0	0
4.0-8.0	9	7	5
>8	315	315	314
Overall Sensitivity	100%	97%	98%
Overall Agreement**	---	98%	99%

* number of isolates
** agreement with Sensititre microdilution panel

Of the 85 isolates of CoNS evaluated, 53 (62%) were resistant to oxacillin with 52 (98%) isolates correctly characterized as oxacillin resistant using Sensititre MIC panels. The single "false-susceptible" result occurred in an isolate that was repeatedly *mecA* PCR positive, yet the MIC consistently reproduced at ≤ 0.25 µg/ml (susceptible using CLSI interpretations). Colony counts performed from the MIC panel for this isolate confirmed an appropriate inoculum was present in the panel. Three false-negative *mecA* PCRs were noted, all of these were positive when repeated after MIC values were known; only one of these isolates had an MIC of 1.0 µg/ml, while the remaining two isolates had MICs of >8.0 µg/ml, and both of these had PCR completed directly from positive blood culture bottles, while repeat testing was performed from growth on solid medium.

CONCLUSION

Susceptibility testing remains one of the most important functions performed in the clinical microbiology laboratory. Commercially available susceptibility products along with instrumentation have been developed, and these systems have helped laboratories gain efficiencies in completing these studies. To generate even faster results, some of the commercial systems have utilized "rapid" technology for identification and susceptibility testing of aerobic and facultative organisms. However, increasing and emerging resistance, often by novel mechanisms, continues to be described in multiple bacterial species; accurate and consistent detection of these resistant strains has continued to challenge the laboratory and current susceptibility testing methods, more specifically, the "rapid" susceptibility testing systems (7).

For many bacteria, particularly the gram positive cocci, a full 24 hours of incubation is required to generate accurate susceptibility data (3,4,7). Issues like these have required many laboratories to maintain multiple susceptibility testing methods to accommodate the variety of organisms encountered with appropriate methodology to accurately detect resistance. We previously reported on the accuracy of the ARIS 2X-Sensititre system compared to Vitek®, Pasco microdilution panels, and E-test strips, for Gram negative and Gram positive organisms, including fastidious bacteria. Indeed, the Sensititre ARIS 2X System was the only system that supported the susceptibility testing of all organisms in the study (5).

Staphylococci are somewhat unique, in that knowledge of oxacillin susceptibility or resistance is often sufficient to initiate appropriate therapy. However accurate detection of oxacillin resistance using phenotypic methods requires 24 hours of incubation. Our study was two fold. First, we coupled real-time PCR and genotypic susceptibility testing, which could be generated rapidly, with phenotypic studies that employed microdilution panels and overnight incubation. The false-negative *mecA* PCR results obtained using the direct specimen testing protocol were of concern. Review of test data showed that most of the discordant *mecA* results were obtained when PCR testing was completed on blood culture bottle samples (sensitivity = 96%) versus testing performed from solid medium (sensitivity = 99%). The apparent decrease in sensitivity on tests performed using blood culture broth has resulted in protocol modifications in an effort to increase the microbial load prior to PCR testing. Positive blood culture bottles are now allowed to incubate off-line to enhance biologic amplification until the PCR is set up later in the day. In addition, the inoculum used for the initial dilution has been increased to 2.0 µl. These validations, while still in progress, look promising in decreasing false-negative direct *mecA* PCR results from blood culture samples.

Secondly, and most importantly, we evaluated the accuracy of Sensititre microdilution panels for the phenotypic detection of oxacillin resistance in both SA and CoNS. Sensititre microdilution panels closely approximate the reference microdilution procedure described by the CLSI, and they performed exceptionally well in the current evaluation, correctly characterizing the oxacillin phenotype for all 674 isolates of SA, and for 84 of 85 strains of CoNS (99.7% accuracy).

The Sensititre ARIS 2X System provides clinical laboratories with an automated incubation and reading platform for microdilution susceptibility panels that is reliable, reproducible, and cost effective.

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