PROSPECTIVE EVALUATION OF *STAPHYLOCOCCUS AUREUS* ANTIBIOTIC RESISTANCE PATTERNS FROM RESPIRATORY SPECIMENS IN ICU PATIENTS

Andrew C. Bernard, MD; Associate Professor; Department of Surgery; University of Kentucky College of Medicine; Lexington, KY

Sue Overman, MA, SM (ASCP); Chief Medical Tech; UK Healthcare Clinical Microbiology; Department of Pathology and Laboratory Medicine; University of Kentucky College of Medicine; Lexington, KY

P. Shane Winstead, PharmD; Assistant Professor; Department of Pharmacy Practice and Science, University of Kentucky College of Pharmacy; Lexington, KY

Anna K. Rockich, PharmD; Assistant Professor; Department of Surgery, University of Kentucky College of Medicine; Lexington, KY

Paul A. Kearney, MD; Professor; Department of Surgery, University of Kentucky College of Medicine; Lexington, KY

Craig Martin, PharmD; Associate Professor; Department of Pharmacy Practice and Science; University of Kentucky College of Pharmacy; Lexington, KY

Julie A. Ribes, MD, PhD; Associate Professor and Director of Clinical Microbiology; Associate Director of Hospital Laboratories; Department of Pathology and Laboratory Medicine; University of Kentucky College of Medicine; Lexington, KY

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Address correspondence to: Andrew C. Bernard, M.D.

Department of Surgery, Section on Acute Care Surgery University of Kentucky College of Medicine

800 Rose Street, C-234 Lexington, Kentucky 40536-0298

Office Phone: 859-323-6346, Ext 224 859Fax: 859-323-6840 acbern00@uky.edu

ABSTRACT

Background: *Staphylococcus aureus* is the leading pathogen responsible for hospital-acquired pneumonia (HAP). Vancomycin is the primary antimicrobial choice for methicillin-resistant *S. aureus* (MRSA) HAP worldwide, but *S. aureus* isolates have been reported susceptible or even resistant to vancomycin.

Objectives: This study compares automated and non-automated susceptibility patterns for MRSA isolates to vancomycin to determine comparability of testing methods for this drug.

Methods: Respiratory samples submitted to Clinical Microbiology from patients in the ICU were plated onto sheep blood agar and chocolate agar media and visually inspected at 12-24 hours. The presence of *S. aureus* was determined serologically. *S. aureus* isolates were subcultured for susceptibility testing to detect MRSA respiratory isolates. Consecutive MRSA respiratory isolates were analyzed for susceptibility and minimum inhibitory concentration (MIC) using E-test strips and BD Phoenix automated system testing for vancomycin and the vancomycin alternative agents teicoplanin, linezolid and tigecycline.

Results: Ninety-five MRSA respiratory isolates were collected. Three were obtained by bronchoalveolar lavage and the remainder by protected alveolar lavage. There was no heteroresistance detected by E-test at 24 hours, but there were 4 isolates at 48 hours demonstrating elevated vancomycin MIC values of 6 ug/ml.

Conclusions: Vancomycin heteroresistance was not a problem in this series of isolates. Agreement between vancomycin automated MICs and E-test MICs was not universal. Although under-calls were few based upon automated MIC, several instances were indeed identified. These data show that Phoenix under-reports vancomycin MIC values for MRSA compared to E-test methodology.

Keywords: *Methicillin-resistant Staphylococcus aureus; antibiotic resistance; hospital-acquired pneumonia*

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) has become the leading pathogen responsible for hospital-acquired pneumonia (HAP) and is responsible for about 20% of cases (NNIS System Report, 1999). *S. aureus* may be either susceptible or resistant to oxacillin/methicillin resistant *S. aureus* (MRSA). Oxacillin resistance rates are increasing, and the average rate of resistance to oxacillin currently exceeds 55% on average nationwide (Jones, et al., 2004; Rosenthal, et al, 2010).

Presence or absence of risk factors for MRSA determines whether or not MRSA drug therapy is used (American Thoracic Society, 2005). If a patient is thought to have pneumonia and they possess risk factors for MRSA, then antimicrobial therapy active against MRSA will be used empirically and "de-escalated" if objective culture data does not demonstrate MRSA. Empiric or culture-based therapy for MRSA HAP includes one of the following: 1) the glycopeptide vancomycin, or 2) the oxazoladinone linezolid.

Adequate empiric antibiotic therapy improves outcome in patients with pneumonia (Alvarez-Lerma, 1996; Dupont, et al., 2001; Kolleff, et al., 1999). Vancomycin is the primary antimicrobial choice for MRSA HAP worldwide. It is relatively inexpensive in per-dose pricing, generally effective, and has a favorable safety profile. However, *S. aureus* isolates which are incompletely susceptible or even resistant to vancomycin have been reported (Smith, et al., 1999). These vancomycin-intermediately susceptible *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) isolates are rare but increasing (Plipat, et al., 2005). Since the first report of VISA in the United States in 1999 (Smith, et al., 1999), other reports have documented the existence of *S. aureus* isolates with incomplete susceptibility to vancomycin, termed as vancomycin "heteroresistance" (Plipat, et al., 2005).

Heteroresistant VISA (hVISA) isolates are those in which the prevalent colonies are vancomycin susceptible but in which a subpopulation of organisms demonstrate phenotypes which are intermediate or resistant to vancomycin [Plipat, et al., 2005]. HVISA has been proposed as an etiology for vancomycin treatment failure in some patients. These hVISA populations may exhibit vancomycin-susceptibility when analyzed by automated testing because automated systems are incapable of identifying rare hVISA colony forming units (CFUs) within single large inoculums (Tenover, et al., 2004). Without more detailed testing, the true incidence of hVISA isolates is unknown. No large direct comparison of automated and non-automated MRSA isolate susceptibilities to vancomycin has been reported. This investigation sought to determine the prevalence of pulmonary MRSA that are resistant or heteroresistant to vancomycin, and the prevalence of MRSA resistance to other frequently used antibiotics (i.e., linezolid, tigecycline, and teicoplanin), in a major tertiary referral center ICU setting. Vancomycin susceptibility testing results for the Phoenix automated panel (Phoenix, BD Biosciences) and the E-test strip method (BioMerieux) were directly compared; alternative agents to vancomycin (i.e., teicoplanin, tigecycline, and linezolid) were also assessed.

2. MATERIALS AND METHODS

This investigation was reviewed and approved by the institution's Medical IRB. Respiratory samples were collected between January 2, 2009 and April 27, 2010 by bronchoalveolar lavage (BAL) or protected alveolar lavage (PAL, CombiCath, KOL Bio Medical Instruments, Chantilly, Virginia). Samples were submitted to the Clinical Microbiology Laboratory and were cultured on sheep blood agar plates and chocolate agar plates incubated in a CO_2 incubator at

35°C. These plates were visually inspected at 12-24 hours. The presence of *S. aureus* was determined and *S. aureus* isolates were subcultured to fresh plates for susceptibility testing. Consecutive MRSA isolates (n=100) were analyzed using E-test strips (bioMerieux, Macry L'Etoile, France) and automated microbroth dilution testing (BD Phoenix Automated Microbiology System, Franklin Lakes, NJ). *S. aureus* isolates that were oxacillin susceptible were excluded from further analysis.

For standard E-test susceptibility testing, isolates were diluted in broth to 0.5 McFarland turbidity, and 0.2 ml was spread onto a 90mm Brain Heart Infusion (BHI) agar plate. The plates were dried briefly prior to applying the E-test strips. Plates were incubated at 35°C, read at 24 hours, and confirmed at 48 hours. Since evidence suggests that sensitivity and specificity for the detection of hVISA are highest if the isolates are fresh and if they are set at 2.0 McFarland turbidity (Walsh, et al., 2001; Liu & Chambers, 2003), this was the method that was used. Linezolid and teicoplanin E-test strips were obtained from Pfizer (New York, NY), and tigecycline E-test strips were obtained from Wyeth (Madison, NJ).

E-test MICs were determined by visual inspection by laboratory microbiologists according to laboratory policy and procedure. An immediate reporting mechanism was established to respond to any evidence of antimicrobial heteroresistance so that alteration in therapy could be made as clinically indicated; no visible heteroresistance was reported during the study period. Microbiologists reading E-test results carefully examined plates for evidence of hazes, feathery growth, or heteroresistant microcolonies, according to the manufacturer's instructions. The interpretation of the test is as follows: If the MIC values for vancomycin and teicoplanin were <6 mcg/ml, the *Staphylococcus* was considered susceptible to vancomycin. MIC values ≥ 32 mcg/ml were considered resistant for vancomycin and teicoplanin. An isolate was considered positive for heteroresistance if vancomycin and teicoplanin MIC values were ≥ 8 mcg/ml, or if the teicoplanin MIC value alone was ≥12 mcg/ml. Positive results based on a teicoplanin result of 12 or 16 mcg/ml were confirmed by repeating the test. Susceptible break points of ≤ 4.0 mcg/mL and ≤ 0.5 mcg/mL were used for linezolid and tigecycline, respectively.

3. RESULTS

One hundred respiratory *Staphylococcus aureus* isolates were collected. Ninety-five percent (95%) were MRSA, indicating a 5% rate of protocol deviation in the microbiology lab. Three were obtained by BAL and the others (92/95, 96.8%) by PAL. MRSA MIC values for vancomycin are shown in Table 1. Using the standard E-test methodology (testing at 0.5 McFarland turbidity), there were no vancomycin MIC values greater than 2 mcg/ml. Using 24-hour incubation and heteroresistance methodology with 2.0 McFarland turbidity, all isolates demonstrated MIC values of ≥ 2 mcg/ml with 7/92 (7.6%) demonstrating MIC values of 6 mcg/ml. At 48 hours, heteroresistance testing demonstrated MIC values of 6 mcg/mL for 3/95 isolates (3.2%). No E-test results were ≥ 8 mcg/ml by any method. Vancomycin MIC results by Phoenix ranged from 0.5 mcg/ml to 4 mcg/ml; 98.9% of MRSA isolates had MIC values of ≤ 2 mcg/ml (91/92 isolates tested)

Table 1. MRSA MICs for Vancomycin				
MIC Result (mcg/ml)	**Standard Methodology E-test n=95**	**Heteroresistance Methodology E-test at 24 Hours n=92**	**Heteroresistance Methodology E-test at 48 Hours n=95**	**Phoenix Microbroth Dilution n=92**
0.5	-	-	-	7/92 (7.6%)
1	12/95 (13%)	-	-	63/92 (68.5%)
1.5	47/95 (49%)	-	-	-
2	36/95 (38%)	23/92 (25%)	11/95 (11.6%)	21/92 (22.8%)
3	-	62/92 (67.4%)	54/95 (56.8%)	-
4	-	7/92 (7.6%)	27/95 (28.4%)	1/92 (1.1%)
6	-	-	3/95 (3.2%)	-

Teicoplanin MIC results using E-test are shown in Table 2. As with vancomycin, results varied by method used with heteroresistance methodology and longer incubation showing higher MIC values overall. No teicoplanin heteroresistance (MIC values ≥ 12 mcg/ml) was seen.

Table 2. MRSA MICs for Teicoplanin			
MIC Result (mcg/ml)	**Standard Methodology E-test n=95**	**Heteroresistance Methodology E-test at 24 Hours n=92**	**Heteroresistance Methodology E-test at 48 Hours n=95**
≤ 0.5	44/95 (46.3%)	2/92 (2.2%)	2 (2.1%)
0.75	15/95 (15.8%)	-	-
1	10/95 (10.5%)	1/92 (1.1%)	-
1.5	15/95 (15.8%)	2/92 (2.2%)	2/95 (2.1%)
2	9/95 (9.5%)	11/92 (12.0%)	3/95 (3.2%)
3	1/95 (1.1%)	33/92 (35.9%)	25/95 (26.3%)
4	-	31/92 (33.7%)	33/95 (34.7%)
6	1/95 (%)	10/92 (10.9%)	20/95 (21.1%)
8	-	2/92 (2.2%)	10/95 (10.5%)

Tigecycline E-test MIC values are shown in Table 3, while those for linezolid are shown in Table 4. At the time of the study, neither tigecycline nor linezolid were on the Phoenix susceptibility panel so these results are not available for comparison.

Table 3. MRSA MICs for Tigecycline Standard Methodology E-test (n=95)	
MIC Result (mcg/ml)	**Number of Isolates (%)**
0.0	1/92 (1.1%)
0.06	3/9 (3.2%)
0.09	19/95 (20%)
0.125	12/95 (12.6%)
0.13	7/95 (7.4%)
0.19	18/95 (18.9%)
0.25	15/95 (15.8%)
0.28	1/95 (1.1%)
0.38	7/95 (7.4%)
1	8/95 (8.4%)
1.5	2/95 (2.1%)
2	2/95 (2.1%)

Table 4. MRSA MICs for Linezolid Standard Methodology E-test (n=95)	
MIC Result (mcg/ml)	**Number of Isolates (%)**
0.25	1/95 (1.1%)
0.38	1/95 (1.1%)
0.5	6/95 (6.3%)
0.75	21/95 (22.1%)
1	30/95 (31.6%)
1.5	9/95 (9.5%)
2	21/95 (22.1%)
3	5/95 (5.3%)
4	1/95 (1.1%)

4. DISCUSSION

While the prevalence of MRSA among all *S. aureus* isolates is increasing, *in vitro* evidence of growing resistance of MRSA isolates to vancomycin is also increasing. *In vitro* susceptibility testing for any organism and antimicrobial is expressed as minimal inhibitory concentration (MIC). Vancomycin MICs for MRSA are rising (Wang, et al., 2003). *S. aureus* isolates for which vancomycin MICs are 4-8 μg/mL are classified as vancomycin-intermediate, and isolates for which vancomycin MICs are ≥16 μg/mL are classified as vancomycin-resistant by the Centers for Disease Control (Centers for Disease Control and Prevention).

Automated equipment historically does not report specific vancomycin MIC values below or equal to 2 ug/ml. The limitation with such equipment is that as vancomycin MIC values for MRSA have crept upward over time, we now have automated equipment that reports all MRSA isolates with vancomycin MIC values ≤ 2 μg/mL as susceptible, with no distinction of whether the MIC is 0.5, 1 or 2 ug/ml. Automated equipment generally reports MIC values in doubling dilutions. Furthermore, while the majority of the sample may contain individual cells with vancomycin MIC values in the susceptible range, we now know that susceptibility may vary from one bacterial cell to another within a given sample. Some solitary organisms or CFUs may demonstrate a phenotype in which vancomycin MIC values are higher than the majority of the sample, but their numbers are so small as to be unrecognized in the microbroth dilution assay. This variability in MRSA resistance to vancomycin is termed heteroresistance, and heteroresistance may contribute to treatment failure. Moreover, ideal methods to detect and address vancomycin heteroresistance in *S. aureus* in every institution or laboratory have yet to be established. Data shown here indicate that methodology is clearly critical when it comes to E-testing. The organism concentration, drug used for testing, and length of incubations all play a role in the detection of heteroresistance within a bacterial population.

These data show that Phoenix under-reports vancomycin MIC values for MRSA compared to E-test methodology. Thirty-eight percent of the E-test results showed a vancomycin MIC of 2 mcg/ml while only 23.9% of automated isolates demonstrated vancomycin MIC of 2 mcg/ml. To determine the prevalence of MRSA isolates with marginal susceptibility to vancomycin or heteroresistance was the primary aim of this investigation. None was found. While there were no misses of heteroresistance by Phoenix testing, none were detected by either method employed in this study. These data are limited in that the isolates are derived from a single institution and must therefore be considered local. Admittedly, the sample size of 95 isolates may have been inadequate to detect the rare heteroresistant isolate found at this institution, particularly in our institution where oxacillin resistance rates in *S. aureus* isolates is lower than the National Nosocomial Infections Surveillance (NNIS)-reported national average of 56% (NNIS, 1999).

Since adequate empiric therapy determines outcome, it is imperative that clinicians treat patients at risk for MRSA with antimicrobials certain to eradicate MRSA. Traditional therapy was almost exclusively the glycopeptide vancomycin, but emerging evidence suggests that in some cases vancomycin is not as effective as other available drugs. Kollef *et al.* reported improved clinical cure and survival in patients with MRSA ventilator-associated pneumonia (VAP) who were treated with linezolid compared to those treated with vancomycin (survival odds ratio 4.6 for

linezolid vs vancomycin) (Kollef, et al., 2004)]. Improved microbiologic eradication and reduced mortality has also been shown in MRSA bacteremia treated with linezolid compared to vancomycin (12% mortality with linezolid vs 40% mortality for vancomycin) (Gomez, et al., 2007).

We found that all isolates analyzed had MICs for tigecycline and linezolid that were in the therapeutic range. Chung *et al.* reported the same in a series of hVISA isolates (Chung, et al., 2007), and Hortiwakul *et al.* also reported 100% susceptibility to linezolid in 100 MRSA isolates from patients (Hortiwakul, et al., 2012).

Teicoplanin resistance did not occur in our series. Wilson *et al.* reported reduced susceptibility to teicoplanin, defined by MIC >16, in 3.3% of 643 MRSA strains (Wilson, et al., 2006). Our series was smaller but affirms the findings that teicoplanin resistance among MRSA strains is relatively low.

Isolates in this study were only cultured for a limited period of time, and it is possible that further culture may have revealed evolving resistance pattern. Webster *et al.* reported a MRSA isolate with an MIC changing from ≤ 1 to 4 ug/ml over several months, demonstrating the ability of MRSA to acquire or alter its resistance patterns over times (Webster, et al., 2007).

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