

Phenotypic Detection of Methicillin Resistance in *Staphylococcus aureus* by Disk Diffusion Testing and Etest on Mueller-Hinton Agar[∇]

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Cefoxitin is increasingly recommended for detection of methicillin resistance in *Staphylococcus aureus* (MRSA) when using disk diffusion testing. In this study, 95 *mecA*-negative *S. aureus* isolates and a highly genetically diverse collection of *mecA*-positive *S. aureus* types ($n = 50$) were used to investigate the influence of technical factors such as disk potency, incubation time, and temperature on Mueller-Hinton agar. The use of cefoxitin MIC testing by Etest for the same purpose was investigated under similar conditions. For disk diffusion, the accuracy was high at both 35°C and 36°C using overnight incubation, while incubation at 30°C or 37°C was associated with slightly lower accuracy. Increasing incubation times from 18 to 24 h did not improve accuracy at either temperature. Cefoxitin Etest MICs for *mecA*-positive strains were 6 mg/liter or higher, while cefoxitin Etest MICs for *mecA*-negative strains were ≤ 4 mg/liter. Our findings suggest that the current CLSI zone diameter breakpoints should be adjusted from resistance ($R \leq 19$ mm to $R \leq 21$ mm). In conclusion, cefoxitin disk diffusion testing and Etest MIC testing can accurately predict the presence of the *mecA* gene in *S. aureus*. Testing can be reliably performed using incubation temperatures of 35 to 36°C and incubation times of 18 to 22 h. We suggest MRSA interpretive criteria of susceptible ($S \leq 4$ mg/liter and $R > 4$ mg/liter, corresponding to $S \geq 22$ mm and $R \leq 21$ mm for the 30- μ g disk and $S \geq 17$ mm and $R \leq 16$ mm for the 10- μ g cefoxitin disk. These criteria resulted in only one *mecA*-positive isolate being misclassified as susceptible.

Infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) are an increasing problem worldwide inside and outside of hospitals (3).

Phenotypic detection of MRSA has been problematic ever since its discovery in the early 1960s. The emergence of low-level-resistant MRSA clones acquired in the community has only added to these difficulties.

Detection of the *mecA* gene or its product, penicillin binding protein (PBP2a), is considered the gold standard (5) for MRSA confirmation. Recent investigations suggest that disk diffusion using cefoxitin is superior to most previously recommended phenotypic methods, including oxacillin disk diffusion and oxacillin screen agar testing (8, 14, 19, 21). In 2005, the Clinical and Laboratory Standards Institute (CLSI) published zone diameter (6) breakpoint guidelines for cefoxitin. However, a number of technical issues remain regarding the use of cefoxitin as a predictor for methicillin resistance. The CLSI M100-S15 document stipulates an incubation time of 24 h unless the isolate has a zone diameter of ≤ 19 mm (i.e., resistant), in which case it can be reported after 18 h of incubation. However, in a recent publication, performance was equally good at 18 h of incubation (21).

For methicillin and oxacillin, incubation temperature is known to affect the test results (12, 17). For oxacillin, a max-

imum of 35°C for testing of staphylococci is specified by CLSI. For cefoxitin, high accuracy has been found using standard incubation temperatures, i.e., 35 to 37°C (8, 10, 19–21), but one study showed relatively low sensitivity at 37°C (2). In a recent investigation, zone diameters obtained on IsoSensitest agar with semiconfluent inoculum were dependent on whether the plates were incubated at 35°C or 36°C (R. Skov, unpublished results). Two studies have shown marginally improved accuracy when tests were performed at 30°C compared to 35°C and 37°C (4, 8). The 10- μ g cefoxitin disk has been shown to be superior to the 30- μ g disk with IsoSensitest agar and semiconfluent growth (20).

The CLSI M100-S15 document recommends oxacillin MIC testing for MRSA detection by the MIC method, and no criteria are as yet available for the use of cefoxitin as an alternative (6).

In this study, the influence of incubation time (18 h and 24 h) and temperatures (30°C, 35°C, 36°C, and 37°C) on the performance of 10- and 30- μ g cefoxitin disks and cefoxitin Etest on Mueller-Hinton agar were evaluated for *mecA*-positive and *mecA*-negative *S. aureus*.

MATERIALS AND METHODS

Strains. A total of 146 *S. aureus* strains were included in the study. All isolates were tested for the presence of the *mecA* gene by the EVIGENE MRSA Detection kit (18) (SSI Diagnostika, Statens Serum Institut, Copenhagen, Denmark) using the manufacturer's instructions. The strains selected from a previously tested collection (20) consisted of 95 *mecA*-negative consecutive blood culture isolates and 51 *mecA*-positive isolates from different patients. Only *mecA*-positive isolates which previously had produced inhibition zones with cefoxitin and had distinctly different pulsed-field gel electrophoresis (PFGE) patterns

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(one or more visible band differences) were included. Two recent clinical *mecA*-positive isolates with large cefoxitin inhibition zones and three isolates particularly sensitive to small variations in temperature, kindly provided by Derek Brown, Addenbrooke Hospital, Cambridge, United Kingdom, were also included.

One *mecA*-positive isolate was later excluded from the investigation as population analysis showed it to be a susceptible phenotype (see the population profile analysis), leaving a total of 95 *mecA*-negative and 50 *mecA*-positive isolates.

Three reference strains were included for quality control: one *mecA*-positive strain (*S. aureus* ATCC 43300) and two *mecA*-negative strains (*S. aureus* ATCC 25923 and ATCC 29213).

All strains stored at -80°C were subcultured on two consecutive days using 5% Danish blood agar (SSI Diagnostika) prior to use.

spa typing and MLST. *spa* typing and multilocus sequence typing (MLST) were performed as previously described (7, 11). The *spa* types (t) and MLST sequence types (ST) were assigned through the Ridom (<http://www.ridom.de>) and MLST databases (<http://www.mlst.net>), respectively. Based on *spa* and/or sequence types, the isolates were given a predicted clonal complex annotation.

Susceptibility testing. In the first phase of the study (phase I), all 145 strains were tested by disk diffusion using cefoxitin and Etest (AB Biodisk, Solna, Sweden) using cefoxitin and oxacillin. All methodological variants were assessed using the same inoculum. The inoculum was standardized to 0.5 McFarland turbidity. Disk diffusion was done with 10- μg and 30- μg disks (Oxoid, Basingstoke, United Kingdom) using Mueller-Hinton BBL II agar (Becton Dickinson, Heidelberg, Germany). Agar plates were incubated overnight (18 to 19 h) in ambient air at 30°C , 35°C , and 36°C in stacks no higher than five plates.

Inhibition zone diameters were read from the back of the agar plate using reflected light and calipers to read to the nearest millimeter at the inner zone edge. For isolates with a zone size of >19 mm after 18 to 19 h for the 30- μg cefoxitin disk at 35°C , all plates were further incubated and read after 24 h as specified in M100-S15 (6).

Etest oxacillin MIC testing was performed according to the manufacturer's instructions using Mueller-Hinton BBL II agar supplemented with 2% NaCl (wt/vol) and incubation at 35°C for a full 24 h. Etest cefoxitin MIC testing was done using Mueller-Hinton BBL II agar without NaCl supplementation. Plates were incubated in ambient air at 30°C , 35°C , and 36°C . Cefoxitin MICs were read after 18 to 19 h of incubation. Isolates for which cefoxitin MICs were ≤ 4 mg/liter were incubated further and read after a total of 24 h.

The influence of incubation at 37°C was investigated in phase II using a subset of 54 strains (35 *mecA*-negative and 19 *mecA*-positive strains) incubated simultaneously at 35°C and 37°C . For phase II we selected the most challenging strains, those with the largest cefoxitin zone diameters and those that during phase I had shown results influenced by variations in incubation temperature. For phase II, MICs and zone diameters were read after 18 h and 24 h of incubation.

In both phase I and phase II, *S. aureus* strains ATCC 29213, ATCC 25923, and ATCC 43300 were included for quality control on all test runs. The temperature in the incubators was monitored at 10-min intervals for the entire incubation period using TinytagPlus digital thermometers with accompanying software (Gemini Data Loggers Ltd., Chichester, West Sussex, United Kingdom). The temperature curves showed that with empty incubators the maximum variation during a 12-h test period was $\pm 0.25^{\circ}\text{C}$ (data not shown). With plates on the shelves, the time to reach the preset temperature on each shelf was dependent on the number of plates placed on the shelf. In phase I, when shelves were full, the temperature only reached the preset temperature at the end of the incubation time. This was true for both temperatures. Each strain was subjected to the same conditions for each of the two temperatures. The temperature at the end of incubation was 34.6 to 34.8°C and 35.6 to 36.4°C when incubators were set at 35°C and 36°C , respectively. In phase II the preset temperatures were reached on all shelves within 4 h, and the temperatures were stationary between 34.7 to 35.6°C and 36.8 to 37.1°C when incubators were set at 36°C and 37°C , respectively (detailed data not shown).

Intraassay variation was studied using *S. aureus* ATCC 25923, ATCC 29213, and ATCC 43300 tested for 10 days using two different batches of Mueller-Hinton II agar (BBL) and two different batches of 10- μg and 30- μg disks (Oxoid). Each agar plate was read independently by five technologists, giving a total of 50 measurements per Mueller-Hinton agar/disk combination.

Population analysis. Two *mecA*-positive isolates obtained from Norway (strains 9-8 and 10-22), previously undetectable by any phenotypic cefoxitin method (19, 20), were tested by population analysis profile using various concentrations of cefoxitin and oxacillin in agar. Colonies from a fresh overnight culture were inoculated into 5 ml tryptic soy broth (SSI Diagnostika) and incu-

TABLE 1. Predicted clonal complexes (CC) based on *spa* type of the 50 *mecA*-positive isolates

CC	No. of isolates	<i>spa</i> type(s)
ST1	2	t127
CC5	5	t002, t005
CC8	13	t008, t024, t037, t051, t190, t211
ST15	1	t084
CC22	4	t022, t354, t431, t790
CC30	6	t018, t019, t318, t1209
CC45	5	t015, t065, t126
CC59	3	t216, t437
CC78	2	t186, t1339
CC80	7	t044, t131
CC97	2	t365

bated overnight at 35°C . Tenfold dilutions of the culture were prepared in 0.9% NaCl, and 20 μl from each dilution was spot inoculated in duplicate onto Mueller-Hinton agar plates containing twofold dilutions of oxacillin (0.25 to 128 mg/liter) and cefoxitin (0.25 to 128 mg/liter). The agar plates were incubated a full 24 h at 35°C , and colonies were counted to plot the population analysis profile. Strain 10-22 showed a highly heterogeneous resistance pattern against oxacillin and displayed colonies of up to 64 mg/liter (data not shown). Since strain 9-8 failed to show any colonies at 4 mg/liter in repeat experiments, even in the undiluted samples, it was concluded that this *mecA*-positive isolate was an oxacillin-susceptible phenotype, and it was omitted from further analysis. This is a well-described phenomenon that can be caused by a defect in one or more enzymes needed for an isolate to express oxacillin resistance to, e.g., one of the *fem* genes (1).

Statistics. Statistical differences in MICs obtained at different temperatures and/or incubation times were analyzed by Wilcoxon's matched pairs test, with $P < 0.05$ considered significant. Distributions were reported by medians and ranges. For statistical analyses, the Statistica software program (version 7.0; Stat Soft Inc., Tulsa, TX) was used.

RESULTS

Genotypes. The 50 *mecA*-positive *S. aureus* isolates represented 28 different *spa* types and 12 different clonal complexes/ST groups, as shown in Table 1.

MIC. Etest oxacillin MIC distributions at 35°C after 24 h of incubation and cefoxitin MIC distributions at 30°C , 35°C , and 36°C after 18 h and 24 h of incubation are shown in Table 2. Oxacillin MICs for the *mecA*-negative strains were between 0.125 mg/liter and 4 mg/liter (the oxacillin MIC for one isolate was 4 mg/liter) and for *mecA*-positive strains was between 1 and >256 mg/liter, with a median of 64 mg/liter. For cefoxitin the range was 1 to 4 mg/liter (with only one isolate at 1 mg/liter) and for *mecA*-negative isolates was 4 to >256 mg/liter, with a median MIC of 32 mg/liter for *mecA*-positive isolates regardless of the incubation temperature or time. However, MICs were significantly lower at 36°C than for incubation at 35°C ($P = 0.006$). Similar results were found in phase II (35 to 37°C). There was no difference in MICs obtained at 35°C for incubation for 18 h or 24 h (not significant; $P = 0.1$). There was no difference between MICs obtained at 30°C and 35°C incubation (not significant; $P = 0.64$). Using an MRSA interpretive breakpoint of susceptible (S) ≤ 4 mg/liter and resistant (R) > 4 mg/liter, only one *mecA*-positive isolate (isolate 10-22 from Norway) was incorrectly categorized as non-MRSA after both 18 h and 24 h of incubation at all temperatures (see also data from the population profile analysis for this isolate in Material and Methods). The oxacillin MIC for this isolate was 4 mg/liter. Another *mecA*-positive isolate (isolate 1748) was pheno-

TABLE 2. Oxacillin Etest MIC at 35°C after 24 h of incubation and cefoxitin Etest MIC at 30, 35, and 36°C after 18 h and 24 h of incubation

Drug concn (mg/liter)	No. of observations by test type, <i>mecA</i> status, time, and temp ^a									
	Oxacillin Etest (35°C, 24 h)		Cefoxitin Etest							
	<i>mecA</i> negative (<i>n</i> = 95)	<i>mecA</i> positive ^b (<i>n</i> = 49)	30°C		35°C		36°C		<i>mecA</i> positive (<i>n</i> = 50), 18 h	<i>mecA</i> positive (<i>n</i> = 50), 18 h
			<i>mecA</i> negative ^b (<i>n</i> = 94)	<i>mecA</i> positive (<i>n</i> = 50), 18 h	<i>mecA</i> negative (<i>n</i> = 95)	<i>mecA</i> positive (<i>n</i> = 50), 18 h	<i>mecA</i> negative (<i>n</i> = 95)	<i>mecA</i> positive (<i>n</i> = 50), 18 h		
		18 h	24 h	18 h	24 h	18 h	24 h			
0.125	2									
0.25	13									
0.5	50									
1	26	1			1	1				
2	3		10	10	5	3		6	5	
4	1	5	84	84	1 ^c	89	91	1 ^c	89	90
6 ^d		2			5			3		3
8		5			3			2		1
16		4			11			11		15
32		3			8			16		22
64		11			13			9		5
128		6			5			5		2
≥256		12			4			3		1

^a Values in columns are number of observations for each unique variable.

^b One strain did not grow.

^c Strain 10-22. After a full 24 h of incubation, the cefoxitin MIC was 4 mg/liter at 30°C, 35°C, and 36°C.

^d All intermediate results are reported to the nearest higher concentration except for 6 mg/liter.

typically sensitive to oxacillin (MIC, 1 mg/liter) but was resistant to cefoxitin (MIC, 16 mg/liter).

Disk diffusion. Results for cefoxitin 30- μ g and the 10- μ g disks incubated 18 to 20 h at 35°C are shown in Fig. 1. Except for one isolate (isolate 10-22), all *mecA*-positive isolates gave inhibition zone diameters of ≤ 21 mm for the 30- μ g disk and ≤ 17 mm for the 10- μ g disk in both phase I and phase II.

For *mecA*-negative isolates, zone diameters for the 30- μ g disk were ≥ 25 mm and ≥ 23 mm for phase I and II, respectively, and ≥ 17 mm for the 10- μ g disk in both phases I and II.

Increasing the incubation time from 18 h to 24 h or the temperature from 35°C to 36°C did not affect inhibition zone diameter distributions for either disk for *mecA*-negative or *mecA*-positive isolates (data not shown).

Increasing the temperature from 35°C to 37°C was slightly more problematic; for the 30- μ g disk, a *mecA*-negative strain was classified as positive, and with the 10- μ g disk a *mecA*-positive strain was classified as susceptible. Decreasing the temperature from 35°C to 30°C made one strain falsely susceptible by both disks (data not shown).

Repeated testing of quality control strains. The results for the 10-day repeated testing of *S. aureus* ATCC 29213 (i.e., CLSI quality control strain for routine MIC testing) and *S. aureus* ATCC 25923 (i.e., CLSI quality control strain for routine disk diffusion testing) are shown in Table 3 (all methodological variants were tested with the same inoculum suspension). *S. aureus* ATCC 25923, the quality control strain normally used by the CLSI, on one day (day 5) gave significantly different results from the rest of the nine days both for the 10- μ g and the 30- μ g disks. Omitting the outlier results from day 5, the following medians (ranges) were obtained for *S. aureus* ATCC 25923: 25 mm (22 to 28 mm) for the 30- μ g disk and 20 mm (18 to 22 mm) for the 10- μ g disk. For *S. aureus*

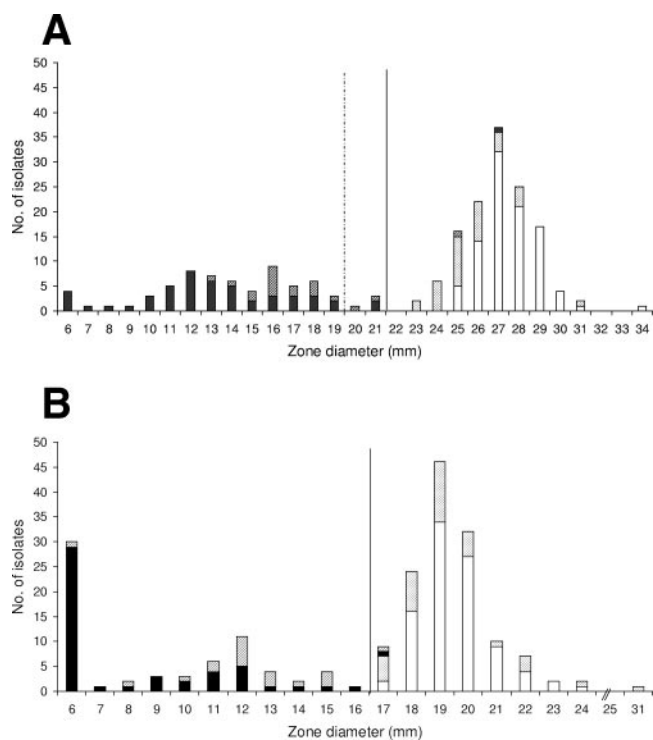


FIG. 1. Zone diameter distribution for 30- μ g cefoxitin disk (A) and 10- μ g cefoxitin disk (B) against 145 *S. aureus* isolates (phase I) and repeat testing with 54 *S. aureus* isolates (phase II) at 35°C and 18 h of incubation. The current CLSI breakpoint for the 30- μ g disk is shown by a dashed line, and the suggested breakpoints are shown by black lines in both figures. □, *mecA* negative, phase I; ▨, *mecA* negative, phase II; ■, *mecA* positive, phase I; ▩, *mecA* positive, phase II.

TABLE 3. Intraassay variation for 10- μ g and 30- μ g cefoxitin disks for *S. aureus* ATCC 29213 and ATCC 25923

<i>S. aureus</i> strain	Disk size (μ g)	Temp ($^{\circ}$ C)	No. of observations with indicated zone diam (mm) ^a												
			16	17	18	19	20	21	22	23	24	25	26	27	28
ATCC 29213	10	35		16 (1)	90 (4)	70 (6)	4 (9)								
	10	37		11 (1)	85 (7)	77 (11)	7 (1)								
ATCC 25923	10	35	(1)	(4)	(9)	19 (6)	78	37	26						
	10	37		(1)	1 (6)	35 (11)	97 (2)	32	15						
ATCC 29213	30	35									3	27 (2)	91 (11)	55 (6)	4 (1)
	30	37									7	37 (8)	89 (9)	39 (3)	8
ATCC 25923	30	35						0 (1)	4 (5)	14 (5)	29 (4)	39	37	15	42 (5)
	30	37			(2)	(1)	(4)	6 (5)	17 (1)	33 (4)	50 (3)	41	20	9	4

^a Ten-day repeat testings were read by five persons using two batches of disks and media, respectively, i.e., 200 observations per variant. Numbers in parentheses refer to the number of observations on day 5 (see the text for an explanation).

ATCC 29213, tighter zone diameter ranges were obtained for both disks, giving medians (ranges) of 25 mm (23 to 27 mm) for the 30- μ g disk and 18 mm (17 to 20 mm) for the 10- μ g disk. There was no difference between batches of agar or disks (data not shown).

DISCUSSION

Cefoxitin disk diffusion testing is now an accepted method for the detection of methicillin resistance in *S. aureus* by an increasing number of reference resistance groups, including CLSI. There are, however, still some unsolved issues, such as the optimal cefoxitin disk content and incubation temperature and time. In this study, a highly diverse collection of *mecA*-positive *S. aureus* isolates (12 different clonal complexes) was used to investigate these technical variants. To increase the challenge, *mecA*-positive strains without inhibition zones around the cefoxitin 10- μ g disk were not included. Furthermore, only strains with unique PFGE patterns (one or more visible band differences) were included.

Results obtained suggest that both cefoxitin inhibition zone diameters and MICs for *mecA*-positive strains are influenced by incubation temperature, although they are influenced to a lesser degree than for oxacillin. Most importantly, detection of MRSA by the cefoxitin-based method was not affected by temperature variations between 35 $^{\circ}$ C and 36 $^{\circ}$ C, while at 37 $^{\circ}$ C disk diffusion gave one false phenotypic categorization. In the International Organization for Standardization (ISO) methodology standard for MIC determinations currently under development (22), the incubation temperature is specified as 34 to

37 $^{\circ}$ C. For oxacillin testing with staphylococci, there is a note stating that the temperature should not exceed 35 $^{\circ}$ C. Although cefoxitin is influenced to a lesser degree, it may be appropriate to include the same note for cefoxitin testing. No advantage was seen with the 30 $^{\circ}$ C incubation temperature as proposed by others (4, 8). Importantly, there was no benefit in extending the incubation time from 18 h to a full 24 h. This is in agreement with the findings of Swenson and Tenover (21). It is a major advantage to clinical laboratories that standard methodology (medium, inoculum, incubation time, and temperature) can be used for the detection of MRSA.

For the 30- μ g disk, our findings suggest MRSA interpretive breakpoints of S \geq 22 mm and R \leq 21 mm, i.e., 2 mm larger than the criteria published by the CLSI (6). This proposal is supported by the results of several previous investigations (Table 4) in which *mecA*-negative isolates all have had zone diameters of 22 mm or more, and occasionally *mecA*-positive isolates exhibiting zone diameters of 20 and 21 mm have been reported (4, 9, 10, 16, 21, 23, 24).

For the 10- μ g disk, the results support corresponding interpretive breakpoints of S \geq 17 mm and R \leq 16 mm. The contents of both disks had comparable sensitivity and specificity for detection of MRSA. The lower disk content produces smaller zones and thereby reduces the interference with results for other antibiotic disks tested on the same agar plate. The higher disk content may have a slightly higher specificity, as reflected by the larger gap between *mecA*-positive and *mecA*-negative zone diameter results.

In our study, cefoxitin MIC testing with Etest was accurate

TABLE 4. Published zone diameter ranges (CLSI 30- μ g cefoxitin disk) for *mecA*-positive and *mecA*-negative *S. aureus* isolates

Reference	No. of isolates (<i>mecA</i> positive/ <i>mecA</i> negative)	Zone diam range (mm) for indicated isolate type			
		<i>mecA</i> positive	<i>mecA</i> -positive outlier	<i>mecA</i> negative	<i>mecA</i> -negative outlier
Swenson and Tenover (21)	202/309	6–19	21, 28	22–>28	
Fernandes et al. (9)	180/418	6–17		22–35	
Velasco et al. (24)	51/51	6–14		\geq 25	17
Pottumarthy et al. (16)	103/100	6–17		23–>35	21
Gueudet and Lemble (10)	49/51	6–20		23–28	
Urbaskova et al. (23)	218/534	6–19		23–35	
Cauweiler et al. (4)	73/82	6–19	23	24–34	

for MRSA detection. The results suggest interpretive breakpoints of $S \leq 4$ mg/liter and $R \geq 8$ mg/liter using CLSI terminology and $S \leq 4$ mg/liter and $R > 4$ mg/liter in EUCAST (the European Committee of Antimicrobial Susceptibility Testing) terminology. However, the cefoxitin MIC mode for *mecA*-negative strains is close to the suggested breakpoints. The proposed breakpoints are supported by data published by other investigators (Felten et al. [8], Fernandes et al. [9], Swenson and Tenover [21], and Votta et al. [M. Votta, D. Turner, B. Turng, T. Wiles, J. Reuben, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. D-47, 2004]). However, in an investigation with many borderline oxacillin-resistant *S. aureus* strains by Swenson et al., cefoxitin breakpoints of $S \leq 6$ mg/liter, instead of 4 mg/liter, and $R \geq 8$ mg/liter were shown to have better specificity but a slightly lower sensitivity (J. Swenson, D. Lonsway, S. McAllister, A. Thompson, L. Jevitt, J. Patel, Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr. D-1732, 2005). In this study, there was no difference in sensitivity or specificity using a breakpoint of $S \leq 6$ mg/liter and $R \geq 8$ mg/liter instead of $S \leq 4$ mg/liter and $R \geq 8$ mg/liter.

In conclusion, this study provides further evidence that cefoxitin is an accurate surrogate marker for the detection of MRSA in routine susceptibility testing for disk diffusion and MIC testing. Incubation temperature should not surpass 36°C. Incubating for a full 24 h did not improve results obtained after 18 h. Hence, standard conditions currently used by clinical laboratories for routine susceptibility testing as described by CLSI can be used, and the cefoxitin disk can be included among other disks relevant for susceptibility testing of *Staphylococcus aureus*. The current CLSI cefoxitin zone diameter breakpoints should be adjusted by a 2-mm increase.

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