ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1972, p. 422-426

Vol. 1, No. 5
Printed in U.S.A.

Identification of Cephalosporin-Resistant *Staphylococcus aureus* with the Disc Diffusion Method

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Received for publication 7 January 1972

Methicillin-resistant strains of *Staphylococcus aureus*, in total 84, representing 16 laboratories in 8 different countries were all resistant to 32 μg of cephalothin per ml with the same typical heteroresistant pattern. With the disc diffusion method, they were easily detected when cephalexin discs were used. With cephalothin discs, on the other hand, 26 to 49% would have been falsely categorized as Group I or II after 24 hr. It is recommended that susceptibility testing of *S. aureus* to cephalosporins by using the paper disc method be performed with 30-μg cephalexin discs on Mueller-Hinton agar without blood. With an inoculum of 10^6 bacteria/ml, an incubation temperature of 30 C, and an incubation time of 24 hr, a zone of less than 10 mm indicates presumptive heteroresistance. This corresponds to the international recommendation with a minimal inhibitory concentration of 32 μg/ml as the upper limit of Group II.

Genetic as well as clinical evidence indicates cross-resistance between penicillinase-stable penicillins and cephalosporins in *Staphylococcus aureus* (3-5, 8, 11-14, 17). This resistance has also been shown to be heterogeneous (3, 11) in that the majority of bacteria may be susceptible whereas a smaller proportion is highly resistant.

The minority of organisms resistant to methicillin typically appear as slow-growing microcolonies, which are more easily detected after incubation at 30 C or on hypertonic medium (1, 9, 10).

This highly resistant minority, when selected out and transferred to medium without antibiotics, reverts to the original state within a few generations and then usually grows at the normal rate (9).

According to recommendations given by a World Health Organization-sponsored group (6), *S. aureus* strains with minimal inhibitory concentrations (MIC) above 2 μg of methicillin per ml should be presumed heteroresistant with an all-or-none interpretation in the disc diffusion test. However, susceptibility to cephalosporin is still given in four categories which may be explained by the fact that the categorization is also printed for species other than *S. aureus*. The purpose of this paper is to define technical modifications of the disc method to make it more suitable for detection of cephalosporin-resistant strains of *S. aureus*.

MATERIALS AND METHODS

Strains. Eighty-four coagulase- and penicillinase-positive strains of methicillin-resistant staphylococci and 49 coagulase-positive and methicillin-susceptible ones, 30 of which also produced penicillinase, were analyzed with regard to cephalosporin resistance.

Methicillin resistance was determined by growth in the presence of 12.5 μg/ml after 48 hr at 30 C (1, 15). Seventy of the methicillin-resistant strains were supplied from 16 laboratories in 8 different countries. Fourteen of the methicillin-resistant strains represented six epidemic outbreaks in five Swedish hospitals.

The 49 methicillin-susceptible strains were isolated from clinical material at the University Hospital of Uppsala, Sweden. All were susceptible to 2 μg/of methicillin per ml.

Media. The following disc diffusion experiments were carried out: (i) Mueller-Hinton (MH) agar (BBL) with 5% defibrinated sheep blood; (ii) Mueller-Hinton agar (Difco); (iii) Mueller-Hinton agar (Difco) with 5% defibrinated sheep blood.

Plate dilution experiments were carried out with: 0.3% meat extract (Difco), 1% peptone (Difco), 1.5% agar (Difco) and 0.5% NaCl in 1,000 ml of distilled water; *p*H 7.3 to 7.4.

Nutrient broth experiments were carried out in the same medium as the plate dilution experiments, except that the agar was omitted.

Antibiotics. The antibiotics used were as follows: (i) sodium methicillin (Belfacillin, Astra), stored at −20 C in distilled water, 0.1 g/ml (each sample thawed and used only once); (ii) sodium cephalothin (Keflin, Eli Lilly & Co.), stored as methicillin; (iii)

cephaloridine (Kefpor, Eli Lilly & Co.), stored as methicillin; (iv) cephalexin (Cephalexin, Glaxo), stored as methicillin but in samples of 0.01 g/ml.

Susceptibility tests. (i) The plate dilution test was carried out as follows. A wire loop was dipped into 10 colonies from an overnight blood-agar plate and used to inoculate 10 ml of broth. After 18 hr at 37 C on a shaking machine, 0.1-ml portions of the undiluted cultures or of 10-fold dilutions in nutrient broth were spread onto nutrient agar plates containing serial dilutions of antibiotic. The results were registered after 24 and 48 hr at 30 or 37 C.

(ii) The paper disc test was performed by the method of Ericsson et al. (6). Inocula of 10^6, 10^5, or 10^4 bacteria/ml prepared from 20 to 25 colonies were flooded over the plate. Viable count was made of the inoculum for each experiment. Bacteria at 10^6 ml gave dense but confluent growth. The plates were incubated at 37 or 30 C with cephalothin and cephalexin discs of 15 and 30 μg and methicillin discs of 10 μg each (Biodisk, Sweden). Since these experiments were performed, international and Swedish recommendations about disc content and upper limits of susceptibility have appeared (6). Zone sizes were recorded after 24 hr and were interpreted according to internationally recommended regression curves as reported by Ericsson and Sherris (6) and as shown in Table 1. The regression curve for cephalothin was $y = -2.9\,x + 52.9$ where y is zone size in millimeters and x is ^{2}log MIC.

Penicillinase was assayed as described by Perret (16).

RESULTS

MIC values and heteroresistance to cephalosporins of methicillin-resistant S. aureus determined with the plate dilution method. When tested for cephalosporin resistance, all 84 methicillin-resistant strains grew in the presence of 32 μg of cephalothin or cephalexin per ml and all but 7 grew at 32 μg of cephaloridine per ml. These strains, however, grow at 16 μg/ml.

Twenty-five out of the 84, with at least one strain from each represented laboratory, were further studied with regard to heteroresistance to cephalothin, cephaloridine, cephalexin, and methicillin. Heteroresistance was expressed as the ratio between the number of colonies growing at various concentrations of antibiotic in the medium and the total number of colonies growing without antibiotic. As this part of the study was performed before the international recommendations, the substances were diluted 12.5:25:50 instead of 16:32:64. A varying degree of heteroresistance was documented for all the strains tested (Table 2). For cephalothin, methicillin, and cephalexin, there was a decreasing proportion of resistant organisms with increasing concentration of antibiotic. The MIC values were at 100 μg/ml or more except for two strains, which did not grow at 100 μg of cephalothin per ml. Cephaloridine turned out to be most effective. As for the median strain, a minority of bacteria grew at 25 μg/ml but not at 50 μg/ml. All strains grew in the presence of 12.5 μg/ml. A final MIC for cephaloridine was, however, difficult to obtain for many of the strains. At 50 μg/ml, for example, there was often an almost confluent growth of microcolonies with the heaviest inoculum, whereas a 1:10 dilution of the inoculum resulted in no growth. To be recorded as "growth" on medium with cephaloridine at a certain concentration of the agent, 100 colonies or less were required on each plate. This inoculum effect may well be due to the formation of penicillinase (2).

Zone sizes and MIC values for cephalosporins given by the paper disc method. Figure 1 presents the inhibition zones of 84 cephalosporin-resistant strains in the disc diffusion test performed as recommended by Ericsson and Sherris (6), with Mueller-Hinton agar (Difco) containing 5% sheep blood, an inoculum of 10^6 bacteria/ml, and 30-μg discs of cephalothin and cephalexin. The plates were incubated at 30 C, and they were read

TABLE 1. *International and Swedish recommendations of disc content and upper limits of susceptibility*

Antibiotic	Disc content (μg/ml)	MIC, upper limit (μg/ml)		
		Group I	Group II	Group III
Methicillin				
International	10	2	—[a]	—[a]
Swedish	10	2 (10 mm)[b]	—[a]	—[a]
Cephalothin				
International	10	4	32	128
Swedish	30	2 (24 mm)	16 (15 mm)	128 (9 mm)
Cephalexin				
International	10			
Swedish	30	2 (25 mm)	16 (15 mm)	128 (6 mm)

[a] The agent not recommended for use in this group.
[b] Growth at 30 C.

TABLE 2. *Heteroresistance of methicillin-resistant Staphylococcus aureus to methicillin and various cephalosporins; median of 25 strains (for cephalexin, median of 17)*

Antibiotic	No. of colonies growing at various concn of antibiotic in the medium (μg/ml)/no. of colonies growing without antibiotic			
	12.5	25	50	100
Methicillin	1/10	1/10	1/10	1/10
Cephaloridine	$1/10^5$	$1/10^7$	0	0
Cephalothin	1/10	1/10	$1/10^3$	$1/10^6$
Cephalexin	1/1	1/1	1/1	1/10

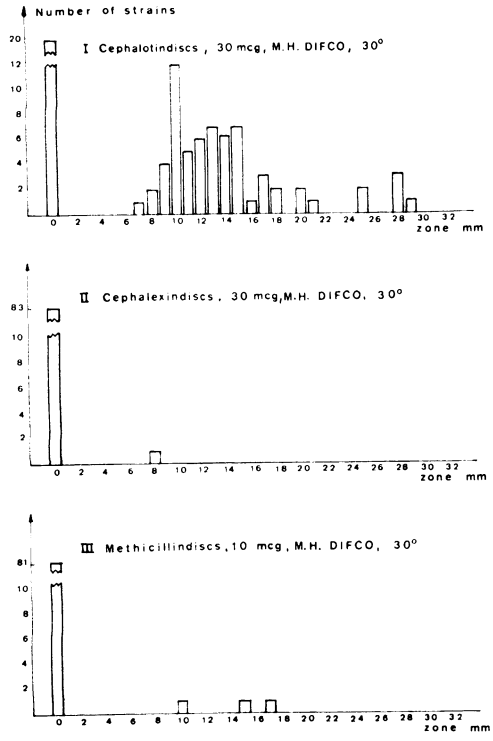

FIG. 1. *Inhibition zones for 84 methicillin-resistant Staphylococcus aureus strains with cephalothin, cephalexin, and methicillin discs after incubation at 30 C on Mueller-Hinton (MH) agar with 5% defibrinated sheep blood.*

after 24 hr. In parallel, susceptibility to methicillin was determined with 10-μg discs. With cephalothin discs, microcolonies commonly appeared as an inner zone. Zone measurement was always made within these colonies, but this phenomenon sometimes caused difficulties in estimating zone limits.

According to the regression curve of Ericsson, 16 μg of cephalothin per ml as the upper limit of susceptibility should correspond to 15 mm and 32 μg/ml to 12 mm. With cephalothin discs, 22 of the 84 strains (26%) would have been categorized as Group I or II if the Swedish recommendations were used (MIC $\leq$ 2 and 16 μg/ml, respectively) and 41 (49%) if the international recommendations were used (MIC $\leq$ 4 and 32 μg/ml, respectively).

With cephalexin discs, on the other hand, heteroresistance was detected for all strains. Only one strain gave a measurable zone of 8 mm.

Influence of incubation temperature. When the plates were incubated at 37 C instead of 30 C, 70 of the strains (83%) should have been falsely recorded as susceptible with cephalothin discs and 16 μg/ml as upper limit (Fig. 2). Corresponding figures with cephalexin discs were two (2%). All but six of the strains grew up to the cephalexin disc, giving no inhibition zone at all. For methicillin, 42 of the 84 strains were recognized as susceptible compared with three at 30 C.

Influence of incubation time. As longer incubation increases the chance for resistant microcolonies to appear, the plates were read again after 48 hr. The number of strains recognized as Group I or II at 30 C was reduced from 22 to 11 with the Swedish and from 41 to 26 with the international recommendations.

Influence of medium. According to recommendations by the World Health Organization group, Mueller-Hinton medium with 5% defibrinated sheep blood was used throughout this study. There was no significant difference of results on Difco Mueller-Hinton and BBL Mueller-Hinton medium. When blood was excluded there was, however, a tendency towards larger zones around the cephalothin discs, 42% of the investigated strains giving zones of 30 mm or more at 30 C and 24 hr. For cephalexin, on the other hand, the results were not significantly influenced by the exclusion of blood.

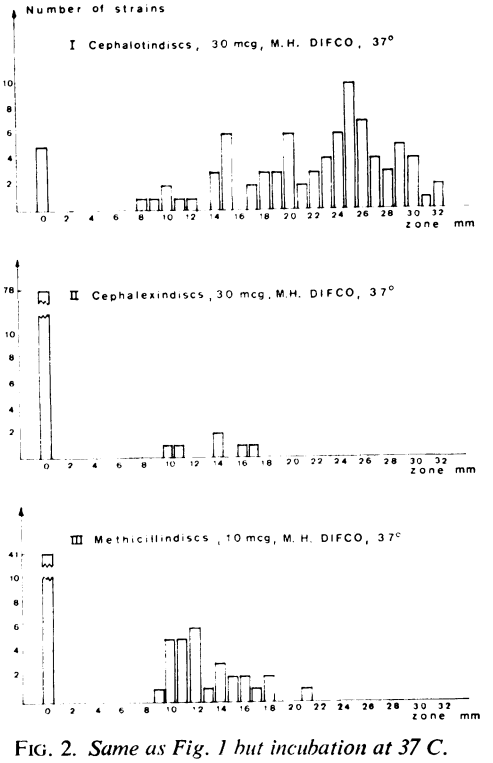

FIG. 2. *Same as Fig. 1 but incubation at 37 C.*

Influence of disc content. Discs containing 15 μg of cephalothin and cephalexin, respectively, gave results comparable with 30-μg discs. A large number of strains were recorded as susceptible. As no regression curves were given by the manufacturer, the difference could not be analyzed in more detail.

MIC values to cephalosporins of 49 methicillin-susceptible S. aureus determined with the plate dilution method. The effect of various cephalosporins on methicillin-susceptible strains is shown in Table 3. Cephalothin was most effective with MIC values below 2 μg/ml for the great majority of strains. Only one grew at 2 μg/ml. For cephaloridine, on the other hand, MIC values of 2 μg/ml or more were recorded for 13 strains, all penicillinase producers. This is in contrast to the results with methicillin-resistant strains, which are all penicillinase producers but were more susceptible to cephaloridine. To cephalexin only 30 of the 49 strains were recorded as susceptible or fairly susceptible to cephalexin according to the Swedish recommendations.

TABLE 3. *MIC values of various cephalosporins for 49 strains of methicillin-susceptible Staphylococcus aureus*

Antibiotic	MIC at		
	<2 μg/ml	2–8 μg/ml	≥16 μg/ml
Cephalothin.....	48	1	0
Cephaloridine....	36	12	1
Cephalexin.....	6	24	19

Zone sizes for cephalexin given by the paper disc method. As cephalexin evidently offered the greatest chance to detect staphylococcal heteroresistance to cephalosporins, it was of interest to study the cephalexin zones with cephalosporin-susceptible strains as a control. Therefore, the 49 strains were analyzed under the same conditions as given for the results in Fig. 1, with the exception that no blood was added to the medium. As seen in Fig. 3, there was a zone range between 20 and 35 mm. The zones for cephalothin were 30 mm or more for all strains.

DISCUSSION

The results of this report support the suggestion of a close association between methicillin and cephalosporin resistance in *S. aureus*, with heteroresistance as a typical trait. The strains used represent 16 laboratories in 8 different countries.

For methicillin, an MIC of 2 μg/ml has been recommended as limit for presuming heteroresistance in *S. aureus*. Susceptibility is recorded as resistant or sensitive (6). Our results indicate that similar recommendations should be given for cephalosporins.

If cephalothin were to be used as prototype agent, the same limit could probably be used. In

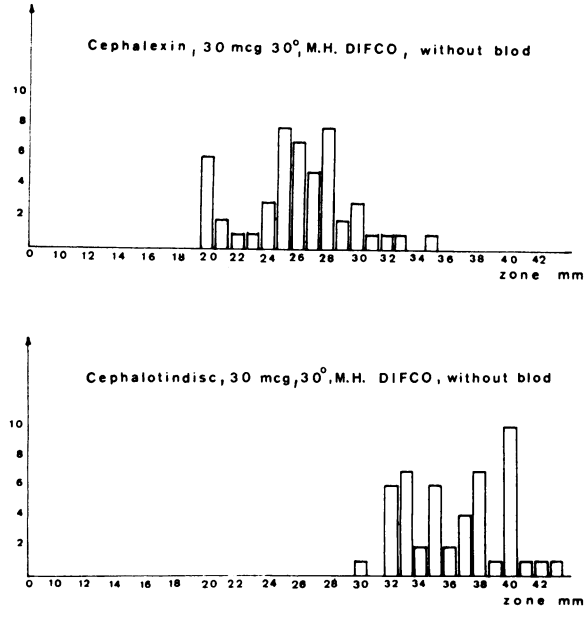

FIG. 3. *Inhibition zones for 49 methicillin-susceptible strains with cephalothin and cephalexin discs after incubation at 30 C on Mueller-Hinton agar without blood.*

the plate dilution test, all methicillin-susceptible strains were inhibited by 2 μg/ml or less, whereas all resistant strains grew in the presence of 32 μg/ml. Cephalothin is, however, not ideal, as many resistant cultures will give zones falsely indicating susceptibility in the disc diffusion test. This problem was further exaggerated when blood was excluded from the medium.

With cephalexin, MIC values of 16 μg/ml or more in plate dilution were recorded for many of the methicillin-sensitive *S. aureus* strains. On the other hand, more clear-cut results were obtained in the paper disc method with zones of 20 mm or more for sensitive and no or small zones for resistant ones. These results were not significantly influenced whether blood was present or not in the medium. As the detection of heteroresistance is probably of primary importance, cephalexin is recommended as prototype cephalosporin.

No discs were commercially available for cephaloridine. However, this drug was the most active on cephalosporin- or methicillin-resistant *S. aureus* strains which were all penicillinase-positive, but it was less effective than cephalothin on penicillinase-producing methicillin-susceptible strains.

From a practical point of view, incubation time and temperature turned out to be essential, whereas disc content or manufacturer of Mueller-Hinton agar seemed to be of less importance.

Whether the laboratory findings are applicable to in vivo conditions cannot be fully evaluated. It is true that intermittent administration of 3 g of cephalothin every 6 hr may give serum peaks of 200 μg/ml and that some of the resistant strains tested did not grow at 100 μg/ml. In vitro, however, organisms with higher resistance are easily selected in the presence of cephalosporin. Literature is controversial on this point. Although Eriksen (7) found methicillin-resistant strains resistant to cephalexin in vitro, he stated that "cephalexin as well as other cephalosporins can be expected to have a clinical effect on methicillin-resistant staphylococci." Kayser (11), on the other hand, thinks "it is impossible to decide which in vitro conditions are relevant. Therefore, methicillin-resistant staphylococci should be regarded as resistant to all penicillins and cephalosporins." Chabbert (3) reported clinical failure of high-dose treatment with methicillin and cephalothin in heteroresistant strains.

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