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Mannitol Salt Agar-Cefoxitin Combination as a Screening Medium for Methicillin-Resistant *Staphylococcus aureus*

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In disk diffusion tests, cefoxitin is now considered a better indicator than oxacillin for the presence of the *mecA* gene in *Staphylococcus aureus*. A logical extension of this work is the incorporation of cefoxitin into media selective for methicillin-resistant *Staphylococcus aureus* (MRSA). This paper describes the development and subsequent testing of mannitol salt agar containing 4 mg/liter cefoxitin with a unique collection of well-characterized MRSA strains, including low-level methicillin-resistant strains and an equal number of known *mecA*-negative *S. aureus* strains. The agar supported the growth of 96.6% of the *mecA*-positive strains in the collection and inhibited the growth of 100% of the *mecA*-negative strains. These results suggest that selective media based on cefoxitin are superior to those based on oxacillin for the detection of MRSA.

Culture media selective for methicillin-resistant *Staphylococcus aureus* (MRSA) have traditionally been based on blood agar, mannitol salt agar (MSA), or Baird-Parker agar containing methicillin or oxacillin alone or in combination with other antibiotics. An enrichment step consisting of culture in nutrient broth with up to 7.5% sodium chloride prior to inoculation of one of the selective agars mentioned above has also been advocated. Countless reports in the literature (2, 3, 5, 7, 10) have described screening media for MRSA, and this possibly reflects the fact that phenotypic methods based on methicillin or oxacillin have never achieved levels of sensitivity and specificity which have been totally acceptable. However, we have continued to base our screening media on these agents simply because we have had nothing better. Recent reports (4, 8, 9) have shown that cefoxitin is a better agent for prediction of methicillin resistance in *S. aureus*, and disk susceptibility testing with cefoxitin now replaces disk susceptibility testing with methicillin and oxacillin in an increasing number of centers. We describe the use of a collection of well-characterized MRSA strains in the development of a selective medium which replaces oxacillin with cefoxitin.

MATERIALS AND METHODS

MSA (DM160D) was from Mast Diagnostics (Merseyside, United Kingdom), cefoxitin sodium salt was from Sigma-Aldrich, and Etest was from AB Biodisk (Solna, Sweden).

A total of 89 *mecA*-negative and 87 *mecA*-positive *Staphylococcus aureus* strains, described below, and three *S. aureus* control strains (two *mecA*-positive strains, ATCC 33591 and ATCC 43300, and one *mecA*-negative strain, ATCC 29213) were used in the study. Pulsed-field gel electrophoresis and/or multilocus sequence typing (MLST) had previously been performed with all *mecA*-positive strains (9, 10).

The following groups of strains were tested in this study: (i) a 100-strain collection of 75 *mecA*-positive *S. aureus* (MRSA) strains and 25 *mecA*-negative *S. aureus* (methicillin-susceptible *S. aureus* [MSSA]) strains described previously (10), which were used in the initial stages in order to set an appropriate concentration of cefoxitin for use in the medium; (ii) 64 *S. aureus* strains that tested negative for the *mecA* gene (of these, 14 were classified as borderline resistant *S. aureus* (BORSA) strains on account of their elevated MICs for oxacillin); (iii) 8 *mecA*-positive strains which have proved difficult or impossible to classify as such by the use of cefoxitin disks (9); and (iv) an additional four MRSA strains that were initially classified as BORSA strains but that were later found to be positive for the *mecA* gene.

The presence or absence of the *mecA* gene and the presence of the *nuc* gene were determined for all strains by PCR (9, 10).

The MICs for the 100-strain collection were determined by using Etest on Iso-Sensitest Agar (Oxoid, Basingstoke, United Kingdom).

MSA plates were prepared with cefoxitin at concentrations of 2, 3, or 4 mg/liter. The test strains were applied at concentrations of 10^2 and 10^5 viable organisms in the following way: the suspensions were prepared by using fresh overnight cultures on blood agar, matched to a McFarland standard, and diluted in phosphate-buffered saline to suspensions such that when 1 μl was applied with a disposable loop and spread over an area of approximately 6 cm by 2 cm, a count of either 100 or 100,000 colonies was achieved. A control plate of blood agar was used for each strain and dilution. Incubation was carried out in air at 35 to 37°C for 48 h, and the result was read at 18 and 48 h. The growth of any visible colonies after incubation was recorded as a positive result.

The remaining strains were tested as described above, but only on MSA containing 4 mg/liter cefoxitin.

RESULTS

The MIC results for the 100-strain collection were as follows: all 25 *mecA*-negative strains exhibited cefoxitin MICs of ≤2 mg/liter. Seven *mecA*-positive strains had MICs of 4 mg/liter, and the remaining 68 *mecA*-positive strains had MICs >4 mg/liter.

MSA plates with 2 mg/liter cefoxitin allowed the growth of all MRSA and MSSA strains after 48 h incubation. MSA with 3 mg/liter cefoxitin allowed the growth of four MSSA strains and all of the MRSA strains. MSA containing 4 mg/liter cefoxitin allowed the growth of none of the 25 MSSA strains and all 75 MRSA strains. We therefore chose to further evaluate a concentration of 4 mg/liter cefoxitin.

The growth of all strains at 18 h and at 48 h is shown in Table 1. The performance of the medium for the eight strains that were the most difficult to grow is shown in Table 2.

DISCUSSION

Methicillin or oxacillin has been the agent of choice in selective media for *mecA*-positive *S. aureus* for over 20 years.

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TABLE 1. Growth of 89 MRSA and 90 MSSA strains on MSA containing 4 mg/liter cefoxitin after 18 and 48 h incubation and with two inocula

Incubation period and strain	No. of strains that grew/total no. of strains tested at the following inoculum:	
	10^2 CFU	10^5 CFU
18 h		
mecA positive	54/89	80/89
mecA negative	0/90	0/90
48 h		
mecA positive	86/89[a]	86/89[a]
mecA negative	0/90	0/90

[a] See strains 3, 6, and 8 in Table 2.

Mannitol and salt have often been combined as an aid in identification and a selective agent, respectively. Mannitol salt agar with 1 mg/liter oxacillin was shown to have a sensitivity of 90.7% and a specificity of 96.0% (10) for the strains (100-strain collection) used in this study. A similar medium, oxacillin resistant screening agar (1), which is also based on mannitol, salt, and lithium chloride but which contains 2 mg/liter oxacillin, was shown to have a sensitivity of 74% when it was used to test patient specimens plated directly, although its sensitivity increased when it was combined with an enrichment broth.

In recent years cephalosporins have been reported to be particularly successful when they are used as an alternative to oxacillin. A phenyl red mannitol broth containing aztreonam and ceftizoxime (11) allowed the growth of all reference MRSA strains tested, although only a small number of strains were tested. Disks containing the cephamycins cefoxitin and moxalactam on Mueller-Hinton agar were shown to be 100% sensitive and specific for the differentiation of MRSA and MSSA (4). By the use of Iso-Sensitest agar and a 30-μg cefoxitin disk, a similarly high sensitivity and specificity were reported for a total of 457 *S. aureus* strains, many of which exhibited low-level resistance to oxacillin (8). A recent report that described the use of a chromogenic medium and cefoxitin (6) indicated that this medium showed a sensitivity of 89% and a specificity of 99.5% for the detection of MRSA in clinical material.

In our work, the typing of the *mecA*-positive *Staphylococcus aureus* strains (by pulsed-field gel electrophoresis and/or MLST) was used only to ensure that the MRSA strain collection was as heterogeneous as possible. Our results demonstrate that cefoxitin is far superior to oxacillin when it is used in this particular mannitol salt medium. By the use of a 48-h incubation and a 10^2-CFU/ml inoculum, the sensitivity and specificity of the cefoxitin MSA plate method were 100% (compared to 90.7% with oxacillin) and 100% (compared to 96.0% for oxacillin), respectively, when the comparison was made for the strains used in both studies. Over and above the strains used for the initial calibration of the medium, we chose a collection of MRSA strains which have proved difficult or impossible to categorize correctly by using oxacillin or cefoxitin (9). These strains give inhibition zones which fall within ±3 mm of the suggested zone breakpoint for cefoxitin (9). Three of a total of eight strains would have been missed by use of this medium, and five of eight strains would have been missed on oxacillin-containing medium. When we included these eight difficult-to-identify MRSA strains in the calculation, the sensitivity of the cefoxitin medium was 96.0% (85.0% for oxacillin) and the specificity was 100% (96% for oxacillin).

It should be noted, however, that all MSA media are not alike; their salt contents differ, and this may affect the growth of some strains (10). The particular MSA used in the present study contains 3% salt, whereas most other commercially produced MSA media contain 7.5% salt, and the results reported here may not be achievable with other brands.

Based on the cefoxitin MICs for our 100 reference strains, a cefoxitin concentration of 4 mg/liter was chosen. Laboratory studies with a battery of MSSA strains tested repeatedly have shown that the plated medium has a shelf life of 30 days at a temperature of 2 to 8°C. In contrast, selective media containing oxacillin deteriorate after 1 week. Our use of two inocula serves to illustrate the "inoculum effect" on the time to detection. Small numbers of bacteria take longer to produce visible growth, up to 48 h in the present study, while heavier inocula, such as those generated after overnight incubation in enrichment broth, can produce growth on solid media 1 day earlier (1). The choice of the two inocula was based on the expected numbers of bacteria in specimens without enrichment (10^2) and after enrichment (10^5). Without the use of an enrichment stage, our medium requires 48 h of incubation.

In routine use our medium has proved to be robust and reliable. Patient material is plated directly on the medium, and

TABLE 2. Performance of the medium with the eight most-difficult-to-grow MRSA strains after 48 h incubation on MSA with cefoxitin

Strain no.	Strain	Origin	PFGE pattern	MLST type	Cefoxitin MIC (mg/liter)	Growth at 48 h with inocula of 10^2 and 10^5 CFU/ml[a]
1	3615	Denmark, SSI[b]	European CA	80	4	+
2	786	Denmark, SSI	Berlin IV	45	6	+
3	S9200002	Holland	EMRSA 16-like	36	3	−
4	112	France	—[c]	Not typeable	4	+
5	HSL256	France	S. Germany II-like	5	12	+
6	966	Denmark, SSI	—	1	3	−
7	1421	Denmark, SSI	—	152	4	+
8	9-8	Norway	Greece I-like	8	2	−

[a] +, growth; −, no growth.
[b] SSI, Statens Serum Institut.
[c] —, the PFGE patterns of these three strains did not fit those for any internationally recognized strain.

the plates are examined on the following day. Any yellow colonies are tested by a tube coagulase test and a cefoxitin susceptibility test. Those strains that test positive for coagulase and that are resistant to cefoxitin are further tested by the MRSA latex test, and a presumptive identification of MRSA is made. At this point, many laboratories would use a PCR test for the *mecA* gene for confirmation, and in areas where MRSA is frequently detected, it may be acceptable to trust the results of the cefoxitin disk test. Plates which are negative at 18 h are incubated for a further 24 h before they are discarded. The number of *S. aureus* strains which are mannitol negative is not known, but as far as we have been able to ascertain, the number is low and none of the strains in the collection (which were not selected by using MSA medium) were mannitol negative. However, ever more frequently patient specimens contain methicillin-resistant (and cefoxitin-resistant) coagulase-negative staphylococci (CoNS). These are capable of growth on the medium, but *S. epidermidis* produces pink colonies, which are easily distinguishable from the yellow (mannitol-positive) colonies of *S. aureus*. A number of CoNS other than *S. epidermidis* are mannitol positive and therefore produce yellow colonies. In our setting and based on the results for all samples screened for 1 year, the problem occurs in 10% of the samples. With the low prevalence of MRSA in our area of Sweden (<1%), most of the secondary work is with the CoNS. In an area with a higher prevalence (e.g., 10 to 40% in many areas of Europe), MRSA will completely dominate the findings and CoNS will not be perceived as a problem. Irrespective of the prevalence of MRSA, since one has to do the cefoxitin test at this point anyway, the addition of a few negative coagulase tube tests which can be discarded before further work is done is of little consequence.

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