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# Development and Evaluation of a Chromogenic Agar Medium for Methicillin-Resistant *Staphylococcus aureus*

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**We describe here the development and evaluation of MRSA ID, a new chromogenic agar medium for the specific isolation and identification of methicillin-resistant *Staphylococcus aureus* (MRSA). We used S. aureus ID (bioMérieux, La Balme Les Grottes, France) and supplemented it with various antimicrobials, including cefoxitin, ciprofloxacin, oxacillin, and methicillin. Cefoxitin proved to be superior to the other antimicrobials for the selection of MRSA from other strains of *S. aureus*. MRSA ID (consisting of S. aureus ID supplemented with 4 mg of cefoxitin/liter) was evaluated by the use of 747 swabs from various clinical sites. All specimens were also cultured on CHROMagar MRSA and oxacillin resistance screening agar base (ORSAB) and in selective mannitol broth (SMB). A total of 85 MRSA strains were isolated by a combination of all methods. After 22 to 24 h of incubation, 80% of the MRSA strains were isolated as green colonies on MRSA ID, compared with 59 and 62% of the strains that were isolated as colored colonies on CHROMagar MRSA and ORSAB, respectively. After 48 h of incubation, 89, 72, and 78% of the MRSA strains were isolated on MRSA ID, CHROMagar MRSA, and ORSAB, respectively. Sixty-five percent of the strains were isolated by growth in SMB. The specificities of MRSA ID, CHROMagar MRSA, ORSAB, and SMB were 99.5, 99.3, 97.9, and 92.8%, respectively, after 22 to 24 h of incubation. We conclude that MRSA ID is a sensitive and specific medium for the isolation and identification of MRSA.**

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a nosocomial pathogen of major worldwide importance (12) and is an increasingly frequent cause of community-acquired infections (23). Despite much debate, laboratory-based screening for MRSA colonization of patients and health care workers remains a cornerstone of infection control measures to limit the spread of this organism (26).

A wide range of methods has evolved for the detection of MRSA in the clinical laboratory (1, 6, 7, 10, 11, 19–21, 27). Cultural methods using selective media are predominantly used and usually employ oxacillin or methicillin to differentiate MRSA from methicillin-sensitive *S. aureus* (MSSA). Mannitol salt agar supplemented with oxacillin is widely used (10, 18) but has shown limited sensitivity (7, 11) and specificity (6, 28) in some studies. A modified version of mannitol salt agar, oxacillin resistance screening agar base (ORSAB), is more selective due to the presence of lithium chloride and polymyxin and contains aniline blue as a pH indicator (1, 28). Independent studies using ORSAB have revealed similar limitations regarding sensitivity and specificity (1–3).

Resistance to ciprofloxacin has been identified as a surrogate marker for the detection of MRSA, and this agent has been used successfully to supplement Baird-Parker medium (7, 17) and mannitol broth (11). These methods are limited, however, since they cannot detect ciprofloxacin-sensitive MRSA strains, which may occur in some areas (14). Our in-house laboratory method uses a selective mannitol broth (SMB) that utilizes colistin, aztreonam, ciprofloxacin, and sodium chloride as selective agents. MRSA strains are visualized by the acidification of phenol red due to the fermentation of mannitol and/or trehalose. This method was previously reported to be superior to the use of Baird-Parker medium plus ciprofloxacin, and mannitol salt agar plus 4 mg of oxacillin/liter for the isolation of MRSA (11).

Media employing chromogenic enzyme substrates have been developed for the isolation of *S. aureus* and offer a high degree of sensitivity and specificity compared to conventional methods (5, 9, 24). Two studies have reported the adaptation of CHROMagar Staph aureus for the specific isolation of MRSA by the inclusion of methicillin or oxacillin. Merlino et al. (20) examined the inclusion of either agent and found the adapted media to be effective for the growth of multidrug-resistant MRSA strains but less effective for the growth of community-acquired MRSA strains. Kluytmans et al. (16) examined the utility of CHROMagar Staph aureus supplemented with 4 mg of oxacillin/liter and showed that the medium had a high specificity but a low sensitivity for MRSA after 24 h of incubation. Neither of these studies examined the performance of these media with pathological samples. Another chromogenic medium, S. aureus ID, was recently developed and has been shown to have a high sensitivity and specificity for the isolation of *S. aureus* (24). *S. aureus* forms green colonies on this medium due to the production of alpha glucosidase, and the medium is highly selective against nonstaphylococci, including enterococci. The primary aim of this study was to adapt S. aureus ID in order to develop an effective medium for the specific isolation of MRSA from clinical specimens. The secondary aim of the study was to compare this medium with our in-house laboratory method and with other commercially available screening media.

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## MATERIALS AND METHODS

**Culture media.** The ingredients of S. aureus ID were provided by bioMérieux, La Balme Les Grottes, France. The medium was prepared in accordance with the manufacturer's instructions and then cooled to 50°C. Cefoxitin, methicillin, and oxacillin were obtained from Sigma Chemical Company, Poole, United Kingdom. Ciprofloxacin was obtained from Bayer Diagnostics, Newbury, United Kingdom. Each antimicrobial was weighed out according to its potency and prepared as double dilutions in sterile distilled water at a concentration range of 320 to 1.2 mg/liter. A 1-ml aliquot of each dilution was mixed with 19 ml of S. aureus ID medium and poured into a petri dish. This produced agar plates with a final concentration range of 16 to 0.06 mg/liter for each antimicrobial. Media that incorporated methicillin and oxacillin were supplemented with a final concentration of 2% sodium chloride. As a control, S. aureus ID plates without antimicrobials were also prepared (with and without 2% sodium chloride).

ORSAB (CM1008) was obtained from Oxoid Ltd., Basingstoke, United Kingdom, and was supplemented with an antibiotic (SRO195) in exact accordance with the manufacturer's instructions. CHROMagar Staph aureus (TA672) was obtained as a dehydrated medium from M-Tech Diagnostics, Warrington, United Kingdom, and was supplemented with a designated batch of antibiotic (X018) provided by the supplier to produce CHROMagar MRSA. Culture plates of each type were prepared with 20 ml of agar in each plate. SMB was prepared as previously described (11).

**Bacterial strains.** A collection of 36 strains representing the most frequently encountered MRSA types isolated in Europe were provided by the Health Protection Agency Colindale, London, United Kingdom, as freeze-dried cultures. The collection included strains isolated in Belgium, Finland, France, Germany, and the United Kingdom. Another MRSA strain, NCTC 11939, was included as a control. A collection of 21 MSSA strains was compiled comprising 18 consecutive wild strains isolated from blood cultures in our laboratory and three further control strains, NCTC 4163, NCTC 8530, and NCTC 6571.

Each strain was reconstituted and inoculated onto an individual Columbia agar plate (Oxoid). All plates were incubated aerobically overnight at 37°C. Colonies from each bacterial strain were then suspended in an aliquot of sterile deionized water and corrected to a density equivalent to McFarland standard 1.0 by use of a Densimat (bioMérieux). Each suspension was then diluted 1:30 in sterile deionized water to produce a suspension of approximately $10^7$ CFU/ml. Using a multipoint inoculator (Mast Laboratories, Bootle, United Kingdom), we inoculated 1-μl volumes of each diluted bacterial suspension onto the surface of S. aureus ID plates containing the various dilutions of antimicrobials described above. The suspensions were further diluted 1:100, and these dilutions were inoculated in an identical fashion. In summary, each strain was tested at final inocula of approximately 10,000 and 100 CFU per spot. All strains (at both inoculum concentrations) were also inoculated onto CHROMagar MRSA and ORSAB. All media were then incubated aerobically for 48 h at 37°C. After 24 and 48 h, the media were examined and scored as either positive or negative for growth and coloration of each test strain. All tests were performed in duplicate to examine their reproducibilities.

A large batch of S. aureus ID containing 4 mg of cefoxitin/liter was prepared and stored for 8 weeks at 4°C. Batches of ORSAB and CHROMagar MRSA were prepared as described above and stored simultaneously. On day 1 and after 2, 4, 6, and 8 weeks, each type of medium was inoculated with 10 MSSA and 10 MRSA strains, using an inoculum of approximately 10,000 CFU per spot as described above. After 24 and 48 h of incubation, the media were examined and scored as either positive or negative for growth and coloration of each test strain. All tests were performed in duplicate.

**Clinical samples.** A total of 747 swabs referred to our department for MRSA screening was used in this study. These swabs were obtained from the following sites from 205 different patients: the nose (192), the throat (180), axillae (209), the perineum (119), and wounds (47). Each swab was emulsified in 750 μl of sterile physiological saline (0.85%), and 50-μl aliquots of the resulting suspension were inoculated onto ORSAB, CHROMagar MRSA, and S. aureus ID supplemented with 4-mg/liter cefoxitin (MRSA ID). A 50-μl sample was also inoculated into 2 ml of SMB. This procedure ensured that each medium received an equivalent inoculum. All media were incubated in air at 37°C. After 22 to 24 h of incubation, any samples of SMB that showed a color change (i.e., appeared orange or yellow) were subcultured (50 μl) onto Columbia blood agar, which was then incubated for 22 to 24 h in air at 37°C. All culture plates were interpreted by two laboratory staff members after 22 to 24 h and again after 48 h of incubation. Media of each particular type were read independently of each other without knowledge of the results obtained on other media.

**Identification of MRSA.** Mauve colonies on CHROMagar MRSA, green colonies on MRSA ID, and blue colonies on ORSAB were regarded as presumptive MRSA isolates and were subcultured onto Columbia blood agar. Any shade of the expected color was considered a positive result. Uncolored colonies were not investigated further. Confirmation of MRSA isolates was achieved by a combination of tests, including the use of the Slidex Staph Plus latex reagent (bioMérieux UK, Basingstoke, United Kingdom), the tube coagulase test (15), and the Mastalex latex reagent (Mast Laboratories) for the detection of penicillin-binding protein 2a (PBP2a) (4). Any colonies resembling staphylococci on Columbia blood agar (subcultures from SMB broth) were also tested by the protocol described above.

TABLE 1. Numbers of control MRSA strains showing growth and coloration on ORSAB, CHROMagar MRSA, and S. aureus ID supplemented with various antimicrobials

| Medium | No. of MRSA strains (no. after 48 h) showing characteristic | | | |
|---|---|---|---|---|
| | Large inoculum[a] | | Small inoculum[b] | |
| | Growth | Color | Growth | Color |
| CHROMagar MRSA | 37 | 37 | 35 (36) | 35 |
| ORSAB | 37 | 35 (36) | 35 (37) | 30 (33) |
| S. aureus ID with antimicrobial | | | | |
| No antibiotics[c] | 37 | 35 (37) | 37 | 35 (36) |
| Cefoxitin (4 mg/liter) | 37 | 35 (37) | 37 | 34 (36) |
| Ciprofloxacin (1 mg/liter) | 34 | 32 | 34 | 27 (32) |
| Methicillin (2 mg/liter) | 37 | 35 (36) | 36 | 27 (35) |
| Oxacillin (0.5 mg/liter) | 37 | 35 (36) | 34 (35) | 24 (34) |

[a] The large inoculum was approximately 10,000 CFU/spot.
[b] The small inoculum was approximately 100 CFU/spot.
[c] Results without antimicrobials were the same with or without 2% salt.

## RESULTS

**Determination of optimal selective agent for isolation of MRSA.** Table 1 shows the optimal concentrations of antimicrobials incorporated into S. aureus ID. These were the minimum concentrations of antimicrobials required to prevent the growth of all MSSA strains within 48 h of incubation. All of the MRSA strains grew well on S. aureus ID without added antimicrobials and generated the characteristic green coloration, although two strains required 48 h for the color to develop. With a large inoculum (approximately 10,000 CFU/spot), the antimicrobials had little impact on the growth or color of MRSA strains except for three strains that were sensitive to ciprofloxacin, for which the MIC was 0.25 mg/liter. With a small inoculum (approximately 100 CFU/spot), some antimicrobials had a strong impact on the growth of MRSA strains, particularly on their ability to demonstrate alpha glucosidase activity (Table 1). For example, methicillin, ciprofloxacin, and oxacillin prevented the formation of green colonies by 26, 26, and 34% of the MRSA strains, respectively, after 24 h of incubation. In contrast, cefoxitin was the only agent to allow the growth of all MRSA strains with a small inoculum and only affected the coloration of a single MRSA strain (3%) after 24 h of incubation. Based on these results, MRSA ID was formulated by supplementing S. aureus ID with 4 mg of cefoxitin/liter.

With a large inoculum, CHROMagar MRSA supported the growth of all 37 MRSA strains, which all generated mauve coloration after 24 h of incubation (Table 1). With a small inoculum, 35 strains (94.6%) grew and generated mauve colonies after 24 h of incubation. ORSAB was less effective than CHROMagar MRSA, as only 35 strains (94.6%) grew and produced blue coloration after 24 h of incubation with large

TABLE 2. Numbers of MRSA strains isolated from 747 clinical samples on different media

| Medium | No. of MRSA strains (no. after 48 h) | % Sensitivity | | % Specificity | |
|---|---|---|---|---|---|
| | | 22–24 h | 48 h | 22–24 h | 48 h |
| Any[a] | 85 | | | | |
| MRSA ID | 68 (76) | 80 | 89 | 99.5 | 85.6 |
| CHROMagar MRSA | 50 (61) | 59 | 72 | 99.3 | 92.1 |
| ORSAB | 53 (66) | 62 | 78 | 97.9 | 93.1 |
| SMB | 55 | 65 | NA[b] | 92.8 | NA[b] |

[a] Total number of strains isolated.
[b] NA, not applicable.

TABLE 3. Numbers of MRSA strains from different specimen types isolated on different media

| Medium | No. of MRSA strains isolated from specimen type (no. after 48 h) | | | | |
|---|---|---|---|---|---|
| | Axillae (209 swabs) | Nose (192 swabs) | Perineum (119 swabs) | Throat (180 swabs) | Wound (47 swabs) |
| MRSA ID | 5 (7) | 25 (26) | 14 (15) | 14 (17) | 10 (11) |
| CHROMagar MRSA | 5 (7) | 20 (25) | 8 (9) | 10 (12) | 7 (8) |
| ORSAB | 3 (4) | 21 (26) | 8 (10) | 11 (15) | 10 (11) |
| SMB | 4 | 21 | 13 | 9 | 8 |
| Total | 6 (9) | 28 (30) | 15 (17) | 16 (18) | 11 (11) |

inocula and only 30 strains (81%) grew and produced blue colonies with small inocula after 24 h of incubation. All three agar media showed a consistent performance after 8 weeks of storage at 4°C, as all MSSA strains were effectively inhibited and the growth and coloration of MRSA strains were unaffected by the storage. All of these findings were shown to be consistent when the tests were repeated.

**Evaluation of MRSA ID with clinical samples.** Eighty-five swabs from 43 patients yielded confirmed strains of MRSA on one or more media within 48 h of incubation. All strains that agglutinated the Slidex Staph Plus latex reagent also gave positive results in the tube coagulase test and the PBP2a latex agglutination test. No MSSA strains were isolated on any of the test media. Table 2 shows the number of MRSA strains that were isolated by each of the media. A total of 68 isolates (80%) were apparent as green colonies on MRSA ID after 22 to 24 h of incubation, and another 8 isolates were recovered after 48 h of incubation. Of the nine strains that were not recovered on MRSA ID, all were isolated in small numbers and produced no more than two colonies on any other agar medium. Three of these strains were isolated by the use of SMB only. The performance of MRSA ID was superior to that of both CHROMagar MRSA and ORSAB, which recovered 50 (59%) and 53 (62%) of the isolates, respectively, after 22 to 24 h of incubation.

The specificity of MRSA ID after 22 to 24 h (99.5%) was also superior to that of the other methods used (Table 2). The specificities of all agar media decreased after incubation for 48 h, most notably for MRSA ID. Any shade of green coloration was considered a positive result, and a large number of coagulase-negative staphylococci produced pale green colonies only after 48 h of incubation. In practice, these were easily distinguishable from MRSA, which generated intense green colonies after 48 h of incubation.

Table 3 shows the sites from which MRSA strains were isolated and the relative sensitivities of the different media for different specimen types. For example, there was little difference between CHROMagar MRSA, MRSA ID, and ORSAB for the isolation of MRSA from nasal swabs after 48 h of incubation. However, MRSA ID appeared to be superior to all other media for the isolation of MRSA from perineal swabs, possibly due to the impact of the competing flora. Table 4 shows that certain MRSA isolates were detected by one method only and that some patients were determined to be positive for MRSA by one method only. For example, of 85 MRSA isolates, 7 were only detected on MRSA ID within 22 to 24 h of incubation. Similarly, three patients were only determined to be positive for MRSA within 22 to 24 h when MRSA ID was used. This was comparable to one patient who was positive by ORSAB only and two patients who were positive by the use of SMB only. For 12 of 43 positive patients, MRSA remained undetected by CHROMagar MRSA within 22 to 24 h, and similar results were obtained by the use of SMB.

## DISCUSSION

In this study, 62% of the MRSA strains were recovered by the use of ORSAB after 22 to 24 h of incubation, and this rate increased to 78% after the plates had been incubated for 48 h. Apfalter et al. (1) also examined the performance of ORSAB at 24 and 48 h and found that the sensitivity of ORSAB increased from 50.8 to 68.2%. Blanc et al. (3) found that 38% of blue MRSA colonies on ORSAB were visible only after 48 h of incubation. In the present study, CHROMagar MRSA was slightly less sensitive than ORSAB after both 24 and 48 h of incubation. There are no reports of the performance of CHROMagar MRSA with pathological specimens. SMB is an in-house enrichment method that detects MRSA on the basis of mannitol and/or trehalose fermentation (11). In this study, the sensitivity of SMB was 65%, which correlates exactly with that reported in a previous study (11).

MRSA ID showed a substantially better performance than any of the other media tested, and its sensitivity after 22 to 24 h of incubation was superior to that of both CHROMagar MRSA and ORSAB incubated for 48 h. It should be noted that in order for a comparison of methodologies to be possible, dilution of the sample inoculum was required so that equivalent inocula could be delivered to each medium. Had the samples not been diluted, it is possible that the sensitivities of all of the media would have been higher.

Several factors contribute to the successful isolation of

TABLE 4. Numbers of MRSA isolates detected on one medium only and numbers of patients determined to be positive by one medium only

| Medium | No. of MRSA strains (no. after 48 h) | No. of positive patients (no. after 48 h) | No. of positive patients who were undetected (no. after 48 h) |
|---|---|---|---|
| MRSA ID | 7 (5) | 3 (1) | 5 (2) |
| CHROMagar MRSA | 0 (1) | 0 | 12 (7) |
| ORSAB | 1 (1) | 1 (0) | 8 (4) |
| SMB | 4 (3) | 2 | 12 |

MRSA on selective media. These include the ability of the medium to support the growth of MRSA (especially with small inocula), the sensitivity of the indicator system, and the effective inhibition of the competing bacterial flora. The results for pure control strains at small inocula suggest that cefoxitin may be a superior selective agent to oxacillin with respect to the growth of MRSA. On S. aureus ID supplemented with oxacillin at 0.5 mg/liter and with 2% salt, 3 strains of MRSA failed to grow and 13 strains failed to generate coloration after 24 h of incubation. On ORSAB (which contains 2 mg of oxacillin/liter), two strains of MRSA failed to grow and seven failed to generate blue coloration after 24 h of incubation. The presence of 5.5% salt in ORSAB may also have contributed to the failure to isolate some MRSA strains from clinical samples (13). It is difficult to speculate on the reasons for the limited performance of CHROMagar MRSA, as the ingredients of this medium are not disclosed.

There has been renewed interest in the use of cefoxitin for the differentiation of MRSA from MSSA. Mougeot et al. (22) demonstrated that cefoxitin was more effective than oxacillin plus salt for the detection of MRSA strains by disk susceptibility testing. Felten et al. (8) compared a standardized oxacillin disk susceptibility test with a cefoxitin disk susceptibility test against 83 MRSA strains, including 26 strains with low-level resistance. They reported absolute discrimination of all of these strains from 69 MSSA strains by use of the cefoxitin disk test at 37°C with a small inoculum. With the same semiconfluent inoculum, the oxacillin disk test showed a sensitivity of 41% for the detection of MRSA. The authors also reported that a concentration of 4 mg of cefoxitin/liter allowed the growth of all MRSA strains and the inhibition of all MSSA strains (8). Skov et al. (29) also demonstrated 100% sensitivity and 99% specificity for the cefoxitin disk diffusion test for the detection of methicillin resistance in 190 "difficult" strains. In comparison, the standardized oxacillin disk test recommended by the Swedish Reference Group for Antibiotics showed a sensitivity of 78%. These results suggest that MRSA strains grow much more readily in the presence of cefoxitin than oxacillin, possibly due to the enhanced induction of PBP2a by cefoxitin (25). MRSA ID, which exploits the apparent advantages of cefoxitin, has been shown to be an effective medium for the isolation of MRSA from clinical samples and compares well with other available media. Further studies are required to confirm the utility of this medium with a wider range of clinical samples and further collections of unrelated MRSA strains.

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