A comparison of three semi-selective media for the isolation of methicillin-resistant *Staphylococcus aureus*

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Summary. During the 1980s the emergence of multiple antibiotic-resistant strains of *Staphylococcus aureus* posed new problems for infection control worldwide, many of which still remain. In investigations of outbreaks of infection, the laboratory has a key role in identifying infected or colonised patients and staff. The rapid isolation and accurate identification of the causative organisms are essential for the implementation of appropriate control measures. Speed and accuracy in identification, by colonial morphology, is often difficult to achieve in the presence of a mixed population of commensal bacteria.

To this end, the sensitivity of three media for the isolation of methicillin-resistant *S. aureus* (MRSA) from simulated clinical specimens was compared. Initial colonial recognition of MRSA was enhanced on methicillin-milk agar when compared with that on other media.

Introduction

Various techniques have been developed over the last decade for the identification of methicillin-resistant *Staphylococcus aureus* (MRSA). Generally they have been adapted from methods which are now widely established in biotechnology. Gene probes,¹ polymerase chain reaction² and monoclonal antibody techniques³ provide greater accuracy but at present remain beyond the economic resources of the average diagnostic laboratory. Traditional but slower culture methods are usually the only available option. These techniques are on occasion unreliable, because they depend on the subjective visual assessment of the colonial appearance of organisms. Thus, the choice of a primary isolation medium becomes critical. The Combined Working Party of the Hospital Infection Society and the British Society for Antimicrobial Chemotherapy in their "Guidelines for the control of epidemic methicillin-resistant *Staphylococcus aureus*" (EMRSA)⁴ recommended the use of either methicillin-blood agar or methicillin-nutrient agar for the isolation of this organism. Local experience highlighted difficulties in the early recognition of suspected MRSA colonies with either medium. A review of published reports suggested that colonial recognition might be simplified by changing to alternative media previously developed for the routine isolation of staphylococci. Of those available, milk agar⁵ had the advantage that it was easy and economical to produce. It was also noted that on a medium containing full fat milk, the majority of *S. aureus* strains produce colonies with enhanced pigmentation.⁶ This is a result of the

interaction between the carotenoids present in the bacteria and the phospholipids and lipids in the milk. The pigmentation ranges in colour from cream to orange and is a useful identification marker for the organism in the presence of a heavy mixed flora.

The purpose of this study was to compare the ease of identification and isolation of MRSA on methicillin-milk agar with that on methicillin-blood agar and on Chapman's medium⁷ with added methicillin.

Materials and methods

Isolation media

Preliminary work suggested that the most reproducible results in terms of colony size, colour intensity and ease of colonial identification were found on a medium containing ultra-heat treated full-fat milk, which is readily available from any supermarket. Methicillin-milk agar was prepared from Taiyo Agar (Lucas Meyer) 2% supplemented with milk 33% v/v, NaCl (GPR; BDH) 4.5% w/v, Nutrient Broth No. 2 (Unipath) 2.5% v/v and methicillin (Sigma)⁴ 4 mg/L. Taiyo agar is made from Japanese seaweed and is used in this laboratory as a gelling agent. Chapman's medium was prepared from Taiyo agar 1.5% supplemented with Lab Lemco (Unipath) 0.1% w/v, Bacteriological Peptone (Unipath) 1% w/v, NaCl 7% w/v, Mannitol (Analar; BDH) 1% w/v, phenol red solution (BDH) 0.0025% v/v and methicillin 4 mg/L. Methicillin-blood agar was prepared from Columbia Agar (Unipath) 3.5% supplemented with defibrinated horse blood 8% v/v and methicillin 4 mg/L. All plates were dried overnight at 37°C and stored at 4°C until required.

Table I. Comparison of the viable counts of MRSA on the three selective media after incubation for 48 h under optimal laboratory conditions

Test organisms	Average viable count (cfu/ml)				
	Original inoculum	Blood agar	Methicillin-blood agar	Methicillin-milk agar	Chapman's medium with methicillin
MRSA (16 strains)	10 ²	5.0 × 10 ²	0.5 × 10 ²	6.0 × 10 ²	0.9 × 10 ²
	10 ⁴	3.5 × 10 ⁴	0.9 × 10 ⁴	5.0 × 10 ⁴	0.8 × 10 ⁴
	10 ⁶	5.0 × 10 ⁶	0.8 × 10 ⁶	3.5 × 10 ⁶	0.8 × 10 ⁶
EMRSA (14 strains)	10 ²	4.0 × 10 ²	0.7 × 10 ²	2.0 × 10 ²	0.5 × 10 ²
	10 ⁴	3.0 × 10 ⁴	0.7 × 10 ⁴	5.0 × 10 ⁴	0.8 × 10 ⁴
	10 ⁶	2.5 × 10 ⁶	0.8 × 10 ⁶	1.0 × 10 ⁶	0.8 × 10 ⁶

Organisms

Epidemic MRSA strains (EMRSA) and sporadic isolates (SMRSA) were supplied by the Staphylococcal Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London. Clinical isolates of *Proteus* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Flavobacterium* spp., aerobic spore-bearers and methicillin-resistant coagulase-negative staphylococci (MRCNS) were used to prepare the simulated clinical specimens.

Recovery of pure cultures of MRSA

Dilutions of each test MRSA strain were prepared in sterile Ringer's solution (quarter-strength) to give working concentrations of *c.* 10², 10⁴ and 10⁶ cfu/ml. Plates were inoculated with an automatic pipette and were read after incubation under optimal conditions for 20 and 48 h: methicillin-blood agar at 30°C, methicillin-milk agar and Chapman's medium at 35°C, which are the optimal temperatures for the growth of *S. aureus* on these media.^{7,8}

Recovery of MRSA from simulated clinical specimens

The specimens were prepared to resemble, as closely as possible, the mixed flora previously observed during outbreak investigations. Overnight broth cultures of each organism were diluted in sterile Ringer's solution (quarter strength) to give a working concentration of *c.* 10⁸ cfu/ml. The simulated clinical samples were prepared individually by adding varying volumes of these dilutions to sterile quarter-strength Ringer's solution. Plates were inoculated directly from the sample tube with an automatic pipette to minimise sampling error and plated for single colonies. A panel of 10 laboratory staff read the plates after incubation for 20 and 48 h. The panel were unaware of the contents of the specimens. All were shown pure isolates of MRSA on each of the test media prior to the reading. Results were compared by means of a formula described by Goldschmidt and co-workers.⁹

Sensitivity of the test =

$$\frac{\text{Number of true positives}}{\text{Number of true positives} + \text{false negatives}} \times 100$$

Confirmatory tests for identification

All suspected isolates of MRSA were subjected to confirmatory identification tests. A commercial co-agglutination test (Staphylase; Unipath) was the initial screening test for isolates from both methicillin-milk and methicillin-blood agar. The "Rapidec" test (bioMérieux, France) was used to confirm identification if the co-agglutination test was equivocal. Methicillin resistance was confirmed by a standard disk sensitivity test by Stokes method¹⁰ on isoSensitest agar (Unipath) supplemented with lysed horse blood 5%. Plates were incubated overnight at 30°C.⁸ The initial confirmatory identification test from Chapman's medium was the antibiotic sensitivity test followed by the co-agglutination test from the isoSensitest agar.¹¹

Results

On methicillin-milk agar MRSA colonies vary in colour from cream to orange. The colour intensity deepens as incubation is prolonged. MRSA isolates on Chapman's medium produce yellow colonies provided that they use mannitol. On methicillin-blood agar, the pigmentation of MRSA colonies varies between strains and even within the same strain.

The results of the study on the recovery of pure MRSA isolates from the various media are shown in table I. The variation in results is not significant and is compatible with sampling error.

Seventy simulated clinical specimens were used in the main study, 29 (41%) of which contained MRSA either in pure or mixed culture (table II). This is far in excess of the percentage isolation rate usually seen in outbreak investigations. The results of the study are shown in table II. A statistical comparison by the Kruskal-Wallis analysis of variance shows that methicillin-milk agar was more sensitive for the isolation of MRSA than methicillin-blood agar after incubation for 20 and 48 h ($p = < 0.001$ and $0.005-0.001$ respectively). Although there was no statistically significant difference between the sensitivities of methicillin-milk agar and Chapman's medium after incubation for 20 and 48 h ($p = 0.975-0.05$) (table II),

Table II. Comparison of the isolation of MRSA from simulated clinical specimens expressed as the sensitivity of the test medium

Composition of specimen	Sensitivity of test					
	Methicillin-blood agar incubated for		Methicillin-milk agar incubated for		Chapman's medium incubated for	
	20 h	48 h	20 h	48 h	20 h	48 h
MRSA 10^2 – 10^3 cfu/ml	None		None		None	
+ MRCNS	7	33	71	100	11	84
+ coliform/s	33	33	100	100	100	100
+ mixed flora	0	0	25	100	25	25
MRSA 10^4 – 10^5 cfu/ml	58	92	89	100	89	92
+ MRCNS	50	71	78	100	57	86
+ coliform/s	53	65	93	100	100	100
+ mixed flora	62	88	63	100	12	100
MRSA 10^6 – 10^7 cfu/ml	100	100	100	100	79	100
+ MRCNS	61	89	100	100	86	100
+ coliform/s	0	0	100	100	100	100
+ mixed flora	50	100	100	100	100	100

methicillin-milk agar was the only medium on which all the members of the panel were able to identify all the specimens containing MRSA (table II).

Discussion

The choice of initial identification tests relies on the user's ability to recognise a wide range of organisms by their colonial morphology. Specific tests on all isolates are rarely possible because of the cost and time involved. The choice of agar is, therefore, of fundamental importance, since it may facilitate presumptive identification at minimal cost in the shortest time. This applies particularly in large outbreaks when the onus is on the laboratory to identify any colonised or infected staff and patients as soon as possible to ensure that the appropriate infection control procedures are initiated rapidly.

Local experience in MRSA outbreaks suggested probable failures in isolating and identifying low numbers of MRSA in the presence of a heavy mixed commensal flora despite pre-incubating the specimens in salt broth 7%.¹² The aim of this study was to find a suitable isolation medium that would overcome these problems.

The advantage of methicillin-milk agar over Chapman's medium is that the colonial pigmentation does not rely on carbohydrate fermentation, which may be a variable factor. Colonial pigmentation on methicillin milk agar is more uniform in colour and easier to detect in the presence of a heavy mixed flora than on methicillin blood agar. Colonies of MRCNS on methicillin blood agar may closely resemble MRSA in colour; thus, the choice of colony for the co-agglutination test may be difficult. If an MRCNS strain uses mannitol, the colonies will resemble MRSA

on Chapman's medium. On methicillin-milk agar, MRCNS were, with one exception, white. Pigmented colonies of *S. sciuri*, an unusual skin commensal, superficially resembled those of MRSA on both methicillin-milk and methicillin-blood agar but all confirmatory identification tests for MRSA were negative.

The results from the viable count studies showed there was little to choose between the three media after incubation for 48 h. After incubation for 20 h, counting was difficult because of small colonial size; this was particularly noticeable on Chapman's medium. However, it is recommended that Chapman's medium is incubated for 36 h before the plates are examined.⁷ This has implications for its usefulness in outbreak investigations when the onus is on the laboratory to produce an early and accurate report. During this work, some difficulty was experienced with excessive variation in colonial morphology—this has also been reported by other workers.¹³ This problem was more noticeable with methicillin-blood agar and Chapman's medium.

Most of this work concentrated on the isolation and identification of MRSA, under laboratory controlled conditions, from a range of simulated clinical specimens. The sensitivity of each of the test media was determined after incubation for 20 and 48 h. The only medium to achieve 100% sensitivity was methicillin-milk agar after incubation for 48 h, although it has been suggested that overnight incubation is sufficient.⁴ In our experience, a primary incubation time of 20 h improves colonial pigmentation on both methicillin-milk and methicillin-blood agar.¹⁴ This facilitates the initial identification of MRSA, hence improving the sensitivity of the test. The failure to identify any colonised or infected person will prolong an outbreak with accompanying cost implications for the laboratory and hospital concerned.

All colonies isolated on methicillin-milk agar, which were subjected to co-agglutination tests proved to be MRSA. Eighty-five percent of confirmatory tests on isolates from Chapman's medium were positive but only 65% from methicillin-blood agar. This confirms that the initial colonial recognition of MRSA on methicillin-milk agar is easier than on either of the other media. Therefore, methicillin-milk agar represents a saving in laboratory time and costs by reduced use of confirmatory tests.

In this laboratory, methicillin milk agar is now used as a primary isolation medium for MRSA. It still requires further validation by its application in a large outbreak, but results so far have been encouraging. During a small outbreak, methicillin-milk and methicillin-blood agar were used in parallel and, in two cases, colonisation with MRSA was detected only on methicillin-milk agar. This supports the original hypothesis that MRSA may not be recognised easily solely by colonial morphology on methicillin-blood

agar when present in a heavy mixed commensal flora. Methicillin-milk agar is now accepted by the staff as a valuable medium for identification and isolation of MRSA.

Recently the tendency in microbiology has been towards the use of chemically defined media for the identification and isolation of an ever increasing range of bacteria which may cause infection. In this study, the use of methicillin-milk agar was a re-appraisal of an old established medium, although ultra-heat treated milk was used in place of sterilised milk. It returns to the concept of a "simple" medium which is relatively cheap to produce, and, in this laboratory, has proved to be a significant practical advance in the laboratory management of MRSA outbreaks.

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