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Application of Lipovitellin-Salt-Mannitol Agar for Screening, Isolation, and Presumptive Identification of *Staphylococcus aureus* in a Teaching Hospital

JOHN MERLINO,[1]* R. GILL,[2] AND G. J. ROBERTSON[1]

Department of Microbiology and Infectious Diseases, Concord Repatriation General Hospital, Concord, New South Wales, 2139,[1] and Department of Cell and Molecular Biology, University of Technology, Sydney, New South Wales, 2065,[2] Australia

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Lipovitellin-salt-mannitol (LSM) plate medium was examined for its ability to directly isolate, recover, and presumptively identify *Staphylococcus aureus* from 418 clinical specimens. The criteria for medium evaluation included colony morphology reactions, selectivity, and ease of isolation. For 298 specimens used for screening, LSM agar medium was compared with the other conventional media used, mannitol salt agar (MSA), 5% horse blood agar (HBA), and phenolphthalein phosphate agar (PPA), to detect and recover *S. aureus* and methicillin-resistant *S. aureus*. The results indicated that LSM agar is more effective than MSA, HBA, or PPA for the recovery and isolation of *S. aureus* and methicillin-resistant *S. aureus*. On a replicator multipoint inoculation system, we compared the reactions on LSM agar, MSA, and DNase agar of 227 different strains of staphylococci, which included 178 different strains of *S. aureus* and 49 different strains of coagulase-negative staphylococci isolated from clinical specimens. By using both the lipovitellin precipitation activity and mannitol fermentation characteristics, LSM agar gave a 100% correlation in presumptively identifying *S. aureus*. LSM agar may be an alternative plate medium for large hospitals that perform extensive screening for the detection and isolation of *S. aureus*.

Staphylococcus aureus is a common isolate in clinical laboratories. In 1961 (1) the first case of methicillin-resistant *S. aureus* (MRSA) was documented. Since that time, such strains have become a rapidly emerging clinical and epidemiological problem worldwide (14). The isolation, recovery, and identification methods used in any routine clinical laboratory are crucial for the detection of these microorganisms.

On lipovitellin-salt-mannitol (LSM) agar, most strains of *S. aureus* produce a yellow (acid) zone due to the acidification of mannitol and, in addition, produce an opaque zone around the colony due to the presence of lipovitellin lipase activity (4, 6, 8).

From studies carried out by Shah and Wilson (11, 12), the egg yolk factor resulting in the opacity reaction in the medium is a lipase. This lipase has a requirement for fatty acid acceptors such as Ca^{2+} ions, which are present in the egg yolk saline. Calcium may also be contributed by phosvitin, a polyelectrolyte protein that is present in the egg yolk as an insoluble calcium salt and that is converted to the soluble sodium salt with the loss of calcium. The lipase from *S. aureus* acts on the lipid moiety (acyltriglyceride), resulting in alterations in the solubility of lipovitellenin, a low-density lipoprotein (15). Lipolysis is optimal at pH 8. The optimal pH for the opacity reaction is either 5.5 or 8. The protein (vitellenin) in lipovitellenin has an isoelectric point at pH 5.5. Shah and Wilson (11) proposed that slight alterations in the stabilizing lipid due to lipolysis and the very low solubility of vitellenin may drastically alter the solubility of lipovitellenin at pH 5.5. The *S. aureus* lipase enzyme is also able to hydrolyze coconut oil, peanut oil, olive oil, and egg yolk oil.

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, Concord Repatriation General Hospital, Hospital Rd., Concord, NSW, 2139, Australia. Phone: (02) 736 6642. Fax: (02) 736 7868.

The present study examined the use of LSM agar in a routine clinical laboratory for the recovery and identification of *S. aureus* and MRSA. It should be made clear that the investigation that was undertaken is not for the screening of MRSA only, but is for the detection of both methicillin-susceptible *S. aureus* and MRSA. The study was divided into three parts: part 1 describes the colonial morphology of *S. aureus* and other organisms that may grow on LSM agar; part 2 evaluates the effectiveness of LSM agar as a selective medium for the isolation and recovery of *S. aureus* in comparison with those of 5% horse blood agar (HBA), phenolphthalein phosphate agar (PPA), and mannitol salt agar (MSA); and part 3 compares the accuracy of reactions of *S. aureus* on LSM agar, MSA, and DNase agar with the accuracy of reactions of coagulase-negative staphylococci by a multipoint inoculation technique (replicator).

(Part of this study was submitted by Rylee Gill as her M.S. thesis at the Department of Cell and Molecular Biology, University of Technology, Sydney, Australia.)

MATERIALS AND METHODS

Specimens and bacterial strains. The specimens (n = 120) used in the study were from routine clinical material received for examination at the Department of Microbiology and Infectious Diseases at Concord Repatriation General Hospital. The specimens included ulcer swab, surgical wound, drained fluid, and bone and tissue samples, eye material swabbings or scrapings, sputum specimens, and blood culture specimens.

Screening specimens (n = 298) included nose and groin swab specimens routinely obtained from preadmission or preoperative patients. Separate nose and groin swab specimens were taken and were inoculated onto separate LSM agar plates and other routine media. The isolation of *S. aureus* from one or both sites from the same patient was counted as only one positive recovery.

The bacterial strains used in this study also included frozen (−70°C) stock organisms previously isolated and identified from routine clinical specimens. This included 178 different strains of *S. aureus* and 49 different strains of coagulase-negative staphylococci. Stock cultures were thawed and inoculated onto HBA plates. Following incubation at 37°C for 18 to 24 h in air, single colonies were subcultured onto a second HBA plate, which was incubated under condi-

tions identical to those described above. Growth from this plate was used to prepare inocula for the third part of this study.

Sampling and inoculation procedures. With the exception of nose and groin specimen screenings in which one swab specimen was taken from each of two separate sites of the same patient, all other specimens tested were from separate sites of different patients. No multiple swab specimens were included in this survey. The pattern of inoculation was in a set order: routine media first and then the LSM medium. Once the primary inoculation was made with a loop (from pus, blood, sputum, etc.) or a swab, a loop or a straight wire was used to spread the material to achieve isolated colonies.

Culture media. The LSM agar plate medium prepared in this study contained MSA (CM85; Oxoid, Victoria, Australia), with 20 mg of egg yolk per ml added to each plate. Each 90-mm-diameter petri dish contained 18 to 20 ml of agar medium.

For screening only, LSM agar was compared with PPA, MSA, and HBA (Columbia base; CM331; Oxoid). PPA plates were prepared with nutrient agar base (CM3; Oxoid) to which 1% phenolphthalein diphosphate-tetrasodium salt (P-9875; Sigma, St. Louis, Mo.) solution was added. MSA plates (CM85; Oxoid) were prepared according to the manufacturer's instructions.

S. aureus ATCC 25923 was used as a quality control strain to monitor batch variability. HBA was used to test organism viability.

Identification methods. Methods of identification included Gram stain reactions, morphology, and catalase (3% [wt/vol] hydrogen peroxide; UN2014; BDH, Kilsyth, Victoria, Australia), mannitol salt fermentation, slide, and tube coagulase (lyophilized rabbit plasma; bioMerieux, Marcy l'Etoile, France), and DNase (CM321; Oxoid) activities (8). API20 Staph galleries (bioMerieux systems) were used for confirmatory identification in some circumstances when conflicting results were obtained.

Conditions and procedure of testing. Two LSM agar plates were added to the testing medium regimen routinely set up in our department, and a comparison of recoveries for each specimen was made at 30 and 37°C (13). The plates were examined at 24 and 48 h, with an additional reading at 30 h. Colonies were presumptively identified from LSM agar as *S. aureus* by catalase and colony morphology reactions (e.g., fermentation of mannitol and lipase precipitation activity). All mannitol-negative *S. aureus* strains were detected by positive catalase and lipase activities. Mannitol-positive, coagulase-negative staphylococci were detected by removing a single colony from an LSM agar plate and observing no lipase activity (opaque precipitation) on the surface of the plate.

Susceptibility testing. The susceptibility patterns of the isolated organisms were confirmed with a replicator-agar plate multipoint inoculation system. Alternatively, a disc agar diffusion method of the National Committee for Clinical Laboratory Standards with (5-μg methicillin discs (Oxoid systems) was also used (13). *S. aureus* ATCC 25923 was used as a quality control strain to monitor antibiotic potency.

Multipoint inoculation system. The multipoint inoculation technique used is the method currently used at Concord Repatriation General Hospital, as described by Funnell et al. (5). Isolated colonies were suspended in 5.0 ml of 0.1 M phosphate-buffered saline (BR14a; Dulbecco A tablets; Oxoid) to a concentration approximating 10^8 CFU/ml, and the inocula were then pipetted into appropriate sterile wells (inoculum pot). The inoculum pot was positioned onto a Mast inoculator to which an inoculum head was affixed. The head carried an array of 36 free-floating stainless steel ground replicating pins (1 mm in diameter) in a configuration identical to that of the wells of the inoculum pot. Each pin transferred 10^4 CFU of inoculum to the surface of the culture medium. The LSM agar, DNase agar, and MSA plates were dried (the lids were kept ajar) at 35°C for 1 h prior to inoculation.

Statistical analysis. The results of isolation and recovery of *S. aureus* on LSM agar and other conventional testing media were compared by using the chi-square test.

RESULTS AND DISCUSSION

Description and growth of *S. aureus* and other organisms on LSM agar. For the purpose of obtaining a description and other growth characteristics, 120 clinical specimens were directly tested on LSM agar and other routine testing media. From these specimens, 40 *S. aureus* and 5 MRSA strains were isolated; these were later confirmed by tube coagulase and susceptibility testing. After 24 h of incubation *S. aureus* colonies appeared yellow on LSM agar. Precipitation occurred in the medium surrounding *S. aureus* colonies because of lipase activity. Increased precipitation (larger zones) was observed after overnight incubation at 37°C than after overnight incubation at 30°C. The degree of precipitation increased with time. This, together with a positive catalase reaction, made the identification of *S. aureus* isolates easy. MRSA strains were indistinguishable from methicillin-susceptible *S. aureus* on LSM agar. Coagulase-negative staphylococcal colonies appeared white-pink, and the surrounding agar remained pink. No precipitation was apparent, and the catalase reaction was positive. Enterococcal isolates appeared as clear, pink, tiny colonies with a yellow background; no precipitation was observed. Although some enterococci ferment mannitol, they were clearly differentiated from the other organisms with a negative catalase test. Coryneforms, usually referred to as diphtheroids, appeared as pinpoint-like colonies; the background remained pink and gave a positive catalase reaction with no precipitation.

TABLE 1. Recovery of *S. aureus* and MRSA during screening for MRSA after 24 h of incubation

Organism(s) type	No. of *S. aureus* strains recovered from 298 specimens	
	LSM agar	Conventional media[a]
S. aureus	35	21
MRSA[b]	6	4
Total	41	25

[a] Conventional media included HBA, PPA, and MSA.
[b] The susceptibilities of the MRSA isolates were determined by both agar disc diffusion and agar dilution after isolation.

Pseudomonas spp., *Proteus* spp., other members of the family *Enterobacteriaceae*, and *Candida* spp. did not grow on LSM agar or MSA. On LSM agar and MSA most other organisms were inhibited by the high level of salt (7.5% NaCl), as described by Chapman (3), making easier the isolation of *S. aureus* from ulcers and swab specimens from superficial sites, where colonization with other commensal organisms may prove to be difficult to determine.

As stated previously from observations of LSM agar at 30 and 37°C, we found that precipitation was influenced by temperature and time. Precipitation was greater around *S. aureus* colonies at 37°C than at 30°C at 30 h. This, however, did not influence our results, since growth and colonial morphology at 24 h were sufficient to recognize the *S. aureus* strains isolated on LSM agar. However, on MSA alone, the *S. aureus* colonies were smaller and needed 30 to 48 h of incubation before the full colony size was reached, and then the only indicator was mannitol fermentation. From this we concluded that the lipovitellin in LSM agar may act as an indicator of an organism and may provide added enrichment for the growth of *S. aureus.*

LSM agar as a screening agar. In the next part of the study LSM agar was compared with the HBA, PPA, and MSA plate media routinely used in our laboratory for the isolation of *S. aureus* and MRSA. From 298 nose and groin swab specimens received, 41 different strains of *S. aureus* including 6 different MRSA strains were isolated on LSM agar. With our routine plating media, only 21 strains of *S. aureus* and 4 different MRSA strains were isolated; these identifications were confirmed by antibiotic disc diffusion and replicator agar plate dilution methods. The use of LSM agar resulted in an increased percent recovery, and the rate of isolation of *S. aureus* and MRSA on LSM agar compared with those on the media that we routinely used is presented in Table 1. Statistical analysis of the data in Table 1 by the chi-square test indicates that the P value ($\chi^2 = 4.362$ with 1 degree of freedom) between LSM agar and the conventional media is <0.05, making the difference between the two groups of media significant. Therefore, in this study, LSM agar was a better medium than HBA, MSA, and PPA for the recovery and isolation of *S. aureus* and MRSA.

TABLE 2. Ability of three media to differentiate coagulase-positive staphylococci from coagulase-negative staphylococci after 24 h of incubation

Medium	% of isolates showing positive reaction		Accuracy of medium for discriminating *S. aureus* and CNS[a]	
	S. aureus (n = 178)	CNS (n = 49)	Sensitivity (%)	Specificity (%)
LSM agar	100[b]	0	100	100
DNase agar	98.9	6.0	98.9	94.0
MSA	98.9	20.5	98.9	79.6

[a] CNS, coagulase-negative staphylococci.
[b] Two strains did not produce a strong reaction after 24 h; they only produced yellow zones. However, following 30 to 48 h of incubation, precipitation was detected. Two strains produced an egg yolk precipitation reaction but did not ferment mannitol.

Differentiating *S. aureus* and coagulase negative staphylococci on a multipoint inoculating system. LSM agar was compared with MSA and DNase agar by using a replicator multipoint inoculating system (2, 10). Of 178 different strains of *S. aureus* tested, 100% gave a positive lipovitellin reaction on LSM agar, while 98.9% positive reactions were obtained on both MSA and DNase agar plate media. When we tested 49 different coagulase-negative staphylococcal species using the same procedure, we found that MSA gave 20.5% positive reactions (fermentation of mannitol) and 6% false-positive DNase agar reactions. No lipovitellin activity was observed with the coagulase-negative staphylococcus species, indicated as in Table 2.

As indicated in Table 2, no statistically significant difference was found between MSA, DNase agar, or LSM agar in identifying *S. aureus*. There was also no significant difference between DNase and LSM agars in differentiating coagulase-negative staphylococcal species and *S. aureus*. However, the results indicate that LSM agar is significantly better than MSA in distinguishing coagulase-negative staphylococci and *S. aureus*. LSM agar medium showed a sensitivity of 100% and a specificity of 100% in differentiating *S. aureus* and coagulase-negative staphylococci. MSA showed a sensitivity of 98.9% but a specificity of only 79.6% in identifying *S. aureus*.

It was also noticed that the growth at the inoculum site was much larger in diameter on LSM agar than MSA for *S. aureus* strains, confirming results from an earlier part of our survey and other studies (7) that lipovitellin may provide added enrichment for the growth of these organisms.

A recent study (14) of 53 microbiology laboratories affiliated with university or tertiary care hospitals in 10 European countries reported an MRSA screening program with divided agar plates, half of which consisted of MSA and the other half of which consisted of Mueller-Hinton agar supplemented with 4% NaCl and 6 mg of oxacillin per liter. Results from our replicator studies and other studies (9, 16) reinforce the fact that although fermentation of mannitol has been described as a characteristic for the differentiation of coagulase-positive and coagulase-negative staphylococci, the use of MSA plates requires secondary confirmation testing with colonies from the primary plates. LSM agar may also have an added advantage over Mueller-Hinton agar supplemented with 4% NaCl and oxacillin, since studies by Zonby and Starzyk (16) pointed out that the MRSA screen plate medium does not inhibit some gram-negative bacilli such as *Proteus* spp. and *Pseudomonas* spp., decreasing the percent recovery during direct screening, making it more time-consuming for the technologist on the bench.

In this report we propose that the action of lipase activity on egg yolk and the addition of 7.5% sodium chloride to the phenol red-mannitol-agar results in an improved medium for the recognition of coagulase-positive staphylococci, while it inhibits most gram-negative bacteria. Our findings were very similar to those obtained in other studies (7, 9, 16) and showed that LSM agar allows for the good growth of all staphylococcal species, while it inhibits most gram-negative rods. The medium turns yellow for mannitol-positive organisms and gives an opaque (precipitated) zone around colonies that are lipovitellenin positive. *S. aureus* colonies also take on a bright yellow pigment. Samples which contained mixed populations of coagulase-positive and coagulase-negative staphylococcal strains were easily differentiated on culture.

In summary, LSM agar in our laboratory proved to be more effective than MSA, HBA, and PPA medium in the isolation and recovery of *S. aureus* and MRSA from clinical specimens. No significant difference was found between MSA, DNase agar, or LSM agar in identifying *S. aureus*. Nor was there a difference between DNase agar and LSM agar in differentiating coagulase-negative staphylococci from *S. aureus*. LSM agar was, however, significantly better than MSA in distinguishing mannitol-positive coagulase-negative staphylococci. The egg yolk providing the lipovitellin reaction is a definite asset to the modified medium and, for laboratories, may be an alternative to the more expensive commercially available differential screening agars.

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