

NEW SELECTIVE AND DIFFERENTIAL MEDIUM FOR COAGULASE-POSITIVE STAPHYLOCOCCI ALLOWING RAPID GROWTH AND STRAIN DIFFERENTIATION¹

SYDNEY M. FINEGOLD AND EDWARD E. SWEENEY

Departments of Medicine, Veterans Administration Center and University of California Medical Center, Los Angeles, California

Received for publication October 12, 1960

Staphylococcal infection and cross-infection in hospitals is a very important problem. Investigation and management of such infection and cross-infection would be facilitated by a simple, rapid, and reliable method for the primary isolation of *Staphylococcus aureus* from body fluids and exudates, from feces, from carriers, from the air, and from various surfaces and fomites in the hospital environment. It is common for staphylococci to be present in mixed culture with a variety of other organisms in many of these sites. The total number of organisms present may be very large (10^8 to 10^{10} per gram in the case of feces, for example) and staphylococci may form a relatively small percentage of the total bacterial population. Furthermore, when staphylococci are present in mixed culture with certain gram-negative bacilli, the staphylococci may be suppressed so that recovery is difficult or impossible except by use of selective media. Such media offer certain other advantages. A high degree of selectivity allows larger samples to be examined without the danger of overgrowth by extraneous organisms. Where mixtures of organisms are not a problem, a selective medium may provide more rapid presumptive and definitive identification of organisms than non-selective media.

Chapman (1945, 1946) has proposed media that are selective for staphylococci and have some value in differentiating between coagulase-positive and coagulase-negative strains. These media are selective chiefly because of a high sodium chloride content; differentiation is based on pigmentation and acid production from mannitol. A more selective medium using tellurite and lithium chloride and a high pH

¹ This work was supported by U. S. Public Health Service Research grant E2662 and the V. A. Cooperative Study Group for Staphylococcal Infections.

was introduced by Ludlam (1949). Gillespie and Alder (1952) utilized an egg-yolk medium with a high sodium chloride content; on this medium most coagulase-positive staphylococci develop halos of opacity about the colonies.

Several media have been developed which assist rapid identification of pathogenic staphylococci. Duthie and Lorenz (1952) incorporated human fibrinogen and plasma in plates; colonies of coagulase-positive staphylococci are surrounded by opaque rings of fibrin on this medium. Other differential media utilize the phosphatase reaction (Barber and Kuper, 1951) and deoxyribonuclease activity (Weckman and Catlin, 1957) for detecting pathogenic staphylococci.

A number of improvements and combinations of the above mentioned selective and differential media have been proposed more recently. There is still need for a better selective and differential medium. There have been reports of inconsistencies between different batches of some of the selective media mentioned above. These media may either fail to yield growth from small inocula or fail to give quantitative recovery from measured inocula. Other defects in certain media include: relatively poor selectivity, slow growth of coagulase-positive staphylococci, atypical morphology or pigmentation (as compared to nonselective media), the need for surface streak technique, and the need to subculture before doing the coagulase test. Some of the differential tests in certain of these media are positive with organisms other than staphylococci and even with some coagulase-negative staphylococci.

Recently a new screening test and selective medium for detection of "epidemic" strains of *S. aureus* was reported by Moore (1960). This medium inhibits the growth of most coagulase-negative staphylococci and many coagulase-positive strains (apparently chiefly "nonepidemic" strains). Many coliforms are inhibited

TABLE 1
*Phage types tested on polymyxin
staphylococcus medium*

3A, 3B, 3C, 54	42B, 52A, 80, 81
3A, 3B, 3C, 71	42B, 80, 81
3A, 7, 42B, 42D, 54, 73, 77	42D
3A, 7, 42D, 54, 73, 77	42D, 54, 73, 77
6, 42B, 42E, 47, 54, 73	42D, 72B, 73
6, 42D, 54	47, 53, 54, 73, 77
6, 42D, 54, 73	47, 54
7, 42B, 42E, 47, 54, 73, 77	47, 54, 77
7, 42B, 47, 54, 73, 77	54
7, 42D, 47, 54, 73, 77	54, 73
7, 42D, 54, 73, 77	54, 77
42B, 42D, 47, 54, 73	77
42B, 47, 54, 73	80, 81

and swarming is prevented with most strains of *Proteus*.

The present report concerns a new selective and differential medium for coagulase-positive staphylococci based on a mechanism apparently differing from those operating in media previously reported. This new medium seems to offer a number of important advantages over previously available media.

MATERIALS AND METHODS

Nutrient agar (BBL) was used as a base for the selective medium. Polymyxin B standard powder (Burroughs Wellcome Company) was weighed and added to the nutrient agar base either before or after autoclaving. Cycloheximide (Acti-Dione) was routinely added in a concentration of 400 $\mu\text{g}/\text{ml}$. *Staphylococcus* medium No. 110 (BBL) and mannitol salt agar (BBL) were used according to the manufacturer's recommendations.

Phenolphthalein diphosphate agar plates were prepared from nutrient agar (BBL) and the sodium salt of phenolphthalein phosphate (Delta Chemical Works, Inc.). They were used according to the methods of Barber and Kuper (1951). Slide and tube coagulase tests were performed according to the Recommended Procedures for Laboratory Investigation of Hospital-Acquired Staphylococcal Infections, Proceedings of the National Conference, 1958. Positive, weakly positive, and negative controls were run concurrently. Sensitivity tests were performed using a plate dilution technique with serial twofold dilutions of polymyxin incor-

porated in nutrient agar plates. The inoculum consisted of a standard loopful of a 10^{-2} dilution of a 24-hr culture in brain heart infusion broth (BBL). Plates were incubated at 37 C for 24 hr and the minimal inhibiting concentration was considered the lowest concentration with no growth. Mannitol fermentation was checked by inoculating phenol red mannitol agar (BBL) plates with a loopful of growth from a 24-hr culture on blood agar and then incubating for 24 hr at 37 C and noting acid production.

All microorganisms used in this study were isolated from either clinical specimens or the hospital environment within the past 3 years. The coagulase-positive staphylococci included a wide variety of phage types (Table 1) and a number of nontypable strains.

RESULTS

Table 2 gives the results of coagulase tests, mannitol fermentation studies, and polymyxin B sensitivity tests with 207 strains of staphylococci and micrococci. Of 117 strains of coagulase-

TABLE 2
*Polymyxin B sensitivity of staphylococci
and micrococci*

No. of Strains	Coagulase	Mannitol Fermentation	Minimal Inhibitory Concn Polymyxin
115	+	+	$\mu\text{g}/\text{ml}$ >100.0*
2	+	-	>100.0
11	-	+	50.0
15	-	+	25.0
7	-	+	12.5
6	-	+	6.2
3	-	+	<6.2
5	-	-	100.0†
22	-	-	50.0
12	-	-	25.0
6	-	-	12.5
1	-	-	6.2
2	-	-	<6.2

* One strain grew with only minute colonies in the presence of any amount of polymyxin.

† Four of the five strains grew with only minute colonies and all of the strains showed some reduction of growth on plates containing 50 $\mu\text{g}/\text{ml}$ of polymyxin.

positive staphylococci (*S. aureus*) tested, 2 of which were mannitol-negative, all but 1 grew optimally in the presence of 100 $\mu\text{g/ml}$ of polymyxin (although 1 strain grew only with minute colonies). Ninety strains of coagulase-negative staphylococci (including both mannitol-positive and mannitol-negative strains) were inhibited completely by 100 $\mu\text{g/ml}$ or less of polymyxin B. In other words, nutrient agar containing 100 $\mu\text{g/ml}$ of polymyxin B supported the growth of all strains of *S. aureus* tested and would not permit growth of any *Staphylococcus epidermidis* or micrococci during a 24-hr incubation period. We have some preliminary evidence which suggests that micrococci or *S. epidermidis* may appear on these plates if they are incubated for 48 to 72 hr or longer. The margin of safety with polymyxin is small. Twenty-one of 27 strains tested failed to grow in the presence of 200 $\mu\text{g/ml}$ and 2 of 9 strains were completely inhibited by 150 $\mu\text{g/ml}$.

Eighty-six strains of gram-negative bacilli (including *Escherichia coli*, Klebsiella, Aerobacter, intermediate coliforms, paracolons, and Pseudomonas) were tested and all were completely inhibited by 100 $\mu\text{g/ml}$ or less of polymyxin B. Ten of 11 strains of *Proteus mirabilis* and 15 strains of *Proteus vulgaris* grew on nutrient agar containing polymyxin B in a concentration of 50 $\mu\text{g/ml}$. However, the colony size was reduced to at least one-half of that noted on control nutrient agar plates after 24 hr of incubation. Incubation for an additional 48 hr (37 C) largely eliminated the difference in colony size. Of further interest is the fact that spreading did not occur with any of these *Proteus* strains on either plain nutrient agar or that containing polymyxin, whereas all strains exhibited typical swarming on blood agar plates set up concurrently.

TABLE 3

Effect of autoclaving on sensitivity of staphylococci to polymyxin B

Minimal Inhibitory Concn:	No. of Strains
Higher after autoclaving*	8
Same after autoclaving	31
Lower after autoclaving*	1

* Difference never more than one twofold dilution.

Sensitivity tests have not been performed with other microorganisms, but *Bacillus* species have been noted in cultures of the hospital environment utilizing nutrient agar plates containing 100 $\mu\text{g/ml}$ of polymyxin B. These organisms retain their typical and very characteristic morphology. Studies of the hospital environment indicate that strains from this source, in contrast to laboratory stocks, may be inhibited by 100 $\mu\text{g/ml}$ of polymyxin. Seventy-five $\mu\text{g/ml}$ is a satisfactory concentration.

Studies with plating of 10-fold serial dilutions of broth cultures of *S. aureus* on media containing various concentrations of polymyxin and on control media indicate that small inocula (of the order of 50 to 200 cells) resist the action of polymyxin as well as large inocula.

Coagulase-positive staphylococci grow rapidly on polymyxin nutrient agar plates so that good

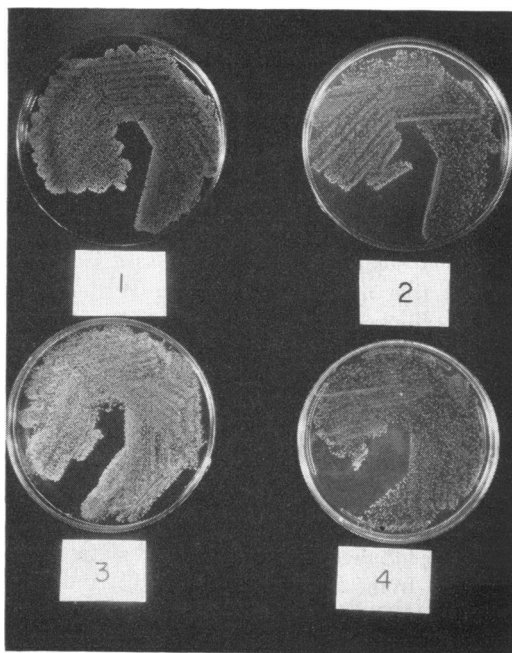


Fig. 1. Nasal swab right nostril S. W. 1, Blood agar; 2, nutrient agar; 3, staphylococcus medium no. 110; 4, polymyxin staphylococcus medium. All plates incubated at 37 C for 24 hr. Virtually pure growth of *Staphylococcus aureus* on plates 1, 2, and 3. Pure growth of *S. aureus* on plate 4. Difference in amount of growth may be attributed to different size inocula since separate swabs were used for each culture. Subject's nasal septum deviates to right.

colony size is achieved within 24 hr. Colony morphology is entirely typical, although when there is crowding on plates, colonies are small. Pigmentation is generally well developed in 24 hr on this medium. There are some differences in degree of pigmentation with different strains, and this (together with other features of colony morphology) enables one to pick up different strains of *S. aureus* when several are present on one plate.

Slide and tube coagulase tests performed directly from the polymyxin staphylococcus medium give results identical with those set up from control nutrient agar plates. Phenolphthalein diphosphate, incorporated in the polymyxin nutrient agar plates in a final concentration of 0.01%, did not interfere with the selectivity or other desirable features of the polymyxin medium, and phosphatase tests were positive with 18 strains of *S. aureus* on these, as well as on control phenolphthalein diphosphate plates.

Cycloheximide incorporated in the polymyxin

staphylococcus medium in a concentration of 400 $\mu\text{g}/\text{ml}$ served to cut down the overgrowth of various saprophytic fungi when plates were held for several days. Cycloheximide did not have any adverse effects on the polymyxin nutrient agar plates nor did it interfere with the performance of coagulase tests directly from this medium or with the phosphatase reaction when phenolphthalein diphosphate was incorporated.

Table 3 summarizes the results of a study comparing the sensitivity of 40 strains of staphylococci (mostly coagulase-negative) to polymyxin B under two circumstances—with the polymyxin added prior to autoclaving (121 C steam pressure for 15 min) and with the polymyxin added aseptically to the autoclaved, cooled nutrient agar. There is relatively little loss of polymyxin activity during the autoclaving (as evidenced by higher minimal inhibitory concentrations).

Brief comparisons of the polymyxin staphylococcus medium with commonly used selective media such as staphylococcus medium no. 110 and mannitol salt agar were made. The poly-

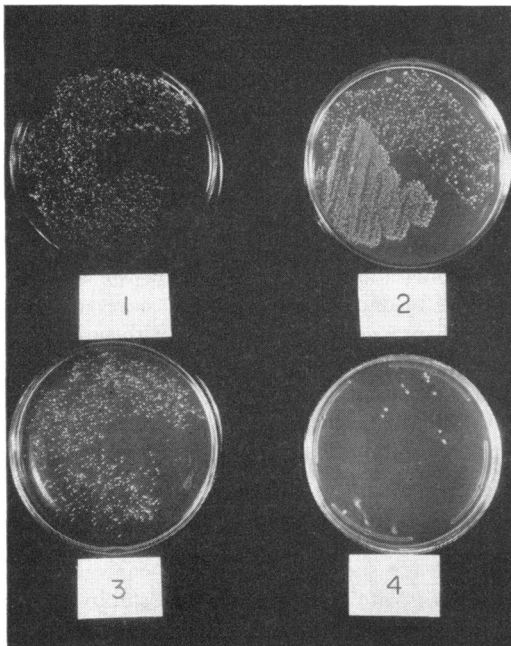


Fig. 2. Nasal swab left nostril S. W. (same subject as in Fig. 1). 1, Blood agar; 2, nutrient agar; 3, staphylococcus medium no. 110; 4, polymyxin staphylococcus medium. All plates incubated at 37 C for 24 hr. Plate 4 shows only *Staphylococcus aureus* (15 colonies). The other plates show many other organisms in addition.

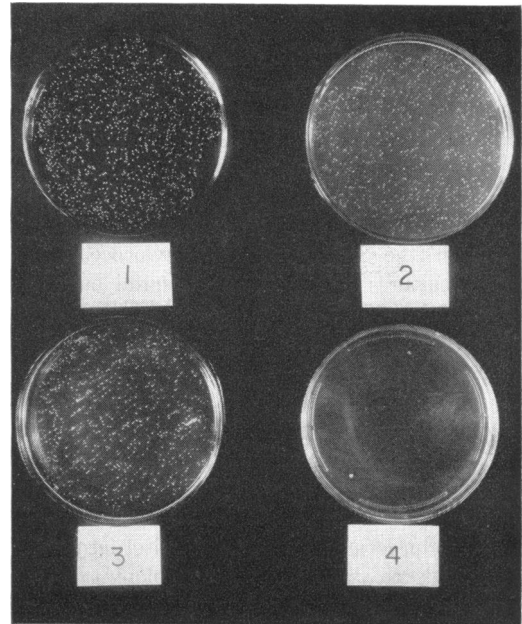


Fig. 3. Nasal swab right nostril E. N. 1, Blood agar; 2, nutrient agar; 3, staphylococcus medium no. 110; 4, polymyxin staphylococcus medium. All plates incubated at 37 C for 24 hr. One colony of *Staphylococcus aureus* on plate 4; other plates show moderate growth of other organisms.

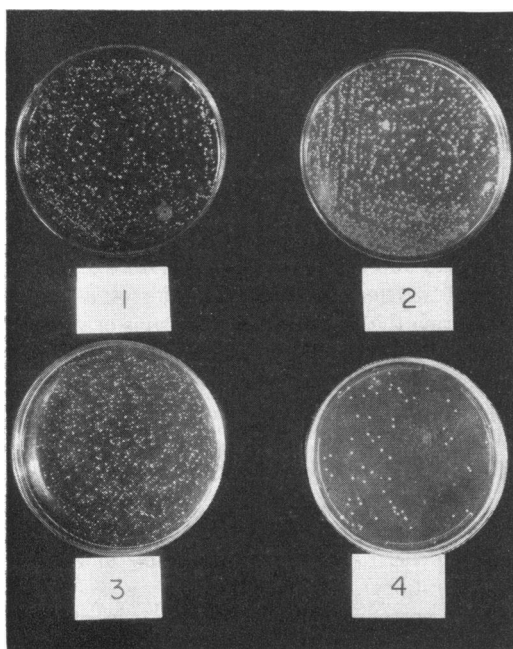


Figure 4. Culture of floor from general surgical ward (4 sq. in. area swabbed with swab moistened in sterile saline). 1, Blood agar; 2, nutrient agar; 3, staphylococcus medium no. 110; 4, polymyxin staphylococcus medium. All plates incubated at 37 C for 24 hr. Plate 4 shows a pure growth of *Staphylococcus aureus*. Other plates, in addition to an equivalent number of *S. aureus*, show large numbers of a variety of other microorganisms.

myxin medium showed several distinct advantages in the isolation of staphylococci from mixed cultures and from the hospital environment. In these studies the vast majority of colonies on the polymyxin medium, after 24 hr of incubation, have been coagulase-positive staphylococci. Growth is much slower on the other media and pigment develops slowly. Many colonies of organisms other than coagulase-positive staphylococci will grow on the high sodium chloride content media (Figs. 1 to 4). Acid produced as a result of mannitol fermentation tends to diffuse from the colony so that difficulties in interpretation may arise on crowded or semicrowded mannitol salt agar plates. Lack of correlation between mannitol fermentation and coagulase production is illustrated in Table 2.

DISCUSSION

Studies by several groups (Pulaski et al., 1949; Kagan et al., 1951; Hirsch, McCarthy,

and Finland, 1960) have clearly indicated that virtually all strains of *E. coli*, *Aerobacter*, *Klebsiella*, paracolon bacilli, *Pseudomonas*, *Salmonellae*, *Shigellae*, and *Haemophilus influenzae* are sensitive or relatively sensitive to polymyxin B. *Proteus* species, on the other hand, are generally quite resistant to this agent. Relatively few studies have been done with staphylococci, but the group as a whole has been considered polymyxin resistant. Hirsch et al. (1960) found only 1 of 26 strains of *S. aureus* inhibited by 100 $\mu\text{g}/\text{ml}$. Pulaski et al. (1949), on the other hand, found 6 strains of staphylococci sensitive to from 1.2 to 5.0 $\mu\text{g}/\text{ml}$ of polymyxin B; these strains were not further characterized.

The present study points out some significant differences in sensitivity to polymyxin B of *S. aureus* and of *S. epidermidis* and micrococci. This difference, together with the inhibitory effect of polymyxin B on most gram-negative bacilli, forms the basis of the selective medium for coagulase-positive staphylococci herein described. The use of a simple nutrient agar base itself is important in preventing swarming of *Proteus* and in allowing rapid and typical growth and pigmentation of *S. aureus*. The optimal amount is 75 μg of polymyxin B per ml of medium; stability is such that the polymyxin may be added before autoclaving. Cycloheximide may be added to hold down fungal growth, if it is desired to keep plates for several days after 24 hr incubation. However, the optimal time for reading and subculturing polymyxin staphylococcus medium plates is 24 hr, since bacteria other than *S. aureus* may appear with prolonged incubation. The phosphatase reaction may be used with this medium (by adding phenolphthalein diphosphate), but there is no necessity for this as the great majority of organisms appearing on the polymyxin medium are coagulase-positive staphylococci.

The polymyxin staphylococcus medium seems to be ideally suited for processing clinical specimens to isolate coagulase-positive staphylococci from mixtures of organisms and for environmental and carrier surveys for *S. aureus*. The medium is both selective and differential. Desirable characteristics which the polymyxin staphylococcus medium seems to offer include:

1) Selectivity—to avoid overgrowth or inhibition of *S. aureus* by other organisms and to minimize the amount of subculturing and identification.

2) Rapid and maximal growth of coagulase-positive staphylococci (24 hr).

3) Good growth from small inocula.

4) Rapid pigmentation of certain *S. aureus* strains—helpful in strain differentiation. Routine clinical specimens have been shown to contain two different strains of coagulase-positive staphylococci (Rosenblum and Jackson, 1959) and certainly cultures of the hospital environment commonly show several strains of coagulase-positive staphylococci on one plate.

5) Typical colonial morphology of staphylococci (and of *Bacillus* species which persist on this medium).

6) Rapid presumptive screening for coagulase-positive staphylococci—this is inherent in the medium because of its high degree of selectivity. Slide and tube coagulase tests may be run directly from these plates of selective medium.

7) Ease of preparation.

8) Stability.

9) Results readily duplicated in any laboratory.

10) Relatively inexpensive.

11) Should be adaptable to pour plates as well as to surface streak techniques.

Currently available media for staphylococci generally fail in several of the above-listed desired characteristics.

Rather simple modification of the polymyxin medium would make it suitable for several purposes—isolation of *Proteus*, *Mycobacterium tuberculosis*, and pathogenic fungi. It is likely to be useful also in the isolation of pneumococci and streptococci from mixed cultures.

SUMMARY

A simple selective and differential medium for coagulase-positive staphylococci is described. This medium allows rapid growth and strain differentiation and has other advantages over some of the media currently used for the same purpose. The degree of selectivity is such that growth of colonies morphologically resembling staphylococci on this polymyxin staphylococcus medium serves as excellent presumptive evidence of coagulase-positive staphylococci (*Staphylococcus aureus*).

This medium should be useful in the clinical laboratory (both as a primary isolation medium

and as a secondary medium), in carrier surveys, in studies of the hospital environment, and in research studies.

REFERENCES

- Appendix A. 1958 Recommended procedures for laboratory investigation of hospital-acquired staphylococcal infections. Hospital-acquired staphylococcal disease. Proceedings of the National Conference, September.
- BARBER, M., AND S. W. A. KUPER 1951 Identification of *Staphylococcus pyogenes* by the phosphatase reaction. *J. Pathol. Bacteriol.*, **63**, 65-68.
- CHAPMAN, G. H. 1945 The significance of sodium chloride in studies of staphylococci. *J. Bacteriol.*, **50**, 201-203.
- CHAPMAN, G. H. 1946 A single culture medium for selective isolation of plasma-coagulating staphylococci and for improved testing of chromogenesis, plasma coagulation, mannitol fermentation, and the Stone reaction. *J. Bacteriol.*, **51**, 409-410.
- DUTHIE, E. S., AND L. L. LORNEZ 1952 Staphylococcal coagulase: mode of action and antigenicity. *J. Gen. Microbiol.*, **6**, 95-107.
- GILLESPIE, W. A., AND V. G. ALDER 1952 Production of opacity in egg-yolk media by coagulase-positive staphylococci. *J. Pathol. Bacteriol.*, **64**, 187-200.
- HIRSCH, H. A., C. G. MCCARTHY, AND M. FINLAND 1960 Polymyxin B and Colistin: activity, resistance and cross-resistance *in vitro*. *Proc. Soc. Exptl. Biol. Med.*, **103**, 338-342.
- KAGAN, B. M., D. KREVSKY, A. MILZER, AND M. LOCKE 1951 Polymyxin B and polymyxin E. *J. Lab. Clin. Med.*, **37**, 402-414.
- LUDLAM, G. B. 1949 A selective medium for the isolation of *Staphylococcus aureus* from heavily contaminated material. *Monthly Bull. of the Ministry of Health*, **8**, 15-20.
- MOORE, B. 1960 A new screen test and selective medium for the rapid detection of epidemic strains of *Staphylococcus aureus*. *Lancet*, **2**, 453-458.
- PULASKI, E. J., H. J. BAKER, M. L. ROSENBERG, AND J. F. CONNELL, JR. 1949 Laboratory and clinical studies of polymyxin B and E. *J. Clin. Invest.*, **28**, 1028-1031.
- ROSENBLUM, E. D., AND J. L. W. JACKSON 1959 Mixtures of coagulase-positive staphylococci and strain variation in specimens from clinical sources. *J. Lab. Clin. Med.*, **53**, 247-252.
- WECKMAN, B., AND B. W. CATLIN 1957 Deoxyribonuclease activity of micrococci from clinical sources. *J. Bacteriol.*, **73**, 747-763.