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Chocolate Agar, a Differential Medium for Gram-Positive Cocci

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Reactions incurred on chocolate agar by gram-positive cocci were correlated with species identity. Darkening and clearing of the medium was usually associated with the species *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus simulans*, and *Streptococcus faecalis*. Yellowing of chocolate agar was associated with alpha-hemolytic species of *Streptococcus*. The study demonstrated that reactions occurring on chocolate agar are useful in identifying gram-positive cocci.

Chocolate agar (CA) is an enriched medium used in clinical laboratories for cultivation of fastidious pathogens, e.g., *Haemophilus influenzae*. It may be supplemented with antibiotics to select for pathogens such as *Neisseria gonorrhoeae* from mixed cultures of bacteria. Colonies of bacteria produce various effects on chocolate agar which are reportedly useful for classifying *Staphylococcus* (6, 7) and *Streptococcus* (1) species.

The taxonomy of gram-positive cocci has been clarified over the last few years through use of new phenotypic and molecular genetic evidence (6, 7, 10, 12, 16). Commercial kits are now available and enable clinical microbiologists to rapidly and accurately identify *Staphylococcus* and *Streptococcus* species encountered in clinical laboratories (5, 15). However, a continuing need exists for updating and improving diagnostic criteria useful for distinguishing among species. The purpose of the study reported here was to describe reactions incurred by gram-positive cocci on CA and to demonstrate the usefulness of these reactions as criteria for distinguishing among *Staphylococcus* and *Streptococcus* species.

Five hundred and thirty-eight *Staphylococcus* strains were tested during the study, including 11 reference strains mentioned elsewhere (7). In addition, 23 *Micrococcus* strains and 121 *Streptococcus* strains were also tested, including 10 reference *Streptococcus* species obtained from R. R. Facklam (Centers for Disease Control, Atlanta, Ga.). Each of the 682 strains were inoculated in 1-cm-long streaks on CA and on sheep blood and gelatin agars. The last medium was used to compare gelatinase activity to reactions observed on CA.

Staphylococcus spp. and *Streptococcus* spp. were identified by criteria and methods described elsewhere (4–7, 9–13, 15, 17, 18). Micrococci were distinguished from staphylococci based on characteristics reported by Schleifer and Kloos (19) and Gunn et al. (8). Gelatinase activity was determined by using starch-gelatin agar as described by Oxborrow and Favero (14). Gelatin hydrolysis was detected after 72 h of incubation at 35°C with acid-mercuric chloride reagent. CA was commercially prepared (BBL Microbiology Systems, Cockeysville, Md.) as described elsewhere (20). Alpha hemolysis was detected using sheep blood agar by the method of Facklam (3).

From the results of this study, it is clear that darkening and clearing of CA is characteristic of *Staphylococcus* spp., rare strains of *Micrococcus* spp., and roughly one-half of the strains of *Streptococcus faecalis* (Table 1). A positive CA reaction was observed as darkening and clearing of the medium beneath and surrounding bacterial growth after an incubation period of 1 to 5 days. Darkening of the medium was first to develop and usually occurred within 48 h of incubation. Clearing of the darkened area was the endpoint of the reaction and developed later but occurred before 72 h of incubation in 87% of the reactive strains. *Staphylococcus aureus*, *Staphylococcus simulans*, and the majority of *Staphylococcus epidermidis* strains cleared CA; however, strains of *Staphylococcus saprophyticus*, *Staphylococcus cohnii*, *Staphylococcus xylosus*, *Staphylococcus capitis*, *Staphylococcus warneri*, *Staphylococcus haemolyticus*, and *Staphylococcus hominis* were usually unable to clear the medium.

Another reaction observed to occur on CA was a yellow color change that was detected beneath and around colonies of streptococci. None of the 562 strains of catalase-positive bacteria produced yellowing of the medium. The yellow color change correlated well with production of alpha hemolysis on sheep blood agar.

Data presented here provide evidence that gram-positive cocci can be presumptively identified by observing reactions that occur around colonies growing on CA. Colonies of gram-positive cocci that change the surrounding medium to a yellow color are typically one of several species of streptococci, namely *Streptococcus mitis*, *Streptococcus sanguis I*, *Streptococcus milleri*, *Streptococcus faecium*, and *Streptococcus mutans*. These species are frequently alpha-hemolytic on sheep blood agar. Colonies of staphylococci do not cause yellowing of CA medium.

Catalase-negative gram-positive cocci that darkened and cleared CA were identified as *S. faecalis*. These strains also hydrolyzed gelatin and were further classified as *S. faecalis* subsp. *liquefaciens* (2).

Clinical strains of catalase-positive cocci that darken and clear CA are typically *S. aureus*, *S. simulans*, or *S. epidermidis*. Rare strains of *Micrococcus* spp., *S. haemolyticus*, *S. warneri*, and *S. hominis* may also prove reactive on CA; however, since these bacteria account for no more than 30% of coagulase-negative staphylococci cultured in clinical materials, these reactive strains are rarely encountered. The *S. saprophyticus* species group of bacteria, i.e., *S. saprophyticus*, *S. cohnii*, and *S. xylosus* (11), do not darken and clear CA. Since their primary source in clinical specimens is urine, a specimen which is not cultured on CA, the CA reaction for these species would usually not be useful in the clinical laboratory. Thus, coagulase-negative, catalase-positive colonies of gram-positive cocci growing on CA that darken and clear the medium are most likely identified as *S. epidermidis* or *S. simulans*. Coagulase-negative strains of *S. aureus* would also fit into this category.

Yellowing of CA has been shown by Carlsson (1) to be due to production of peroxides by growing bacteria. Lack of

TABLE 1. Reactions of 604 gram-positive bacteria on CA and gelatin agar

Organism	No. of strains	% showing reactions			
		CA			Gelatinase
		Darkening/clearing	Yellowing	No reaction	
Staphylococcus sciuri sp. nov.	2	100	0	0	100
S. aureus	41	93	0	7	73
S. epidermidis	220	60	0	40	56
S. simulans	12	33	0	67	0
S. warnerii	63	17	0	83	22
S. haemolyticus/hominis	105	14	0	86	13
S. saprophyticus	60	4	0	96	21
S. cohnii	3	0	0	100	0
S. sciuri subsp. *lentus*	2	0	0	100	0
S. xylosus	2	0	0	100	100
S. capitis	28	0	0	100	0
Micrococcus spp.	20	15	0	85	65
M. roseus	3	0	0	100	0
Streptococcus durans	2	0	100	0	0
S. equinus	1	0	100	0	0
S. mitior	18	0	94	6	0
S. sanguis I	8	0	88	12	0
S. faecium	4	0	75	25	0
S. milleri	20	0	55	45	0
S. mutans	8	0	25	75	17
Streptococcus groups A/G	14	0	7	93	7
S. faecalis	28	47	3	50	47
Streptococcus group B	3	0	0	100	0
S. salivarius	7	0	0	100	0
S. bovis	8	0	0	100	0

yellowing by catalase-positive bacteria is explained by inactivation of peroxides by cellular catalases and peroxidases. Since expression of alpha hemolysis is due in part to production of peroxides acting on sheep erythrocytes in sheep blood agar, it is not surprising that alpha hemolysis shows positive correlation with yellowing on CA.

Most strains that produced the proteolytic enzyme gelatinase also darkened and cleared CA (Table 1). Exceptions were noted for the species *S. xylosus*, *S. simulans*, *Streptococcus mutans*, and some micrococci. These strains exhibited either darkening and clearing or gelatinase activity, but not both. Gelatinase activity by streptococci was weak for most species except *S. faecalis*. Several *Staphylococcus* species also hydrolyzed gelatin, but these too were usually weak. The method used in this study to test for gelatin hydrolysis is very sensitive. In fact, most strains were negative when retested with thiogel medium (BBL) which contains 5% gelatin, compared with the 1.5% added to starch-gelatin agar. The increased sensitivity of the latter test medium could account for the many weakly positive gelatinase results obtained with starch-gelatin agar.

The results of this study show that CA is useful for presumptively identifying certain *Streptococcus* and *Staphylococcus* species. Reactions occurring on CA may also be used as ancillary tests for distinguishing among strains of gram-positive cocci cultured from clinical specimens, e.g., isolates of *S. epidermidis* from multiple blood culture bottles derived from a single patient. Results may also be used to suggest early use of a multiple antibiotic regimen as in the case of a *Streptococcus* sp. that clears chocolate agar, e.g., a group D enterococcus.

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