Porphyran Test as an Alternative to Benzidine Test for Detecting Cytochromes in Catalase-Negative Gram-Positive Cocci

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A total of 66 strains of gram-positive cocci, including 21 catalase-negative members of the family Streptococccae and strains of Stomatococcus mucilaginosus, were investigated for the ability to produce porphobilinogen and porphyrin from δ-aminolevulinic acid as an alternative to the benzidine test for detecting the presence of cytochromes. Production of porphobilinogen correlated 100% with membership in the family Micrococcaceae.

The production of catalase by microorganisms is a common biochemical trait used by diagnostic and reference laboratories to differentiate among major groups of gram-positive bacteria. This enzyme, which contains a prosthetic heme group, has the ability to degrade hydrogen peroxide into water and molecular oxygen and is usually detected by the appearance of bubbles on the surface of bacterial colonies which have been exposed to the substrate. The chief usefulness of the test in the clinical laboratory revolves around its ability to distinguish between two major groups of gram-positive cocci: the families Micrococcaceae (catalase positive) and Stomatocccaceae (catalase negative).

An additional test which has been useful in the past in the separation of the above-mentioned groups is the benzidine reaction (1), which detects the presence of iron porphyrins in cytochrome-containing bacteria. The principal advantage of the benzidine test lies in its ability to confirm the presence of catalase-negative Staphylococcus spp. and Stomatococcus mucilaginosus isolates as members of the Micrococcaceae and to distinguish these strains from Aerococcus sp., a member of the Stomatocccaceae which occasionally produces a non-heme catalase but which is invariably benzidine negative.

A modified benzidine test, which is used to separate Staphylococcus from micrococci, has also been described (5). Although both staphylococci and micrococci contain cytochromes, only micrococci and Staphylococcus sciuri contain c-type cytochromes. When all non-covalently linked heme groups are removed with trichloroacetic acid-perchloric acid and acetone-hydrochloric acid before testing with benzidine, only the covalently linked c-type cytochromes remain to give a positive reaction with benzidine.

Over the past several years, the carcinogenic potential of benzidine has been documented, negating its potential usefulness in the laboratory as a phenotypic test. Recently, a confirmatory and parallel test to replace the benzidine assay and supplement the catalase reaction has been sought at the Microbial Diseases Laboratory. One attractive possibility was the porphyrin test of Kilian (6), which was originally developed to demonstrate the ability of non-heme-requiring Haemophilus spp. to produce porphobilinogen (PBG), a porphyrin precursor, and subsequently porphyrin rings from a precursor, δ-aminolevulinic acid. Since cytochrome-containing bacteria which do not require heme for growth should be able to synthesize either PBG or porphyrin rings from the same δ-aminolevulinic acid precursor, I have investigated the possible usefulness of this test as a replacement for the benzidine test.

A total of 66 isolates of gram-positive cocci submitted to the Microbial Diseases Laboratory over a 13-year period were evaluated in this study. Of these isolates, 63 were of human origin and were recovered from diverse clinical specimens. Catalase-negative Staphylococcus aureus and Staphylococcus epidermidis isolates were submitted by local laboratories for catalase testing, as were weakly catalase-positive Aerococcus viridans isolates. The Stomatococcus mucilaginosus cultures were usually catalase negative and were often submitted as Streptococcus or Aerococcus species. Strains of catalase-positive Staphylococcus aureus, Staphylococcus epidermidis, and a variety of Streptococcus spp. were included to ensure the specificity of the test. All isolates received were identified to the genus and species levels by established biochemical criteria (2–4, 7).

Each isolate, previously grown on a heart infusion slant for 24 h at 35°C, was inoculated heavily into 0.5 ml of porphyrin substrate test solution (0.034% δ-aminolevulinic acid–hydrochloride and 0.01% anhydrous magnesium sulfate in 0.1 M phosphate buffer [Sorensen], pH 6.9) and into 0.5 ml of porphyrin substrate control solution (0.01% anhydrous magnesium sulfate in 0.1 M phosphate buffer, pH 6.9). After inoculation, both tubes were incubated for 24 h at 35°C. At the end of this period, the tubes were exposed to a Wood's lamp and evaluated for red fluorescence, which is indicative of the presence of porphyrin rings. After this determination was made, 0.5 ml of the Kovacs modification of Ehrlich reagent (1 g of p-dimethylaminobenzaldehyde, 15 ml of isooamyl alcohol, 5 ml of concentrated HCl) was added to each tube and observed for the appearance of red in the aqueous phase which indicates the presence of PBG, a precursor of porphyrin; the substrate control tubes remained colorless.

Stomatococcus mucilaginosus has mucoid growth and is difficult to emulsify. Preliminary tests with strains of this species gave poor results in the standard test, and the following modification was made. Stomatococcus mucilaginosus cultures were grown in 10 ml of heart infusion broth enriched with 2% glucose and supplemented with 1% Tween 80 to disperse the growth homogeneously. After incubation for 48 h, the cultures were centrifuged and the supernatant was discarded. The sediment was added to the porphyrin substrate and control solutions. These solutions were then
incubated for 48 instead of 24 h. This procedure yielded a positive result in the porphyrin substrate test solution on the addition of Kovacs reagent. It is important that a very heavy suspension of organisms be used in this test.

The results of testing 66 isolates for production of porphyrin rings and PBG are shown in Table 1. Since several of these isolates were characterized biochemically before the carcinogenic nature of benzidine was recognized, benzidine results are available in some cases. All of the *Staphylococcus* spp. and *Stomatococcus mucilaginosus* strains produced PBG from δ-aminolevulinic acid, although only 46% produced porphyrin rings as well. None of the *Streptococcus* spp. or *Aerococcus* sp. produced either PBG or porphyrin rings. Production of PBG correlated 100% with membership in the family Micrococaceae.

This test appears to be 100% sensitive and specific and is an acceptable alternative to the benzidine reaction for the detection of the presence of cytochromes. The test is used in this laboratory to confirm the identity of catalase-negative *Staphylococcus* spp. and *Stomatococcus mucilaginosus* as members of the Micrococaceae.

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**LITERATURE CITED**


