NOTES

A New Medium for the Detection of Gelatin-Hydrolyzing Activity of Human Dental Plaque Flora

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A new medium for the detection of gelatin-hydrolyzing activity of human dental plaque flora by a simplified plate method is described. It is designed to support the growth of obligate anaerobes, facultative anaerobes, and aerobes. This medium can be used for the identification of gelatinase-producing organisms of clinical importance.

Gelatin-hydrolyzing activity of bacteria is generally tested by the gelatin tube method (3, 6). Although this method is useful in detecting the gelatin-hydrolyzing activity of rapidly growing organisms, some difficulty in reading the test is encountered with those organisms which grow slowly and exhibit partial or weak gelatinase activity. In such situations, the tube test may take several weeks before the results are known. The problem becomes more acute in a busy laboratory where several hundred organisms need to be identified every week.

Smith and Goodner (5) studied gelatin agar plate methods for gelatinase activity of rapidly growing facultative bacteria. However, their medium does not support the growth of obligate anaerobes and other members of the dental plaque flora such as *Bacteroides melaninogenicus*, *Campylobacter sputorum*, *Veillonella* species, and *Actinomyces* species, which require specific nutrients for their growth (2, 4; W. J. Loesche, A. I. D. R. 1968, abstr. 475, p. 156).

In the present communication a new medium for the detection of gelatin-hydrolyzing activity of human dental plaque flora by a rapid agar plate method is described. It can be used for the study of aerobic, anaerobic, and facultative organisms of clinical importance and is routinely used in our laboratory for the study of human dental plaque flora under aerobic and strict anaerobic conditions (1). The constituents of the medium referred to hereinafter as gelatin agar (GA) are listed in Table 1. After preparation, the GA medium is poured in sector plates or plain petri plates (100 by 15 mm) which are stored in a refrigerator until used for the gelatin-hydrolyzing activity test. The test is per-

formed as follows. The GA plates are inoculated with the test organisms using either a

TABLE 1. Composition of GA

Constituents	Amt
Agar	15 g
Trypticase	10 g
Gelatin (Difco)	30 g
Sodium acetate	1 g
Sodium formate	1 g
Sodium lactate (60%)	5 ml
Sodium succinate	1 g
Yeast extract	1 g
Sodium chloride	2 g
Glucose	1 g
D-Mannitol	1 g
Potassium nitrate	1 g
Distilled water	945 ml

Autoclave the above at 121 C for 15 min and then add a sterile solution^a containing the following compounds:

Menadione ^b (0.05% stock solution)	2 ml
Dithiothreitol	0.1 g
Sucrose	0.5 g
Cysteine hydrochloride	0.5 g
Sodium phosphate dibasic	1 g
Sodium carbonate (anhydrous)	0.5 g
Distilled water	50 ml

^a Sterilize by using a membrane filter (0.22- μ m pore size) and add to the sterile medium, cooled to 50 C. Final pH, 7.2 $\pm$ 0.1. The solution should be freshly prepared each time the medium is made.

^b Prepared in 50% ethanol in distilled water; filter sterilized and stored in an amber glass bottle in the refrigerator. Commercial source of Menadione: Nutritional Biochemical Corp., Cleveland, Ohio.

broth culture or an agar-grown culture. In either case a small amount of inoculum is placed in the center of each quadrant and incubated at 37 C. At least four organisms per plate can be used for the test. When a broth culture is used as inoculum, care is taken to allow the spot to dry before the plates are inverted for incubation. A known gelatinase-positive bacterial strain and a gelatinase-negative strain are used as controls. The incubation period for rapidly growing organisms is 18 to 24 h. With slow growing organisms, the incubation period is varied between 48 to 72 h after the appearance of growth. At the end of the incubation period the plates are refrigerated for 1 h. Increasing the refrigeration time does not alter the results. If a more rapid test is desired, the plates can be kept in the freezer compartment of the refrigerator for 20 to 25 min. After the plates are taken out of the refrigerator, condensed water and frost are wiped off and they are examined in a diffused light preferably against a dark background. A white opaque or translucent zone is formed around the colonies exhibiting gelatin-hydrolyzing activity. Such a zone is not formed around the colonies that do not hydrolyze gelatin. This is illustrated in Fig. 1. Using known organisms such as *Bacillus*

cereus, *Clostridium histolyticum*, *Streptococcus faecalis*, *Streptococcus mutans*, *Streptococcus sanguis*, and *Actinomyces viscosus*, we have tested this medium by GA plate method and compared it with the gelatin tube method. The results were comparable. The added advantage is that the GA plate method is rapid, sensitive, and highly reproducible. To date we have tested several thousands of dental plaque isolates on this medium. They include *Bacillus* species, *Veillonella* species, *Fusobacterium nucleatum*, *B. melaninogenicus*, *Bacteroides ochraceus*, *Actinomyces* species, *Enterobacter cloacae*, and oral streptococci. The tests were repeated several times to insure consistency of the results. None of the organisms gave false-positive or false-negative results. Prolonged incubation period or reincubation of the plates did not increase the frequency of positive tests.

In a subsequent study, agar plates with varying concentrations of gelatin (1.5, 3, 5, and 8%) were prepared and tested to determine the optimal concentration of gelatin in the medium which would give a distinct opaque zone around the growth of gelatin-hydrolyzing organisms. The zones were not detectable on the plates with 1.5% gelatin. The gelatin hydrolysis zones were most distinct on 3% gelatin agar plates.

FIG. 1. Gelatin hydrolysis test by the gelatin agar plate method. The organisms tested were: *B. melaninogenicus* subsp. *asaccharolyticus* ATCC 25260, *B. melaninogenicus* subsp. *intermedius* B3 and B9 (oral strains); *F. nucleatum* 9-3D (oral strain). The opaque zones around the growth of 25260, B3, and B9 indicate positive reactions (see text for details).

However, there was no remarkable difference between the zones on the plates which had 3 and 5% gelatin, respectively. Plates with 8% gelatin were not suitable as they were off white in appearance and could not be used with reliability to detect the gelatinase activity zone. These results suggest that the concentration of gelatin is important in preparing transparent gelatin agar plates so that the gelatinase-producing colonies can be easily identified by the distinct translucent or opaque zones against a clear background. In this regard, the commercial preparation of gelatin appears to be equally important as it can be the determinant of the opacity or transparency of the medium (5). The gelatin used in this study was obtained from Difco Laboratories (Detroit, Mich.) and is satisfactory for the gelatinase test by the GA plate technique.

The GA plate medium is suitable for a large variety of organisms. It contains certain compounds such as formate, succinate, lactate, and nitrate which are generally required by the dental plaque organisms for their growth and metabolic activity. The presence of vitamin K (menadione) permits the growth of *B. melaninogenicus* on this medium. Inclusion of cysteine hydrochloride and dithiothreitol as the reducing agents lowers the oxidation-reduction potential (Eh) of the medium and makes it a favorable medium for the growth of anaerobes. It is recommended that the GA be used within 1 to 3 weeks of its preparation because older and dry plates tend to become translucent (drying effect) and give a false-positive result.

The shelf life of the medium is increased if the unused plates are stored in the refrigerator. If the gelatinase test is to be performed on the anaerobic organisms, the GA plates should be held in the anaerobic atmosphere for 24 h or more before use. If the medium is desired for testing aerobic organisms, then the ingredients such as vitamin K, dithiothreitol, cysteine hydrochloride, and sodium carbonate can be omitted. However, their presence in the medium does not affect the growth of aerobes.

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