Notes

A NEW AGAR MEDIUM SUITABLE FOR SCREENING OF ANTI-CLOSTRIDIUM AGENTS

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Pseudomembranous colitis is one of the serious infectious diseases with a high mortality. It tends to occur in association with antibiotic therapy, especially with broad spectrum antibacterial agents. The major causative pathogen is the anaerobic bacterium *Clostridium difficile*.[1] This bacterium is extremely sensitive to oxygen, and requires strict anaerobic conditions for growth. Once exposed to air, it cannot grow any further. Therefore, all processes for the isolation, incubation for growth, and preservation of the bacterium have to be carried out in anaerobic boxes or anaerobic chambers. This requirement offers considerable inconvenience in the development and running of screening systems for agents effective to control pseudomembranous colitis.

We describe here a simple and convenient agar medium for growth of *C. difficile*, which can be handled under air for several hours, followed by incubation in an anaerobic chamber.

C. difficile KB-258 (ATCC 9689), and GAM medium (Nissui Co., Tokyo) and nutrient medium (Nissui Co., Tokyo) were used. Agar plates seeded with *C. difficile* KB-258 were incubated anaerobically overnight at 37°C in an BBL anaerobic system (BBL Microbiology Systems, Cockeysville, Md.), in which an atmosphere of H_2 plus CO_2 was generated by a Gas Pak system (BBL, No. 70304).

When *C. difficile* KB-258, seeded in a single layer of GAM agar, was incubated in a BBL anaerobic system, it did not grow at all, even though the GAM agar was put into the anaerobic system immediately after preparation. Whereas, other anaerobic bacteria including *Bacteroides fragilis*, *Fusobacterium varium* and *Clostridium perfringens* grew well under the same conditions.

In order to protect *C. difficile* cells from exposure to oxygen, the effect of a second covering layer was examined. A double layered agar plate was prepared which was made of a bottom layer (5 ml of GAM agar) seeded with *C. difficile*, and an upper layer (10 ml of nutrient agar) as oxygen barrier and nutrient reservoir. This plate contrasts to a conventional double layer in which the upper layer contains a test organism and the bottom layer is for nutrient supply. After preparation of the reverse phase double layer, paper discs previously dipped into antibiotic solutions (1,000 μg/ml) were placed onto the surface of the agar. The plates were put into the BBL anaerobic system, and incubated overnight at 37°C. Under these conditions *C. difficile* grew well, and vancomycin (1,000 μg/ml) formed a clear inhibition zone of about 22~25 mm in diameter. For the growth of *C. difficile*, nutrient medium was superior to GAM medium as the upper layer. Occasionally, bubbles with 3 to 10 mm in diameter were produced. Bubble formation was suppressed to almost nil when L-cysteine HCl and sodium thioglycolate were supplemented simultaneously each at 0.3 and 1.0 mg/ml into the bottom layer and upper layer, respectively.

The double layered agar thus prepared could be preserved under air for not more than 4 hours at 4°C and 2 hours at 20°C before being incubated under anaerobic conditions.

Table 1 shows the activity of various antibiotics active against *C. difficile* KB-258 in the agar medium prepared as above, and with a conventional paper-disk method. Among the antibiotics tested rifampicin gave the largest zone of inhibition.

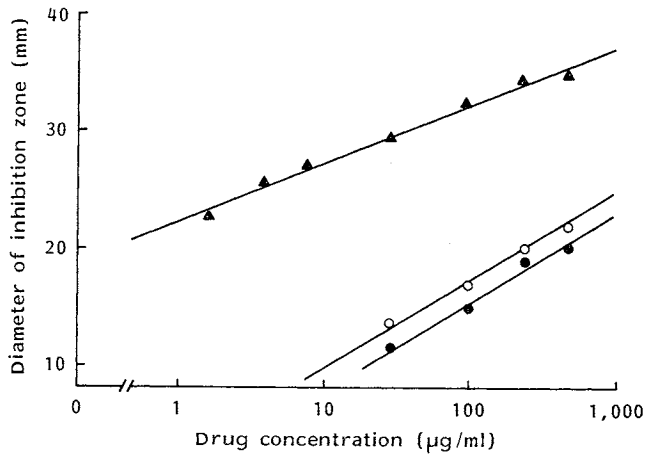
Fig. 1 illustrates typical calibration curves for three known antibiotics. These results demonstrate that the double layered agar medium can be used for detection and determination of anti-clostridium activity of drugs. The new agar medium was proved useful also for growth of

Table 1. Sensitivity of *Clostridium difficile* to various antibiotics.

Antibiotic	Diameter of inhibition zone (mm)*	Antibiotic	Diameter of inhibition zone (mm)*
Ampicillin	14.9	Clindamycin	10.0
Penicillin	15.2	Lincomycin	—
Sulbenicillin	—	Vancomycin	16.0
Erythromycin	15.6	Teicoplanin	16.4
Spiramycin	—	Ristocetin	12.8
Gentamicin	9.7	Rifampicin	32.7
Chlortetracycline	25.4	Metronidazole	19.5

* 100 μg/ml solution, 8 mmϕ paper-disk method.
—: No activity.

Fig. 1. Calibration curves for rifampicin, vancomycin and ampicillin.
▲ Rifampicin, ● vancomycin, ○ ampicillin.



other anaerobic bacteria such as *B. fragilis* and *F. varium*.

This method was applied to a screening program for new antibiotics active against *C. difficile* and other anaerobic bacteria. Screening of 4,730 actinomycetes cultures led to the discovery of three new antibiotics. They are: Clostomicins[2] produced by *Micromonospora echinospora* subsp. *armeniaca* KMR-593, luminamicin[3] and lustromycin[4] produced by *Streptomyces* sp. OMR-59 and SK-1071, respectively. During the course of this screening program, several known antibiotics were found to inhibit the growth of *C. difficile*, which include aurodox,[5] mocimycin,[6] tirandamycin,[7] and lipiarmycin.[8]

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