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Comparison of Blood-Free Medium (Cyclodextrin Solid Medium) with Bordet-Gengou Medium for Clinical Isolation of *Bordetella pertussis*

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Cyclodextrin solid medium (CSM) developed by us was evaluated to be a suitable synthetic medium for the clinical isolation of *Bordetella pertussis* when compared with Bordet-Gengou (BG) medium. The addition of 5 μg of cephalexin (CEX) per ml to CSM not only supported the good growth of *B. pertussis* but also sufficiently suppressed the growth of nasopharyngeal flora. During period 1 of this study, nasopharyngeal specimens from 60 patients with clinical pertussis were inoculated on CSM supplemented with 5 μg of CEX per ml. The isolation rate was 70% (42 of 60). To confirm the efficacy of CSM, another study was performed. During period 2 of this study, nasopharyngeal specimens were cultured on both CSM and BG medium, each with 5 μg of CEX per ml. The comparative isolation rates were 100% (40 of 40 specimens from 29 patients) on CSM with 5 μg of CEX and 65% (26 out of 40) on BG medium with 5 μg of CEX. The excellent efficacy of CSM as measured by the isolation rate was thought to be due to the poor nutrition of this medium for the growth of nasopharyngeal bacteria. CSM retained its efficacy in clinical isolations even after 3 months of storage in a refrigerator. These data led us to conclude that CSM with 5 μg of CEX was much better than BG medium with 5 μg of CEX, determined by both the isolation rate and preservativity considerations, and that CSM with 5 μg of CEX per ml can be successfully used instead of BG medium as a medium for the clinical isolation of *B. pertussis*.

The incidence of whooping cough has decreased since the introduction of pertussis vaccination. However, there are still quite a few small outbreaks of pertussis. Because *Bordetella pertussis* is not easily isolated from clinical specimens, diagnosis is usually made on clinical grounds (2). Although Bordet-Gengou (BG) medium has been widely used for the clinical isolation of fastidious *B. pertussis*, medium containing fresh blood, generally speaking, has a very short shelf life (2, 7). One of the most important factors in the successful isolation of *B. pertussis* is believed to be immediate plating of specimens (7). However, it is economically disadvantageous to keep BG medium at all times in hospitals where specimens are taken only sporadically. Moreover, since plain BG medium has almost no selectivity for the growth of nasopharyngeal flora, supplementation with antibiotics, such as penicillin, cephalexin, or methicillin, is required for clinical isolation (1–3, 7, 8).

A synthetic medium for culturing *B. pertussis* which contains heptakis(2,6-*O*-dimethyl)beta-cyclodextrin (MeCD) was developed by us. The synthetic medium, called cyclodextrin solid medium (CSM), is a modified Stainer-Scholte agar medium supplemented with MeCD which has been proved to be a significant growth stimulant for *B. pertussis* (4, 5).

The study was designed to evaluate whether CSM was suitable as a clinical isolation medium compared with BG medium from the aspects of (i) selectivity for nasopharyngeal flora, (ii) isolation rate, and (iii) shelf life.

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MATERIALS AND METHODS

Culture media. CSM consisted of basic medium and supplement. The basic medium contained the following: 10.7 g of sodium glutamate (mono), 0.24 g of L-proline, 2.5 g of NaCl, 0.5 g of KH_2PO_4, 0.2 g of KCl, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of $CaCl_2$, 6.1 g of Tris, 0.5 g of Casamino Acids (certified; Difco Laboratories, Detroit, Mich.), 1.0 g of MeCD, and 18.0 g of Bacto-Agar (Difco) per liter of distilled water. The medium was adjusted to pH 7.4 and autoclaved at 121°C for 15 min. The supplement preparation for CSM contained 40 mg of L-cysteine monohydrochloride, 10 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of ascorbic acid, 4 mg of niacin, and 150 mg of reduced glutathione per 10 ml of distilled water. This supplement was sterilized by filtration, and 10 ml was added to each liter of basic medium.

The BG medium used was a BG base (Difco) containing 15% fresh defibrinated horse blood.

Patient population and culture method. Children seen at Keio University Hospital and its affiliated hospitals with symptoms of pertussis were from two separate periods, March 1983 to March 1984 (period 1) and April 1984 to October 1985 (period 2). Nasopharyngeal specimens were obtained by using pernasal swabs (Medical Wire and Equipment Co. Ltd., Corsham, Wilts, England). The specimens were directly inoculated and streaked with a standardized threefold streak on CSM supplemented with 5 μg of cephalexin (CEX) per ml during period 1 and on both CSM and BG medium, each supplemented with 5 μg of CEX per ml, during period 2. The inoculated plates were incubated for 5 days at 35°C.

TABLE 1. Comparison of the growth of nasopharyngeal flora on four media[a]

Medium tested	No. of plates with colonization/no. of plates tested (%)
Plain BG	19/20 (95)
Plain CSM	11/20 (55)[b]
BG with CEX (5 μg/ml)	7/20 (35)
CSM with CEX (5 μg/ml)	4/20 (20)[c]

[a] Pernasal swabs from 20 healthy children were cultured at 35°C for 5 days.
[b] Significantly lower than plain BG medium ($P < 0.05$).
[c] Significantly lower than plain CSM ($P < 0.05$).

The CSM plates with 5 μg of CEX per ml were stored in a refrigerator for up to 3 months before use, and the plates of BG medium with 5 μg of CEX per ml were stored for up to 2 weeks.

Identification of *B. pertussis*. The colonies which appeared after 3, 4, or 5 days of incubation were subcultured on plain CSM and plain BG medium. Identification of *B. pertussis* was performed by both Gram staining and an agglutination test with *B. pertussis* antiserum (Difco).

RESULTS

Supplementation with CEX in CSM and BG medium. Five lyophilized stock cultures of recent clinical isolates were inoculated on plates of BG medium for 72 h at 35°C. The harvested cells from these plates were subcultured on BG medium and incubated for an additional 48 h. The harvested cells from these plates were suspended in 1% Casamino Acids (Difco) solution and were adjusted to a concentration of about 5×10^3 cells per ml; then, 0.05 ml of this suspension was spread on CSM and BG medium supplemented with various concentrations of CEX (0, 5, 10, or 20 μg/ml).

These plates were incubated for 5 days at 35°C. All clinical isolates showed full cell growth, determined by both the number of CFU and the size of the colonies on the four media: plain BG medium, BG medium with 5 μg of CEX per ml, plain CSM, and CSM with 5 μg of CEX per ml. Growth of three of the five clinical isolates was clearly suppressed on both media by supplementation with 20 μg of CEX per ml. The effect of 10 μg of CEX per ml in CSM but not in BG medium resulted in slightly smaller colony sizes.

Growth of nasopharyngeal flora. Nasopharyngeal specimens were obtained with pernasal swabs (Medical Wire and Equipment Co. Ltd.) from 20 healthy children aged 0 to 10 years. Each specimen was suspended in 1 ml of broth. A loopful of this suspension was cultured for 5 days at 35°C on plain BG medium, plain CSM, BG medium with 5 μg of CEX per ml, and CSM with 5 μg of CEX per ml. The appearance rate of colonization was 95% (19 of 20) on plain BG medium, 55% (11 of 20) on plain CSM, 35% (7 of 20) on BG medium with 5 μg of CEX per ml, and 20% (4 of 20) on CSM with 5 μg of CEX per ml (Table 1). Plain CSM was nutritionally poor for the growth of nasopharyngeal flora ($P < 0.05$). The addition of 5 μg of CEX per ml gave CSM significant selectivity for the growth of nasopharyngeal flora ($P < 0.05$). Three of four cases colonized on CSM with 5 μg of CEX per ml were identified as *Neisseria* spp.; the other was identified as *Staphylococcus aureus*.

Clinical isolation of *B. pertussis*. In period 1, nasopharyngeal specimens were inoculated on CSM with 5 μg of CEX per ml. *B. pertussis* was isolated from 42 of 60 patients with clinical pertussis (70.0%). In period 2, the organism was detected from 40 nasopharyngeal specimens of 29 patients with clinical pertussis on at least one of the two media inoculated. The comparative isolation rates were 100% (40 of 40) on CSM with 5 μg of CEX per ml and 65% (26 of 40) on BG medium with 5 μg of CEX per ml. When a sufficient number of organisms was present in the clinical specimen, little difference in the isolation rate between the 2 media was observed (100% versus ~94%). However, when there was only a small number of viable *B. pertussis* cells in the clinical specimen, the CSM isolation rate (100%) was superior to that of BG medium (~46%). Overgrowth of nasopharyngeal flora interfered with the successful isolation of *B. pertussis* on BG medium. However, the high selectivity of CSM made it easier to isolate *B. pertussis* even when present only in small amounts (Fig. 1). The superiority of CSM over BG medium in selectivity for nasopharyngeal flora not only resulted in better isolation rates for CSM but made clinical isolation on CSM easier than with BG medium. CSM which had been previously stored in a refrigerator for 3 months retained sufficient efficacy for clinical isolation of *B. pertussis* and showed no difference in quantity or size of colonization from those measured in freshly prepared BG medium. This suggests that the shelf life of CSM is at least 3 months.

DISCUSSION

BG medium has been widely used for clinical isolation of fastidious *B. pertussis*. However, both its restricted shelf life

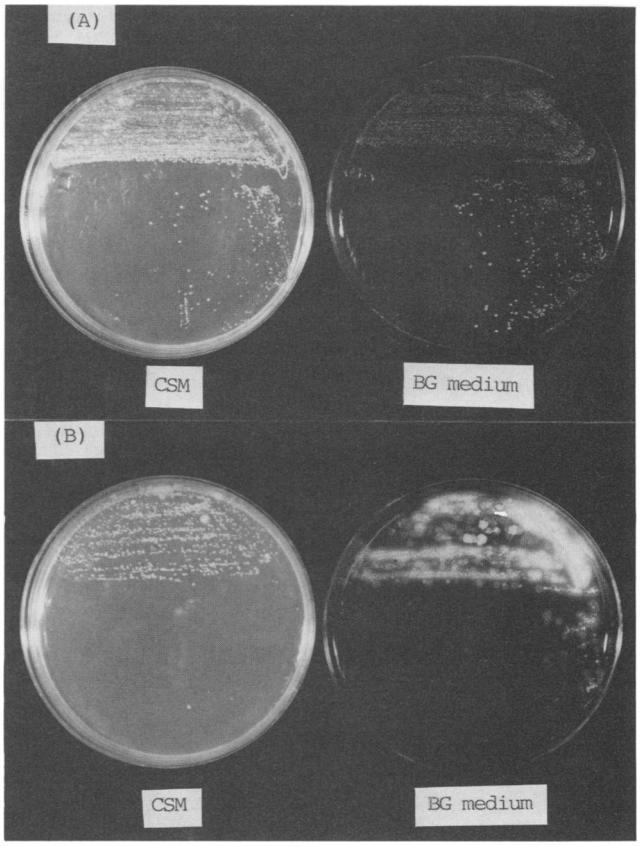

FIG. 1. Comparison of clinical isolation between CSM with 5 μg of CEX per ml and BG medium with 5 μg of CEX per ml. Nasopharyngeal specimens from the same patient were cultured on each medium for 5 days. The amount of isolated *B. pertussis* was ++ (A) and + (B).

and its lack of selectivity for nasopharyngeal flora have adversely affected the isolation of the target organism. Because of the difficulty in the clinical isolation of *B. pertussis*, diagnosis is usually made on clinical grounds (2).

In this study, CSM developed by us was judged to be a suitable synthetic medium for clinical isolation of *B. pertussis* when compared with BG medium. Penicillin, methicillin, and cephalexin have been recommended as suitable supplements for the isolation of *B. pertussis* (1–3, 7, 8). CEX was chosen as a supplement in our study because the drug susceptibility studies of 10 strains of *B. pertussis* showed that they were more resistant to CEX than to the other two drugs (unpublished data). The addition of 5 μg of CEX per ml to CSM did not suppress the growth of *B. pertussis*. This level of CEX gave CSM an excellent suppressive ability against nasopharyngeal flora (Table 1).

The isolation rate of *B. pertussis* on CSM supplemented with 5 μg of CEX per ml was 70% (42 out of 60), which is sufficiently close to rates reported by others (1, 3, 6). When compared with BG medium with 5 μg of CEX per ml, CSM with 5 μg of CEX per ml had a far better isolation rate for the organism. The difference in the suppressive abilities for other residents in the nasopharynx resulted in different isolation rates between the two media tested (Fig. 1).

Other organisms which could be grown on CSM with 5 μg of CEX per ml were identified as some strains of neisseria, staphylococci, and corynebacteria. The three organisms were easily distinguished from *B. pertussis* by both the onset and the appearance of colonization. The rest of the resident organisms in the nasopharynx (alpha-*Streptococcus* spp., *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and *Haemophilus parainfluenzae*) could not grow on plain CSM (unpublished data).

Clinical isolation was successful with CSM that had been stored for 3 months, which suggests that the shelf life of CSM is much longer than that of BG medium. A long shelf life will make it possible to supply the medium whenever nasopharyngeal swabs are taken and to plate the swabs directly on the medium. Direct plating is one of the most important factors in successful isolation of *B. pertussis* (7) and does not require any transport media.

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