Improved selective isolation

of *Bordetella pertussis* by modified cyclodextrin solid medium

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Running title: Selective medium for *Bordetella*

Key words: *Bordetella pertussis*, direct plating, selective isolation, cefdinir,
modified cyclodextrin solid medium

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ABSTRACT

We have developed a modified cyclodextrin solid (MCS) medium using the selective antibiotic, cefdinir. MCS medium exhibited higher sensitivity (95.6%; any culture positive as reference) and greater inhibition of nasopharyngeal flora than Bordet-Gengou agar (65.2%, $P = 0.009$) or cyclodextrin solid medium (39.1%, $P < 0.001$).
Pertussis is an acute respiratory infection caused by *Bordetella pertussis*. Since the introduction of the acellular pertussis vaccine, the number of reported pertussis cases has drastically decreased. However, occasional outbreaks have still been reported (2-4, 7), and adult pertussis has emerged (2,3).

Culturing *B. pertussis* from clinical specimens is the “gold standard” for diagnosis of pertussis, although this remains an insensitive method (12,13). In addition, isolation of clinical strains is required for epidemiological analysis (including phenotypic and genotypic characterization). It is also required for the determination of the appropriate vaccine strain and the antimicrobial susceptibility of isolates in order to control the spread of pertussis (12,13). Bordet-Gengou agar was the standard culture medium for isolation of *B. pertussis*, but has the problem of low selectivity. More selective media, such as BG agar with 40 µg/ml of cephalixin (BG agar), and Regan-Lowe agar with 40 µg/ml of cephalixin (RL agar: charcoal agar based), have been described, however these media have a short shelf life because they contain blood (6,15). Cyclodextrin solid medium with 5 µg/ml of cephalixin (CS medium) does not contain blood products, and is reported to have improved selectivity and a long shelf life (1). However, the detection rate of *B. pertussis* by this medium was lower than that achieved with other conventional media (10). Moreover, β-lactam (especially first generation cepharosporins like cephalixin)-resistant bacteria, such as *Haemophilus influenzae*, *Streptococcus pneumonia*, *Staphylococcus aureus* and
Moraxella catarrhalis have recently emerged (8,11). Therefore, cephalexin may no longer be a suitable selective reagent for isolation of *B. pertussis* from nasopharyngeal specimens. To address this problem, we have attempted to improve the selective isolation of *B. pertussis* when performing direct plating of clinical specimens.

Modified CS medium (MCS medium) was prepared by replacing cephalexin with cefdinir (Asteras Pharma Inc., Tokyo, Japan), which inhibits the growth of *M. catarrhalis* (9,16), vancomycin, and amphotericin B at final concentrations of 4 µg/ml, 8 µg/ml and 4 µg/ml respectively. The concentration of cefdinir was set by the MIC data from 50 clinical *M. catarrhalis* strains and 40 clinical strains of *B. pertussis*.

MCS medium was composed of basic medium and supplement. The basic medium contained the following: 10.7 g of sodium glutamate, 0.24 g of L-proline, 2.5 g of NaCl, 0.5 g of KH₂PO₄, 0.2 g of KCl, 0.1 g of MgCl₂·6H₂O, 0.02 g of CaCl₂, 6.1 g of Tris, 2.5 g of casamino acids (Difco Laboratories, Detroit, MI), 2.0 g of dimethyl-β-cyclodextrin (Teijin Ltd, Osaka, Japan), and 17.0 g of Bacto-Agar (Difco) per liter of distilled water. The supplement preparation for MCS medium contained 40 mg of L-cysteine, 10 mg of FeSO₄·7H₂O, 20 mg of ascorbic acid, 4 mg niacin, and 150 mg of reduced glutathione per 10 ml was added to each liter of basic medium. All the chemical reagents without representation were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For making MCS medium, all of the ingredients except antibiotics were autoclaved for 15 min at 121 °C. After cooling...
down to 50 °C, the sterile stock solution (X100) of each antibiotic was added.

To perform the recovery test of *Bordetella* species on three media, one colony of each strain was streaked on BG agar, CS medium and MCS medium plates and incubated at 35 °C for 5 days. On the fifth day, the growth of each bacterial strain was observed and categorized as no growth, poor growth (<0.1 mm of colony size), or positive growth (≥0.1 mm of colony size). A total of 57 strains of *Bordetella* species (43 *B. pertussis* including *B. pertussis* CCUG 30837T, 3 *Bordetella parapertussis* including *B. parapertussis* CCUG 413T, 2 *Bordetella bronchiseptica* including *B. bronchiseptica* CCUG 219T, 4 *Bordetella holmesii* including *B. holmesii* CCUG 34073T, and 4 strains of *Bordetella avium* CCUG 13726T, *Bordetella hinzii* CCUG 33847T, *Bordetella petrii* CCUG 43448T, and *Bordetella termatum* CCUG 32381T), could be grown on the MCS medium. However, BG agar could not support the growth of *B. avium*, *B. hinzii*, *B. holmesii*, and *B. termatum*, and *B. termatum* grew poorly on CS medium (data not shown).

As nasopharyngeal flora contains various microorganisms which interfere with the growth of *B. pertussis*, we evaluated the selectivity of *B. pertussis* on BG agar, CS medium, RL agar (14) and MCS medium using artificially prepared (“mock”) samples. *H. influenzae* ATCC 49247, *S. pneumoniae* ATCC 49619, *S. aureus* ATCC 43300, *M. catarrhalis* NK-015 (clinical strain, β-lactamase producer), and *Candida albicans* ATCC 24433 were used to prepare an artificial mix of nasopharyngeal flora (NPF).
Final concentrations of $10^4$ to $10^7$ CFU/ml of NPF were mixed with $10^4$ CFU/ml of *B. pertussis* CCUG 30837$^T$. Ten microliters of each aliquot was spread onto each medium. The plates were incubated at 35 °C under aerobic conditions. After 5 days, the growth of NPF and *B. pertussis* was determined (Table 1). *B. pertussis* could not be detected on BG agar or CS medium when cultured with any of the NPF concentrations, and could only be detected on RL agar with only $10^4$ CFU/ml of NPF. However, *B. pertussis* was easily detected on MCS medium with all concentrations of NPF tested.

To evaluate the advantages of MCS medium in the clinical setting, 120 nasopharyngeal specimens using Rayon swab (Seedswab No.2: Eiken Chemical, Tokyo, Japan) were collected, in a blind fashion between December 2001 and November 2002. Samples were collected predominantly from children with symptoms of pertussis. The specimens were sent to our laboratory within two days of collection from institutes participating in the Japanese pertussis surveillance group. All institutes had ethical approval for participation in this study. Each swab was then suspended in 300 µl of normal sterile saline. One loopful (approximately 2 µl) of this suspension was streaked out onto BG agar, CS medium, and MCS medium. Each medium was then incubated in humidified air at 35 °C for seven days. *B. pertussis* was identified by gram staining, oxidase reaction, and agglutination test with polyclonal antisera (Denka Seiken, Tokyo, Japan) (12,13). Identification of other bacterial species was performed according to the Manual of Clinical Microbiology (13). Crude DNA extracts were then
prepared by boiling the remaining 100 µl of each suspension at 100 °C for 15 min. Samples were then centrifuged at 18,500 X g for 5 min, and supernatants were used for PCR. To compare the detection of *B. pertussis* and *B. parapertussis*, nested duplex PCR targeting IS481 and IS1001 was performed as described by Farrell et al (5). The chi-square test was used for statistical evaluation and *P* values of less than 0.05 were considered significant. Table 2 shows the results for detection of *B. pertussis* by culture on each medium or by PCR. Of the 120 samples, 23 (19.1%) were positive on at least one of the three different culture media, and 44 (36.6%) were positive by PCR on at least one of the three media. There were no PCR-negative culture-positive cases. *B. parapertussis* was not detected from any specimens by culture or PCR. The sensitivity of culture on BG agar, CS and MCS medium, was 34.0, 20.4, and 50.0%, when PCR was used as the gold standard, and 65.2, 39.1, and 95.6% by any culture-positive as a reference respectively. The number of *B. pertussis* strains isolated on MCS medium was greater than on CS medium (*P* = 0.003) and also greater than on BG agar, but this was no statistically significant. However, when any positive culture was used as a reference, MCS medium was superior to CS medium (*P* < 0.001) or BG agar (*P* = 0.009). In addition, eight strains were only detected on MCS medium (*P* = 0.01 vs. BG agar, *P* = 0.003 vs. CS medium). Growth of NPF, except *B. pertussis*, from clinical specimens on each of the three media is shown in Table 3. Growth of NPF on MCS medium was significantly less than that on BG agar and CS medium.
Moreover, the number of samples with complete inhibition of NPF on MCS medium (73: 60.8%) was significantly greater than on BG agar (24: 20.0%) and on CS medium (32: 26.6%). Table 4 summarizes the NPF microorganisms from clinical specimens isolated on each medium. Of the three media used, MCS medium yielded the fewest NPF microorganisms other than *B. pertussis* and successfully inhibited growth of all the α-streptococci, *Haemophilus*, *Bacillus*, and *Staphylococcus* strains. MCS medium could inhibit *M. catarrhalis* more effectively than CS medium, probably due to the selective inhibition of β-lactamase-producing *M. catarrhalis* strains by cefdinir (9,16). More α-streptococci, *Neisseria*, *Haemophilus*, *Staphylococcus*, and *Corynebacterium* strains were grown on BG agar than MCS medium, and this result was statically significant. Our results suggest that MCS medium not only inhibited indigenous NPF overgrowth but also supported the growth of *B. pertussis*.

MCS medium was shown to support the growth of *B. pertussis* Tohama and *B. pertussis* Yamaguchi strains for up to six months and could inhibit *S. aureus* IFO 13726, *S. aureus* ATCC 43300, *M. catarrhalis* NK-015 and *C. albicans* ATCC 10231 completely for up to five months when stored at 4-9°C (data not shown). A long shelf life is another benefit of this medium because most clinical microbiology laboratories are infrequently required to culture specimens from pertussis patients (6,15). The cost of MCS medium is similar to that of BG agar or RL agar.

In conclusion, MCS medium improved the selective isolation of *B. pertussis*
from clinical specimens. This culture method will assist future studies aimed at a better understanding of the pathophysiology of pertussis.

We thank the participants from the Japanese pertussis surveillance group for their invaluable contribution to this study.

REFERENCES


### TABLE 1. Effects of nasopharyngeal flora on detection of *B. pertussis* from artificially prepared samples on four media

<table>
<thead>
<tr>
<th>Medium</th>
<th>NPF (10⁷ cfu/ml)</th>
<th>NPF (10⁶ cfu/ml)</th>
<th>NPF (10⁵ cfu/ml)</th>
<th>NPF (10⁴ cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>B. pertussis</em></td>
<td><em>B. pertussis</em></td>
<td><em>B. pertussis</em></td>
<td><em>B. pertussis</em></td>
</tr>
<tr>
<td>RL agar†</td>
<td>3⁺, 0</td>
<td>3⁺, 0</td>
<td>3⁺, 0</td>
<td>2⁺, 1⁺</td>
</tr>
<tr>
<td>BG agar‡</td>
<td>3⁺, 0</td>
<td>3⁺, 0</td>
<td>3⁺, 0</td>
<td>2⁺, 0</td>
</tr>
<tr>
<td>CS medium§</td>
<td>3⁺, 0</td>
<td>3⁺, 0</td>
<td>3⁺, 0</td>
<td>2⁺, 0</td>
</tr>
<tr>
<td>MCS medium¶</td>
<td>0, 2⁺, 0</td>
<td>2⁺, 0</td>
<td>2⁺, 0</td>
<td>0, 2⁺</td>
</tr>
</tbody>
</table>

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*3⁺, growth over all of the inoculated area; 2⁺, growth two thirds of the inoculated area; 1⁺, <10 colonies on the primary inoculated area.*

† NPF: Artificially mixed nasopharyngeal flora composed of *S. aureus* ATCC 43300, *H. influenzae* ATCC 49247, *S. pneumoniae* ATCC 49619, *M. catarrhalis* NK-015 (clinical isolate, β-lactamase producer), and *C. albicans* ATCC 24433 with respective concentrations.

‡ RL agar: Regan-Lowe agar (charcoal agar containing 10% horse blood and 40 µg/ml of cephalexin).

§ BG agar: Bordet-Gengou agar with 40 µg/ml of cephalexin.

¶ CS medium: Cyclodextrin solid medium with 5 µg/ml of cephalexin.

¶ MCS medium: Modified cyclodextrin solid medium.
TABLE 2. Performance characteristics of three media for detection of *B. pertussis* in clinical specimens

<table>
<thead>
<tr>
<th>Reference</th>
<th>Medium</th>
<th>No. (%) of isolates that grew</th>
<th><em>P</em> value</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. (%) on the indicated medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR (44 positive)</td>
<td>CS medium</td>
<td>9 (28.4)</td>
<td>0.003</td>
<td>0.003</td>
<td>20.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>MCS medium</td>
<td>22 (65.2)</td>
<td>0.001</td>
<td>0.001</td>
<td>39.1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Culture (23 positive)</td>
<td>CS medium</td>
<td>9 (39.1)</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>95.4</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* a *P* value, vs. MCS medium.
* b *P* value, vs. MCS medium.
* c PPV, positive predictive value.
* d NPV, negative predictive value.
* e NS, not significant.
* f any culture positive on three media.

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<table>
<thead>
<tr>
<th>Growth of any microorganisms composed nasopharyngeal flora except B. pertussis</th>
<th>MCS medium</th>
<th>P value</th>
<th>BG agar</th>
<th>P value</th>
<th>CS medium</th>
<th>P value</th>
<th>NS, not significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>3+</td>
<td>13 (10.8)</td>
<td>0.02</td>
<td>21 (17.5)</td>
<td>&lt;0.001</td>
<td>4 (3.3)</td>
<td></td>
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<tr>
<td>2+</td>
<td>62 (51.6)</td>
<td>&lt;0.001</td>
<td>44 (36.6)</td>
<td>&lt;0.001</td>
<td>16 (13.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+</td>
<td>21 (17.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24 (20.0)</td>
<td>&lt;0.001</td>
<td>32 (26.6)</td>
<td>&lt;0.001</td>
<td>73 (60.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td></td>
<td>120</td>
<td></td>
<td>120</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* $P$ value, vs. MCS medium.
* $P$ value, vs. MCS medium.
* NS, not significant.

Number (%) of each medium with growth of categorized nasopharyngeal flora on July 13, 2017 by guest
<table>
<thead>
<tr>
<th>Microorganism of nasopharyngeal flora</th>
<th>Number (%) of each medium with categorized nasopharyngeal flora growth</th>
<th>BG agar</th>
<th>CS medium</th>
<th>MCS medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$P$ value$^a$</td>
<td>$P$ value$^b$</td>
<td>NS</td>
</tr>
<tr>
<td>α-streptococci</td>
<td></td>
<td>&lt;0.001</td>
<td>0 (0.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>20 (16.6)</td>
<td>NS</td>
<td>40 (33.3)</td>
<td>0.002</td>
</tr>
<tr>
<td>Gram negative rods</td>
<td>15 (12.5)</td>
<td>NS</td>
<td>21 (17.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Neisseria spp.</td>
<td>15 (12.5)</td>
<td>0.003</td>
<td>5 (4.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Candida spp.</td>
<td>10 (8.3)</td>
<td>NS</td>
<td>9 (7.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Haemophilus spp.</td>
<td>9 (7.5)</td>
<td>0.002</td>
<td>0 (0.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Staphylococcus spp.</td>
<td>7 (5.8)</td>
<td>0.007</td>
<td>24 (20.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Corynebacterium spp.</td>
<td>4 (3.3)</td>
<td>0.04</td>
<td>3 (2.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Bacillus spp.</td>
<td>1 (0.8)</td>
<td>NS</td>
<td>1 (0.8)</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^a$ $P$ value, vs. MCS medium.

$^b$ $P$ value, vs. MCS medium.

* NS, not significant.