Improved Selective Isolation of *Bordetella pertussis* by Use of Modified Cyclodextrin Solid Medium[▽]

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Received 28 January 2009/Returned for modification 26 March 2009/Accepted 2 October 2009

We have developed a modified cyclodextrin solid (MCS) medium using the selective antibiotic cefdinir. MCS medium exhibited higher sensitivity (95.6%; any culture-positive sample as reference) and greater inhibition of nasopharyngeal flora than did 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 (BG 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 cephalexin (cefalexin) (BG agar) and Regan-Lowe agar with 40 µg/ml of cephalexin (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 cephalexin (CS medium) does not contain blood products and is reported to have improved selectivity and a long shelf life (1). However, the rate of detection of *B. pertussis* by this medium was lower than that achieved with other conventional media (10). Moreover, β-lactam (especially narrow-spectrum cephalosporins like cephalexin)-resistant bacteria, such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, *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 strains of *M. catarrhalis* 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 KH_2PO_4 , 0.2 g of KCl, 0.1 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02 g of CaCl_2 , 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 containing 40 mg of L-cysteine, 10 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 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 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), unless otherwise specified. For making MCS medium, all of the ingredients except antibiotics were autoclaved for 15 min at 121°C. After the mixture was cooled down to 50°C, the sterile stock solution (100×) 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 5th day, the growth of each bacterial strain

TABLE 1. Effects of NPF on detection of *B. pertussis* from artificially prepared samples on four media^a

Medium	Growth with concn of NPF:							
	10 ⁷ CFU/ml		10 ⁶ CFU/ml		10 ⁵ CFU/ml		10 ⁴ CFU/ml	
	NPF	<i>B. pertussis</i>	NPF	<i>B. pertussis</i>	NPF	<i>B. pertussis</i>	NPF	<i>B. pertussis</i>
RL agar	3+	0	3+	0	3+	0	2+	1+
BG agar	3+	0	3+	0	3+	0	2+	0
CS medium	3+	0	3+	0	3+	0	2+	0
MCS medium	0	2+	0	2+	0	2+	0	2+

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

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[▽] Published ahead of print on 21 October 2009.

TABLE 2. Performance characteristics of three media for detection of *B. pertussis* in clinical specimens^a

Reference standard and medium	No. (%) of isolates that grew on indicated medium	<i>P</i> value vs MCS medium	No. (%) of isolates that grew only on indicated medium	<i>P</i> value vs MCS medium	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
PCR (44 samples positive)								
BG agar	15 (34.0)	NS	1 (2.2)	0.01	34.0	100	100	72.3
CS medium	9 (20.4)	0.003	0 (0.0)	0.003	20.4	100	100	68.4
MCS medium	22 (50.0)		8 (18.1)		50.0	100	100	77.5
Culture (23 samples positive) ^b								
BG agar	15 (65.2)	0.009	1 (4.3)	0.009	65.2	100	100	92.3
CS medium	9 (39.1)	<0.001	0 (0.0)	0.001	39.1	100	100	87.3
MCS medium	22 (95.6)		8 (34.7)		95.6	100	100	98.9

^a Abbreviations: PPV, positive predictive value; NPV, negative predictive value; NS, not significant.

^b Any culture that was positive on three media.

was observed and categorized as no growth, poor growth (<0.1-mm colony size), or positive growth (≥0.1-mm colony size). A total of 56 strains of *Bordetella* species (43 of *B. pertussis* including *B. pertussis* CCUG 30837^T, three of *Bordetella parapertussis* including *B. parapertussis* CCUG 413^T, two of *Bordetella bronchiseptica* including *B. bronchiseptica* CCUG 219^T, four of *Bordetella holmesii* including *B. holmesii* CCUG 34073^T, and the four strains *Bordetella avium* CCUG 13726^T, *Bordetella hinzii* CCUG 33847^T, *Bordetella petrii* CCUG 43448^T, and *Bordetella termatum* CCUG 32381^T) 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 (NPF) 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 by the use of 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 NPF. Final concentrations of 10⁴ to 10⁷ CFU/ml of NPF were mixed with 10⁴ 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 be detected on RL agar only with 10⁴ 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 were collected using a rayon swab (Seedswab no. 2; Eiken Chemical, Tokyo, Japan), in a blinded 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 2 days of collection from institutions participating in the Japanese pertussis surveillance group. All institutions 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 7 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 × *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

TABLE 3. Growth inhibition of NPF from clinical specimens on three media

Growth of any microorganisms composing NPF except <i>B. pertussis</i> ^a	BG agar		CS medium		MCS medium: no. (%) of samples with growth of categorized NPF
	No. (%) of samples with growth of categorized NPF	<i>P</i> value vs MCS medium	No. (%) of samples with growth of categorized NPF	<i>P</i> value vs MCS medium	
3+	13 (10.8)	0.02	21 (17.5)	<0.001	4 (3.3)
2+	62 (51.6)	<0.001	44 (36.6)	<0.001	16 (13.3)
1+	21 (17.5)	NS ^b	23 (19.1)	NS	27 (22.5)
0	24 (20.0)	<0.001	32 (26.6)	<0.001	73 (60.8)
Total	120		120		120

^a Semiquantitative NPF growth except *B. pertussis*. 3+, growth over all of the inoculated area; 2+, growth over two-thirds of the inoculated area; 1+ <10 colonies on the primary inoculated area; 0, no growth on the primary inoculated area.

^b NS, not significant.

TABLE 4. Growth of NPF from clinical specimens on three media

Type of microorganism in NPF	BG agar		CS medium		MCS medium: no. (%) of samples with categorized NPF growth
	No. (%) of samples with categorized NPF growth	<i>P</i> value vs MCS medium	No. (%) of samples with categorized NPF growth	<i>P</i> value vs MCS medium	
α Streptococci	54 (45.0)	<0.001	0 (0.0)	NS ^a	0 (0.0)
<i>M. catarrhalis</i>	20 (16.6)	NS	40 (33.3)	0.002	20 (16.6)
Gram-negative rods	15 (12.5)	NS	21 (17.5)	NS	19 (15.8)
<i>Neisseria</i> spp.	15 (12.5)	0.003	5 (4.1)	NS	3 (2.5)
<i>Candida</i> spp.	10 (8.3)	NS	9 (7.5)	NS	7 (5.8)
<i>Haemophilus</i> spp.	9 (7.5)	0.002	0 (0.0)	NS	0 (0.0)
<i>Staphylococcus</i> spp.	7 (5.8)	0.007	24 (20.0)	<0.001	0 (0.0)
<i>Corynebacterium</i> spp.	4 (3.3)	0.04	3 (2.5)	NS	0 (0.0)
<i>Bacillus</i> spp.	1 (0.8)	NS	1 (0.8)	NS	0 (0.0)

^a NS, not significant.

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 medium, and MCS medium was 34.0, 20.4, and 50.0%, respectively, when PCR was used as the gold standard and 65.2, 39.1, and 95.6%, respectively, by any culture-positive sample as a reference. The number of *B. pertussis* strains isolated on MCS medium was greater than that on CS medium (*P* = 0.003) and also greater than that on BG agar, but this was not 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 detected only on MCS medium (*P* = 0.01 versus BG agar, *P* = 0.003 versus 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 those 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 and *Haemophilus*, *Bacillus*, and *Staphylococcus* strains. MCS medium could inhibit *M. catarrhalis* more effectively than could CS medium, probably due to the selective inhibition of β -lactamase-producing *M. catarrhalis* strains by cefdinir (9, 16). More α streptococci and *Neisseria*, *Haemophilus*, *Staphylococcus*, and *Corynebacterium* strains were grown on BG agar than on 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 6 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 5 months when stored at 4 to 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.

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