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Enrichment Medium for the Isolation of *Bordetella*

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The development of a specimen collection and transport medium outfit for the rapid laboratory diagnosis of whooping cough is described. The transport medium consisted of a semisolid agar containing charcoal, cephalexin, and defibrinated horse blood. It was also found to be an excellent enrichment medium for the selective isolation of *Bordetella pertussis* and *B. parapertussis* from scantily populated specimens. The investigation of 3,237 specimens that yielded 1,419 positive isolates of *Bordetella*, including 86 *B. parapertussis*, during a 20-month period is presented. A total of 3,076 specimens were processed in the laboratory by using the enrichment medium in addition to the routine procedure. Of these specimens, 757 were submitted in our medium, from which 137 (18%) were positive. Of the 567 specimens received in Amies transport medium, 290 (51%) positive cultures were obtained by the enrichment method only and not by primary culture.

The increased number of clinically diagnosed cases of whooping cough indicates that *Bordetella pertussis* is still very much present in the community and, despite some evidence toward viral implication (3), this organism remains the etiological agent of the whooping cough syndrome.

Isolation of *B. pertussis* has long been a problem for many laboratories (22). Although difficulty in obtaining specimens exists for all, those sited near hospitals have greater access to infected patients than do those in public health laboratories, which obtain specimens chiefly by mail.

Several factors contribute to the difficulty of isolating *Bordetella*, including incorrect collection procedures, delay in transit, overgrowth by other microbial species and/or fungi, the requirement for a specialized isolation medium, and lack of experience in recognizing the organisms.

To improve our isolation rate, it was necessary to discover exactly which of the abovementioned obstacles was responsible for impeding isolation of *Bordetella*. Technical recognition and supportive properties of our isolation medium, Bordet-Genou (Difco B48, Difco Laboratories, Detroit, Mich.), were tested by a "blind quality control specimen" obtained from our stock culture bank. The routine medium was tested against a charcoal medium (Oxoid CM 119 Oxoid Ltd., London) with penicillin (0.3 IU/ml) and 4,4'-diamidinodiphenylamine dihydrochloride at a concentration of 2 μg/ml (M and B 938; May and Baker, Kent, England).

The Bordet-Gengou medium did not support the growth of the organism. The charcoal medium with additives supported diminutive growth of *B. pertussis*, which was overgrown by commensal bacterial flora, thus rendering it unsuitable for isolation. We then evaluated the charcoal medium containing 40 μg of cephalexin per ml (30), which proved to be superior to the same medium containing penicillin and diamidinodiphenylamine dihydrochloride.

The problem of transportation has been examined by many workers (9, 15, 16, 28, 29), but was most clearly presented by Kendrick (18). Some workers have tended to use on-site collection to obviate this problem (13, 23-25, 29). Preston (29) stated that, if a specimen in transport medium was received in the laboratory within 1 day, there would be no appreciable drop in the viability of *Bordetella*. However, if left at room temperature for another 24 h, the percentage of isolation was lowered considerably. This was generally due to overgrowth by other flora. He also stated that the transport and isolation media should be of similar composition.

This report presents the development of a suitable isolation medium by using charcoal agar with and without cephalexin and its further successful development as a transport and laboratory enrichment medium for the isolation of *B. pertussis* and *B. parapertussis*.

MATERIALS AND METHODS

Isolation media. The isolation medium was Oxoid charcoal agar CM 119 and was prepared as per the

instructions of the manufacturer. Cephalexin and defibrinated horse blood were added to final concentrations of 40 μg/ml and 10%, respectively.

Isolation medium without cephalexin was also used as a control. In instances where overgrowth by yeast or fungi was evident after 24 or 48 h of incubation, charcoal agar plates were prepared containing, in addition to cephalexin, 50 μg of amphotericin per ml. The medium was inoculated from the original swab. The plates were then aerobically incubated in a moist atmosphere at 36°C and examined with a stereomicroscope on each of the next 7 days.

Transport medium. The transport medium was the same as the isolation medium, except that only half-strength was used. The cephalexin and defibrinated horse blood concentrations were unaltered. Each Bijoux bottle was filled to the neck, capped securely, and stored at 4°C. They were dispatched, upon request, with a stated 8 weeks expiry date from the day of preparation.

Shelf life determination. The transport medium performance was evaluated as to its shelf life by using the following as indicator organisms: *B. pertussis*, *B. parapertussis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Haemophilus influenzae*.

A standardized suspension in phosphate-buffered saline (pH 7.2) equivalent to a McFarland no. 2 nephelometer standard was prepared for each organism. A 0.1-ml portion of this suspension was absorbed onto a sterile cotton swab and placed in a Bijoux bottle containing the transport medium. Eight sets of transport medium were prepared for each organism and were stored at 4°C. During each of 8 successive weeks, suspensions of each organism were prepared as described, and each was introduced to a set of transport medium. A Miles and Misra (26) viability count was performed on these suspensions by using the charcoal agar medium with and without cephalexin. All bottles were stored at room temperature after swab introduction. At 24- and 48-h intervals, a swab was removed from each bottle in one set of transport media and placed in 4.5 ml of phosphate-buffered saline. The absorbed material was resuspended with a Vortex mixer for 30 s.

***B. pertussis* collection and transportation outfit.** Three methods of collection have been widely used: cough plate, pernasal swabbing, and auger suction.

The poor performance and misuse of the cough plate technique is not disputed and has been abandoned by most workers. Auger suction is excellent for on-site collection and is widely used in hospitals. It is not, however, an ideal method of collection for the physician's office or contact surveys. We felt that the preferred method for these areas would be the pernasal swab. There is, however, apprehension with regard to its use. Such fears as cotton swab detachment during use or injury to the patient due to wire protrusion have been cited (13), but we were able to locate an excellent cotton-tipped wire swab (MW 160 Pro. Lab. Inc., Toronto) that avoided both these dangers completely. Since it was felt that upper respiratory distress was not necessarily caused by *Bordetella* alone (5, 29), we decided to equip our outfit with a Bijoux bottle containing Amies modification of Stuart transport medium (2). In addition to this, we included our medium and applicators for throat swabs, direct smears for fluorescence studies, and pernasal swabs; microscope slides marked for direct fluorescent antibody (FA) and Gram stains; and instructions for use of the outfit.

The swab from Amies transport medium was treated as a throat swab and plated on routine isolation medium.

Enrichment method. All swabs after initial direct plating were replaced in our transport medium, incubated for 48 h, and subcultured onto isolation medium (with and without cephalexin) and examined on each of 5 successive days or less if *B. pertussis* or *B. parapertussis* had been recovered. If swabs were received in Amies transport medium only, these were directly inoculated onto the isolation medium and then inserted into our freshly prepared transport medium and processed by the enrichment method.

Auger suction specimens. Auger suction specimens were inoculated onto isolation medium. Excess secretion was recovered by a sterile swab that was then placed in our medium and processed by the enrichment method. Two "impression" smears were made from the submitted material and processed by the FA technique.

FA technique and slide agglutination. Direct smears prepared in the physician's office were examined by standard fluorescent technique (7) as were smears of microcolonies obtained on isolation medium.

Bordetella were identified by the slide agglutination technique with Wellcome antiserum (AM 12 and 13) and by the standard FA technique (FA bordetella conjugated antisera, Difco no. 2359-56 and 2378-56) and initially were confirmed by growth reactions on MacConkey agar, nutrient agar, citrate, and urea and by its motility. Subsequently, however, these tests were used only in instances of equivocal results such as "rough" serological reactions.

RESULTS

We established the superiority of charcoal agar to Bordet Gengou as an isolation medium. The use of this medium without cephalexin proved fruitful in cases of pure growth of *Bordetella*. In these instances, microcolonies of the organism were evident on the cephalexin-free medium in less than 36 h, whereas growth did not appear on the cephalexin-containing medium until a full 48 h. This indicated a slight retardation of the growth rate by cephalexin. At no time was *B. pertussis* or *B. parapertussis* recovered from the cephalexin-free medium only. Potential overgrowth was observed earlier on the antibiotic-free charcoal agar and allowed preparation of the amphotericin-cephalexin charcoal agar within a 24-h period.

A total of 3,237 specimens were processed from August 1974 to April 1976. From these, 1,419 isolates of *Bordetella* were obtained, as

well as 358 inconclusive results, most of which were due to an overgrowth of fungi. After amphotericin was introduced into the medium, the number of inconclusive results was considerably reduced and caused thereafter only by *Pseudomonas aeruginosa* and other cephalexin-resistant bacteria. The overall isolation rate of 44% encompasses all types of specimens received.

Our medium was used for 1,816 specimens, from which 786 cultures of *Bordetella* were recovered. The isolation rate was 43%, with 165 inconclusive results. Fifty-four specimens were taken from 47 patients and nursing staff involved in a cross-infection outbreak, and 7 of these were resubmitted in our transport medium after the patients underwent chemotherapy. The survey produced 15 positive cultures – a 32% isolation rate. The seven resubmitted specimens were negative.

A total of 97 auger suction specimens were received, from which 38 positive cultures were obtained, giving a 39% isolation rate. This group included six inconclusive results.

The shelf life of the transport medium was established as being 8 weeks (Table 1). The results expressed as a ratio, the inoculum being unity, indicated a reduction in the activity of growth ingredients in the medium. The recovery at 8 weeks was reduced to a point where scantily populated specimens would not survive in transit in medium any older than this. The other indicator organisms continued to be inhibited on cephalexin isolation medium but showed a resurgence when plated on cephalexin-free charcoal agar at 8 weeks, revealing a loss of cephalexin activity in the transport medium.

TABLE 1. *Effect of medium storage time on recovery of B. pertussis and B. parapertussis*

Medium storage time (wk)	Holding time after inoculation (h)	Recovery ratio[a]	
		B. pertussis	*B. parapertussis*
0	24	2.55	0.59
	48	2.00	1.06
1	24	1.75	1.02
	48	ND[b]	0.64
2	24	0.33	ND
	48	0.03	ND
3	24	0.35	0.12
	48	0.18	0.46
4	24	3.27	1.05
	48	ND	ND
7	24	0.63	0.07
	48	0.20	0.10
8	24	0.03	0.11
	48	ND	ND

[a] Ratio = number of colonies recovered from transport medium stored at 4°C before inoculation/original inoculum.

[b] ND, No data.

The *B. pertussis* outfit containing Amies transport medium produced 383 upper respiratory pathogens. Of the 518 specimens received, 92 had pathogens as well as either *B. pertussis*, *B. parapertussis*, or both (Table 2). During the 20-month period, 243 specimens had pathogens but no *Bordetella* isolates. From Amies medium, 156 other pathogens were obtained in conjunction with 580 positive cultures.

The isolation of *Bordetella* obtained by the enrichment method is shown in Table 3. Isolates obtained by enrichment from specimens received in our transport medium numbered 137 from a total of 757 (18%). However, in the case of isolates from Amies transport medium, 290 of 567 *Bordetella* were obtained by enrichment only (51%). From 61 auger specimens, 29 cultures were positive, of which 7 (24%) came by the enrichment route.

The results obtained by fluorescent staining of physician-prepared smears were compared with those of culture isolates (Table 4). A total of 438 specimens were compared, of which 42%, or 184, were positive by either or both methods; 7.5%, or 33, were positive by culture and FA. Thirty-four percent, or 151, were positive by culture only. There was no case of positive FA where the culture was negative. However, of the cultures positive, FA negative, 48 or 10% came from enrichment culture only.

DISCUSSION

Since there are disadvantages to all the transport methods reviewed (5, 14, 18, 19, 24, 27), the choice of transport medium must be based on a successful laboratory growth formula. The formula of Lacey (21) was not selected by reason of its complexity. Preston noted that recovery was not increased by adding penicillin to or removing blood from the transport medium. In our laboratory, we had already proven the efficacy of cephalexin over penicillin and therefore chose to add this to our transport medium. Other workers indicated the need for access to air and moisture (5, 29). In North America, the Jones and Kendrick transport slant is currently recommended as the preferred transport medium (28). But since it complies only with the former requirement, we produced a sloppy agar transport medium differing

TABLE 2. *Upper respiratory pathogens isolated in the presence of Bordetella*

Organism	No. of isolates
Acinetobacter calcoaceticus subsp. *anitratus*	3
Enterobacter sp.	2
Escherichia coli	9
E. coli and *Moraxella*	1
Eikenella corrodens	1
Haemophilus influenzae	23
H. influenzae and *Streptococcus pneumoniae*	2
Klebsiella pneumoniae	4
K. pneumoniae and *S. pyogenes*	1
Moraxella sp.	1
Pseudomonas aeruginosa	3
P. aeruginosa and *Enterobacter* sp.	1
Staphylococcus aureus	10
S. aureus and *H. influenzae*	2
S. aureus and *S. pneumoniae*	1
S. aureus and yeasts	1
S. pneumoniae	13
S. pneumoniae and *E. coli*	1
S. pyogenes	8
S. pyogenes and *Enterobacter* sp.	1
S. pyogenes and yeasts	1
Yeasts	3
Total 518 (*Bordetella*)	92

from the isolation medium only in its half-strength concentration. The cephalexin concentration was maintained at 40 μg/ml. The addition of blood to both isolation and transport media at a concentration of 10% has been stressed (5, 12). Harbour (12) in his studies indicated that blood had an effect on the numbers and size of *B. pertussis* colonies. He stated that lysed, defibrinated horse blood produced smaller but more numerous colonies, whereas fresh, whole defibrinated blood had the opposite effect. The authors considered the latter to be acceptable for an isolation medium.

In the development of the transport medium, based on preliminary results obtained from its use, we not only considered that the medium retained viability of *B. pertussis* and *B. parapertussis*, but that these organisms increased in number while in transit. Analyses of positive cultures showed that when the specimen had been submitted in our transport medium, more isolates were grown in a shorter time than if Amies transport had been used. This could have indicated that the specimens that had been submitted in our medium had a denser number of organisms present at the time of collection than those in the Amies medium. The other possibility was that the *Bordetella* transported in our medium had indeed grown while in transit, whereas those in Amies medium had not. Extrapolation of this latter theory suggested that scantily populated specimens, irrespective of the transport medium, would benefit from preincubation before plating, so we applied this enrichment method to the swabs from Amies transport medium.

We analyzed 688 positive cultures obtained from specimens submitted in either our medium or Amies medium with regard to their time in transit and incubation period before the isolation of *Bordetella*. A total of 375 isolates were obtained from direct plating and 93 from enrichment plating of specimens submitted in our medium. In the case of isolates from Amies medium, the figures were divided almost equally between direct and enrichment plating. At no time was an isolate obtained from direct plating that was not also present on enrichment. The majority of isolates from our transport medium were grown on direct plating within 4 days, whereas those from the Amies medium were divided equally between direct and enrichment methods for the same incubation period.

We considered that if the number of *Bordetella* organisms present were reduced to the point where they required enrichment before successful isolation, then a smear made from such a specimen would be equally devoid of the organism. We further conjectured that if the smear were subjected to FA staining, it would be negative for *Bordetella*. In this respect, we examined the direct FA results in comparison with positive cultures (Table 4). In this analysis we have shown that only 7.5% of our FA-positive results were also culture positive, whereas 34% of the culture positive were FA negative, and of these 10% were obtained by enrichment

TABLE 3. *Isolation rate of total B. pertussis obtained by enrichment method*

Determination	No. of specimens	Total isolates	No. of enrichment isolates	% Total
Our medium	1,832	757	137	18
Amies medium	1,183	567	290	51
Auger suction	61	29	7	24

TABLE 4. *Results of direct FA staining and cultures for Bordetella*

Result	No.	% Total
Positive, either or both methods	184	42
Culture +, FA +	33	7.5
Culture +, FA −	151	34
Culture −, FA +	0	0
Culture −, FA −	254	58
Culture + (Enrichment), FA −	48	10

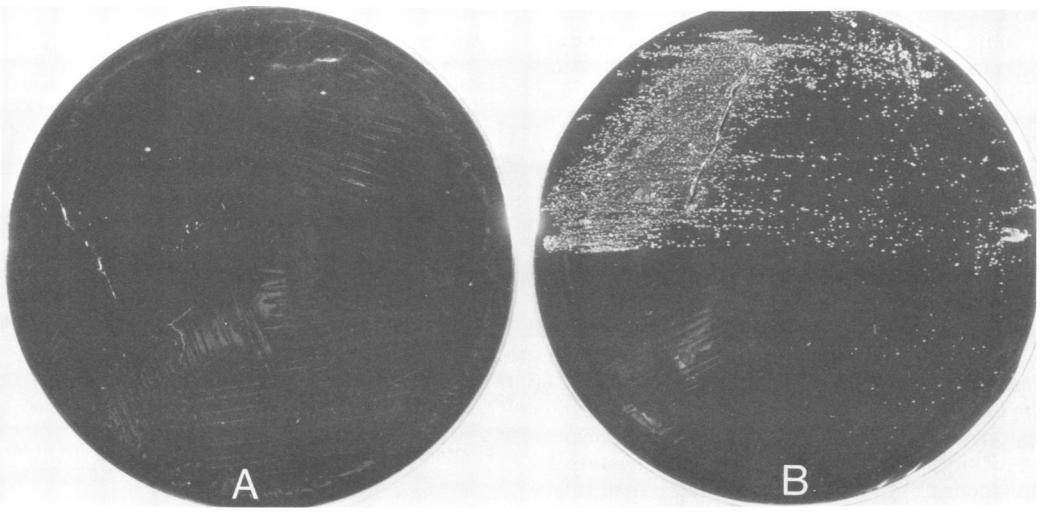

FIG. 1. *Four colonies of Bordetella isolated after (A) 3 days of incubation with direct plate medium and (B) 48 h of incubation with enrichment plate medium.*

only. It is our contention, therefore, that there is misplaced emphasis on the laboratory diagnosis of whooping cough based on fluorescent antibody staining of direct smears.

It is sometimes difficult to verbally capture the contribution that the enrichment method can make to the isolation of *Bordetella*. However, an example of its effect is depicted in Fig. 1, showing 4 colonies that were isolated after 3 days incubation of the direct plate medium. This is compared to the massive growth produced by the same swab incubated for 48 h by the enrichment method. A time differential of 24 h occurred between direct- and enrichment-plate recovery.

Many workers have dealt with the problem of transportation. In 1969, Jones and Kendrick (16) performed studies with Cohen-Wheeler medium. From their results, the solid medium proved to be positive for up to 4 days, with only occasional recovery of *Bordetella* after 7 days, thus verifying the findings of Gastrin et al. (10) with regard to the failure of sparsely inoculated Stuart transport medium to support *Bordetella* after 2 days. However, Preston (29), dealing with a charcoal medium, considered more than a one-day delay to be excessive.

Our findings confirm the results of Jones and Kendrick with regard to the Cohen-Wheeler medium. However, in the case of charcoal modification of Stuart medium (2), our results indicate that recovery, although limited, is still possible after 7 days and disagree with Preston in that after a 3-week delay, it was still possible to obtain a positive isolate.

In the shelf-life experiment, we realized that utilizing the dehydrated medium at half strength also reduced supportive ingredients by half. However, in routine use, shortage of available medium necessitated preparation of the medium from basic ingredients. The agar only was reduced to half strength. The medium prepared in this manner showed no beneficial effect to the survival rate or size of *Bordetella* colonies. The choice of a sloppy agar was made to comply with the need for access to air and moisture (5, 29) and to allow for ease of swab placement. The inclusion of Amies transport medium in our outfit has been justified by the results shown in Table 2. Respiratory distress has been documented as being symptomatic of *B. pertussis* involvement. However, other disorders display similar symptoms (3, 6, 14). It was considered that to develop an outfit capable only of isolating *B. pertussis* and *B. parapertussis* would leave non-*B. pertussis* cases either undetected or delayed in their detection.

Many workers have pursued direct smear FA examination with varying degrees of success (8, 11, 13, 31). It was our experience that although "heavy" positives were obtained by this technique, scant positives were negative. This method also required more examination time than the enrichment method, which enabled rapid isolation of positive cultures. The technique, however, was retained for testing microcolonies. It was equally true that success of the outfit brought such an increase in examination requests that with the time involved in performing the direct smear FA method, the volume could not be coped with. Also, the authors felt results did not warrant continuance of the

direct smear procedure.

Some interesting experience was gained during our 20-month study. Spread of infection within hospitals has been documented by other workers (20, 23, 25). During 1 month of the 20-month period, our laboratory received 27 specimens from patients of the pediatric ward of a local hospital. Of these, 17 specimens yielded *B. pertussis*. A survey of 33 nursing staff produced 12 positive isolates. After treatment, only 1 of the 12 remained positive. But after a change in chemotherapy, a negative result was obtained. This successful eradication resulted in no requests for 2 months thereafter.

Apart from the obvious adult involvement of the previously mentioned nursing staff, various age groups of above 0 to 5 years were encountered since the introduction of the system to this laboratory. Although we had no firm information as to symptoms or whether the patients were contacts only, the question of the carrier state is very real (1, 4, 9, 14, 15, 17, 19, 24, 27). The authors consider that the information gained in this 20-month period adds credence to the school of thought that adults, as much as the 0- to 5-year age group, contribute greatly to the spread of infection. In a randomly selected, but consecutive group of 407 positive cultures, we observed 174 isolates (42.8%) in the 0 to 5 years group; 48 (11.6%) in the 6 to 10 years; 28 (6.8%) in the 11 to 15 years; 11 (2.8%) in the 16 to 20 years; 17 (4.2%) in the 21 to 30 years; and 9 (2.3%) in the 31 to 40 years range. In the above 40 years age group, we encountered 12 (3%) patients whose ages included 52, 65, and 72 years. There were 108 patients (26.6%) of the 407 positives selected in which their age was not divulged.

In conclusion, we feel that adaptation of our transport medium as an enrichment medium establishes a means for the recovery of *Bordetella* from any method of collection. However, the number of negative results derived from direct smear fluorescent staining in culturally confirmed cases, invalidates the use of this technique. Using this technique alone to determine infection is even more of a risk.

The majority of specimens submitted in Amies transport medium only were throat swabbings. This therefore raises the question of the necessity for sophisticated collection, e.g., auger, pernasal. The authors intend to expand their investigation to include a parallel study of throat and pernasal swabs as a means of collection and transportation. We also emphatically recommend the use of our medium as an enrichment method, irrespective of the collection procedure, for the isolation of both *B. pertussis* and *B. parapertussis*.

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