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Effects of Transport Temperature and Medium on Recovery of *Bordetella pertussis* from Nasopharyngeal Swabs

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We compared relative recoveries of *Bordetella pertussis* from simulated nasopharyngeal (NP) specimens incubated in three separate transport media at different temperatures. Transport media included one-half-strength Regan-Lowe (RL.5), Regan-Lowe with one-half-strength agar (RL.5A), and buffered charcoal-yeast extract agar supplemented with alpha-ketoglutarate, lincomycin, and anisomycin (BCYEαLA). For each transport medium, recovery of *B. pertussis* was least efficient after storage at 25°C. The highest recovery of *B. pertussis* from a mixed culture was achieved with RL.5 at 4°C. Overall, RL.5 and RL.5A were comparable as transport media whether held at 4 or 25°C, but fewer organisms were recovered from BCYEαLA. In addition, Regan-Lowe (RL), Bordet-Gengou, and cyclodextrin media were compared as primary isolation media for recovering *B. pertussis* from simulated NP swabs held at 4 and 35°C in RL.5 medium. The highest recovery of *B. pertussis* was obtained on RL primary isolation medium. Bordet-Gengou medium recovered only 80% and cyclodextrin medium recovered <60% of the numbers recovered on RL medium. Based on these results, refrigeration (4°C) of NP swabs shipped in RL.5 transport medium and using RL as the primary isolation medium are recommended for recovering *B. pertussis* from swab specimens.

The low isolation rate of *Bordetella pertussis* from patients with whooping cough has hindered diagnosis of this disease for many years (6, 10, 14, 15; J. Regan, Clin. Microbiol. Newsl. 2:1–3, 1980). Attempts to improve the isolation of *B. pertussis* from nasopharyngeal (NP) swabs have included the use of growth stimulants for *B. pertussis* (7, 8), use of different types of NP swabs (1, 4, 15), development of new transport and primary isolation media (3, 7–10, 14, 16, 17), and incubation of transport and primary isolation media at various temperatures (4, 5, 13, 15). Despite this extensive research, the optimal conditions for the recovery of *B. pertussis* from clinical specimens remain poorly defined.

It is currently recommended that pernasal NP swabs be collected from all patients during a pertussis outbreak (11). If immediate plating of the swab samples is not possible, the samples are usually submerged in one-half-strength Regan-Lowe transport medium (RL.5) and then shipped to a processing laboratory. Swabs are cultured on both Bordet-Gengou (BG) and Regan-Lowe (RL) selective media and then returned to the transport medium for enrichment at 35°C. After being incubated for 48 h, swabs are again cultured on BG and RL media.

The type of NP swab used to collect clinical specimens affects the isolation rate of *B. pertussis*. Dacron, rayon, calcium alginate, and cotton wool swabs have been tested for their ability to maintain *B. pertussis*. Several studies have cited inhibition of growth of *B. pertussis* by fatty acids associated with cotton wool swab material (4, 5, 15). Calcium alginate, Dacron, and rayon swabs have all proved to be superior to cotton wool (4, 5, 15).

Even though the transport of NP swabs is common, shipping and storage conditions are not standardized. If swab specimens are processed the same day as collected, a transport medium may not be necessary (6, 13). However, inhibition of *B. pertussis* by growth of NP flora on swabs held in transport media at ambient temperature has been reported (13, 14). Incubation of swabs at 35°C before shipment can increase *B. pertussis* numbers, but may also permit overgrowth by NP flora. One study showed better recovery of *B. pertussis* from Dacron swabs held for 48 h at 4°C compared with ones held at room temperature (15). Since transportation time for swab specimens may exceed 48 h, shipping temperature needs to be regulated.

We attempted to define optimal conditions for the recovery of *B. pertussis* from NP swabs using only currently accepted media and focusing on the effects that transport and incubation temperatures have on the survival of *B. pertussis*.

MATERIALS AND METHODS

Bacterial strains. *B. pertussis* TX-13 (Centers for Disease Control designation), an isolate passaged fewer than four times on RL, was used in all experiments.

Bacteria used for mixed cultures consisted of Centers for Disease Control stock trains of *Bacillus cereus*, *Candida krusei*, *Staphylococcus epidermidis*, and *Streptococcus salivarius* maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). *S. epidermidis* and *C. krusei* were included because they represent NP flora (11), while *S. salivarius* is a common inhabitant of the throat (2). *B. cereus* was also included because *Bacillus* spp. have often been present in the clinical specimens received by our laboratory.

Transport media. Two RL-based transport media were used. The first (RL.5 medium) was half-strength charcoal agar (Oxoid Ltd., Hampshire, England) with 10% (vol/vol) defibrinated horse blood, 40 μg of cephalexin per ml, and 50 μg of amphotericin B per ml. The second transport medium (RL.5A) contained 10 g of beef extract, 10 g of meat peptone, 10 g of soluble starch, 4 g of charcoal, 5 g of NaCl, 1 mg of nicotinic acid, and 6 g of agar per liter. The same concentrations of antibiotics and blood were used in both media.

Buffered charcoal-yeast extract agar (12) with 1% (wt/vol)

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alpha-ketoglutarate, 3 μg of lincomycin per ml, and 80 μg of anisomycin per ml (BCYEαLA) was also evaluated.

The transport media were aseptically dispensed at 4 ml per tube into sterile plastic screw-cap test tubes (13 by 100 mm). All media were used within 7 days of preparation.

Primary isolation media. Recovery of *B. pertussis* from RL.5 transport medium was studied with RL, BG, and cyclodextrin primary isolation media. RL medium consisted of Oxoid charcoal agar with 10% (vol/vol) defibrinated horse blood and 40 μg of cephalexin per ml (14). Cyclodextrin medium was composed of Stainer-Scholte liquid medium with Casamino Acids (Difco Laboratories, Detroit, Mich.), heptakis (2,6-*o*-dimethyl) β-cyclodextrin, and 1.8% (wt/vol) agar (8). BG agar was prepared from Difco dehydrated BG base (without peptone) plus 1% (vol/vol) glycerol, 15% (vol/vol) defibrinated sheep blood, and 2.5 μg of methicillin per ml.

Trypticase soy agar was used for culturing *B. cereus* and *C. krusei*. *S. epidermidis* and *S. salivarius* were cultured on Trypticase soy agar plus 5% (vol/vol) sheep blood (BBL Microbiology Systems).

NP swab inoculation. *B. pertussis* TX-13 was passaged twice on RL medium before being harvested and suspended in sheep blood. Suspensions were stored at −70°C until required, when aliquots were thawed and spread on BG agar and incubated for 4 days in a humidified 35°C incubator. All four NP flora strains were grown at 35°C for 48 h on appropriate media and then subcultured to RL agar.

Cells were suspended in a solution of 1% (wt/vol) Casamino Acids. Optical densities were measured at 540 nm with a spectrophotometer (model 24; Beckman Instruments, Inc., Fullerton, Calif.) and adjusted to 0.96. Suspensions were diluted to give a final concentration of 5×10^4 CFU/ml for *B. pertussis* and 2.5×10^4 CFU/ml for all other test organisms. CFU were counted after culturing 0.1 ml of each of the respective suspensions on triplicate plates of the appropriate growth medium for each strain.

Because of better adherence of swab head to shaft, Dacron swabs were used in this study. NP swabs were immersed in the suspensions of *B. pertussis* alone or *B. pertussis* mixed with the other test organisms for 10 to 15 s before being placed into a transport medium. Triplicate swabs were used for each temperature and day of incubation. Tubes were held at −20, 4, 25, or 35°C or a combination of these temperatures and examined at 24-h intervals.

To determine numbers of *B. pertussis*, swabs were removed from transport tubes and vortexed individually in 0.9 ml of 1% Casamino Acids for 15 s. Vortexed suspensions were cultured (0.1 ml per plate) by the spread plate method on single plates of each medium being evaluated. Plates were incubated at 35°C in a humidified incubator for 6 days before CFU were counted. All counts are expressed as a mean of triplicate swabs.

RESULTS

Recovery of *B. pertussis* from NP swabs inoculated with an axenic culture in RL.5 transport medium was highest at 35°C. Although *B. pertussis* numbers declined slightly in the first 24 h, multiplication began during the next 24 h and continued until day 8. Serial dilutions of swab suspensions revealed that *B. pertussis* numbers peaked on day 8 at 1.8×10^9 CFU/ml, but decreased 30% to 1.2×10^9 CFU/ml by day 9.

Recovery of *B. pertussis* was similar from NP swabs held at −20 and 4°C in RL.5 medium. In the first 24 h, a >75% decrease in *B. pertussis* CFU was observed for both temperatures (Fig. 1). Throughout the next 8 days, *B. pertussis* numbers were maintained at both −20 and 4°C. There was no significant difference in *B. pertussis* recovery between −20 and 4°C ($P < 0.01$). Recovery of *B. pertussis* from NP swabs was worst at 25°C. *B. pertussis* was not isolated after 48 h from axenic culture (Fig. 1).

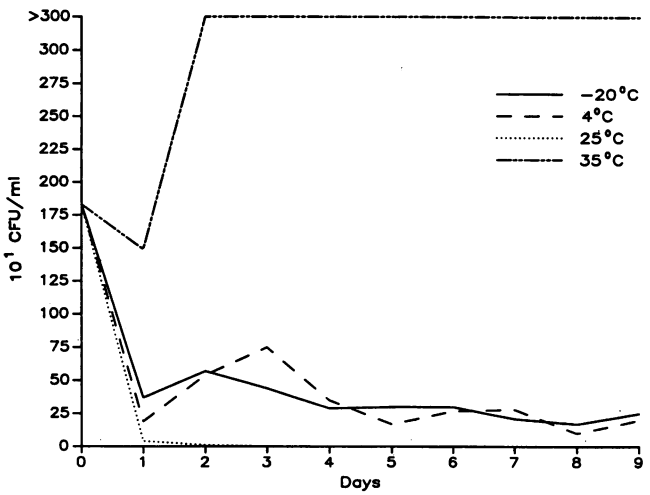

FIG. 1. Recovery of *B. pertussis* from RL.5 transport medium using RL as the primary isolation medium.

The recovery of *B. pertussis* from mixed culture in RL.5 medium was affected the most at 35°C. *B. pertussis* was not isolated from mixed culture at this temperature because of overgrowth by the test organisms. At 4 and −20°C, only *B. pertussis* was recovered from swabs inoculated with mixed culture. The other four test bacteria were not recovered after 48 h. Numbers of *B. cereus*, *C. krusei*, and *S. epidermidis* increased at a slower rate at 25°C than at 35°C, but *B. pertussis* was not isolated after 48 h. *S. salivarius* was not recovered after day 3.

We also examined the effects of a combination of storage temperatures on *B. pertussis* recovery (Table 1). When swabs were held in RL.5 and RL.5A media at 4°C for 24 h and then shifted to 35°C for 48 h, *B. pertussis* CFU declined 20 to 30% during refrigeration, but increased during incubation at 35°C. If swabs were incubated at 35°C for 48 h preceding refrigeration, no decrease in *B. pertussis* numbers was evident. Shifting preincubated swabs to 25°C for 24 h caused a >50% decrease in *B. pertussis* recovery. As before, room temperature incubation had an adverse effect on the recovery of *B. pertussis* from NP swabs held in RL.5 medium.

TABLE 1. Recovery of *B. pertussis* TX-13 from NP swabs held in two transport media at various temperatures

Incubation temp	Transport medium	*B. pertussis* (10^3 CFU/ml)[a]		
		Day 1	Day 2	Day 3
4°C for 24 h shifted to 35°C for 48 h	RL.5	1.90	>3.00	>3.00
	RL.5A	2.26	2.50	>3.00
35°C for 48 h shifted to 4°C for 24 h	RL.5	1.86	>3.00	>3.00
	RL.5A	2.65	>3.00	>3.00
35°C for 48 h shifted to 25°C for 24 h	RL.5	1.75	>3.00	1.18
	RL.5A	2.07	>3.00	1.15

[a] RL medium used for recovery from inoculum of 2.8×10^3 CFU/ml.

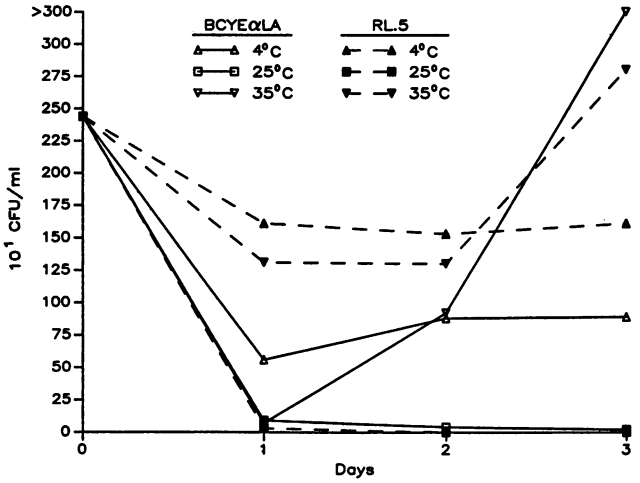

FIG. 2. Recovery of *B. pertussis* from mixed culture in BCYEαLA and RL.5 transport media held at 4, 25, and 35°C and plated on RL medium.

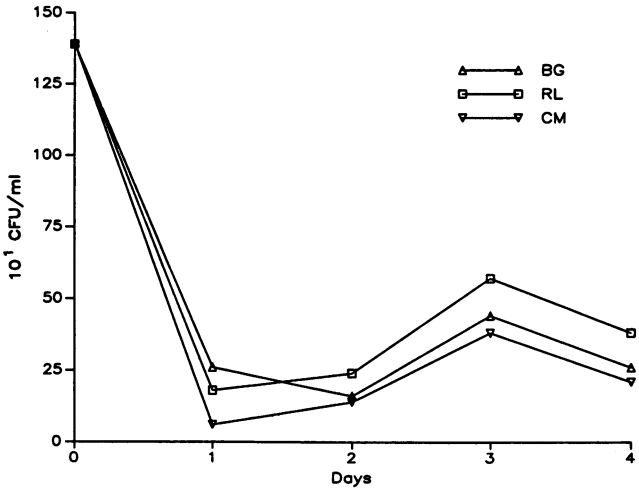

FIG. 4. Recovery of *B. pertussis* from mixed culture in RL.5 transport medium held at 4°C and plated on BG, RL, and cyclodextrin (CM) media.

In a comparison of transport media, recovery of *B. pertussis* from RL.5 medium was 30% higher at 4°C than recovery from BCYEαLA (Fig. 2). At 35°C, recovery of *B. pertussis* from RL.5 medium was more efficient during days 1 and 2, but 10% less efficient than from BCYEαLA on day 3. Viability of *B. pertussis* in these transport media was comparable at 25°C. The antibiotics used in BCYEαLA were no more effective at inhibiting the test flora than those in RL medium.

Recovery of *B. pertussis* from swabs was similar in RL.5 and RL.5A transport media at 4 and 25°C (Fig. 3). When we tested shifting temperatures during incubation, the numbers of *B. pertussis* recovered from RL.5A medium were slightly higher than those recovered from RL.5 medium in some instances, but the differences were not significant ($P < 0.01$; Table 1).

RL medium provided the best recovery of *B. pertussis* from axenic culture NP swabs held in RL.5 medium at 4°C. Recovery of *B. pertussis* on BG agar was only 80% as effective as on RL medium. Cyclodextrin medium was the least efficient medium tested; only 60% was recovered compared with RL medium at 4°C (Fig. 4). Despite an initial 75% decrease in *B. pertussis* numbers in the first 24 h, overgrowth of *B. pertussis* on all media occurred after 48 h at 35°C.

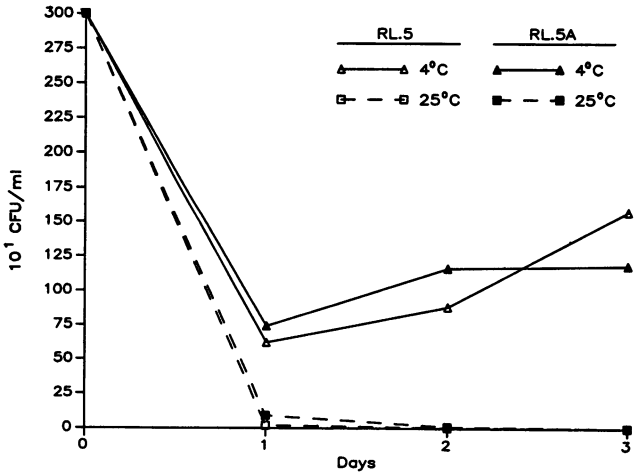

FIG. 3. Recovery of *B. pertussis* from mixed culture in RL.5 and RL.5A transport media held at 4 and 25°C and plated on RL medium.

DISCUSSION

The NP specimens we receive are routinely shipped at ambient temperature, with a transport time ranging from 1 to 3 days. Despite the addition of cephalexin and amphotericin B to our RL transport medium, overgrowth by NP flora is common, making the recovery of *B. pertussis* a difficult task. We studied the effects of transport temperature and medium on the survival of *B. pertussis* to improve the shipping practices used for NP specimens.

Our results indicated that *B. pertussis* survives best in RL.5 transport medium incubated at 35°C, as other studies have shown (4, 5, 11). Although this was the only temperature included in our study that allowed growth of *B. pertussis*, it is only suitable for axenic cultures. In mixed culture, *B. pertussis* was overgrown by the NP test bacteria within 48 h. Preincubation of swabs before transport can increase the numbers of *B. pertussis* in a specimen, but this procedure is valuable only if normal flora can be controlled with antimicrobial agents.

Our results repeatedly identified 25°C as the least desirable storage temperature for NP swabs inoculated with *B. pertussis*. We were unable to recover *B. pertussis* after 72 h of storage in any of the transport media incubated at 25°C. Hoppe et al. (4, 5) reported that 2 days of incubation at 35°C followed by 1 day at 20°C gave them the best recovery of *B. pertussis* from swabs. We found that a shift from 35 to 25°C caused a >50% decrease in numbers of *B. pertussis* recovered within 24 h.

Contrary to reports that 4°C is not a favorable shipping temperature, we were able to isolate large numbers of *B. pertussis* from specimens accidentally refrigerated during shipping. At least one other study (15) has shown that *B. pertussis* can be recovered from Dacron swabs more efficiently at 4°C than at 25°C. After an initial 75% decrease in the first 24 h, we observed a subsequent stability of *B. pertussis* numbers at 4°C for up to 8 days. This stability at

4°C indicates that this temperature is an appropriate shipping temperature for NP swabs.

Regan and Lowe (14) previously stated that use of RL.5A transport medium did not promote a higher recovery rate for *B. pertussis* than RL.5 medium. Our results support this finding at every temperature tested. Considering the ease of preparation of RL.5 over RL.5A medium, there is no advantage to using the latter formulation.

Recent research by Imaizumi et al. (7, 8) with heptakis (2,6-*o*-dimethyl) β-cyclodextrin indicates that cyclodextrin medium is comparable to BG agar in plating efficiency and growth rate of *B. pertussis*. In our study, cyclodextrin medium did not compare well with either BG or RL medium in the isolation of *B. pertussis* from swabs held at 4°C, although results were comparable at 35°C after 48 h. Cyclodextrin is not as effective a growth stimulant in a solid medium as it is in a broth (7). The addition of agar may interfere with the formation of inclusion complexes, which remove toxic inhibitors from cyclodextrin broth.

In conclusion, our data show that when standard RL.5 transport medium is used, RL primary isolation medium is preferred for the isolation of *B. pertussis* from simulated NP swab specimens. Refrigeration of swabs during shipping should also improve primary isolation of *B. pertussis*.

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