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# Inhibition of PCR-Based Assay for *Bordetella pertussis* by Using Calcium Alginate Fiber and Aluminum Shaft Components of a Nasopharyngeal Swab

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**A PCR-based assay for *Bordetella pertussis* was inhibited by using a calcium alginate fiber-tipped swab with an aluminum shaft but not by using a Dacron fiber-tipped swab with a plastic shaft. The calcium alginate fiber component inhibited the assay following storage for less than 1 min in a suspension of $10^3$ CFU of *B. pertussis* per ml, whereas the aluminum shaft component required storage for at least 48 h in order to cause inhibition. We recommend the Dacron swab over the calcium alginate swab for collecting specimens for testing in PCR-based assays.**

Recent studies (5, 6, 9) suggest that PCR-based assays are more sensitive than culture for detection of *Bordetella pertussis* in nasopharyngeal specimens. Primers used for the amplification of target regions within the chromosome of *B. pertussis* and probes used for the detection of the amplified products (5, 6, 9, 14) have been developed from the DNA sequence information that is available for a repeated chromosomal element (5) and the pertussis toxin gene (10). The PCR-based assays have the potential for same-day turnaround time, whereas final isolation and identification with culture generally take 3 to 5 days. Since antibiotic susceptibility testing is not routinely performed with isolates of *B. pertussis*, the PCR may eliminate or reduce the need for culture. Nasopharyngeal swab and aspirate specimens are used with culture and PCR-based assays for detection of *B. pertussis*. For isolation of *B. pertussis* by culture, calcium alginate swabs are superior to Dacron, rayon, and cotton swabs (4, 7, 8). However, the effect of different swab types on PCR-based detection of *B. pertussis* is unknown.

Suspensions of bordetellae were used to assess the effects of calcium alginate and Dacron swabs on a PCR-based assay for *B. pertussis*. Calcium alginate- and Dacron fiber-tipped nasopharyngeal swabs and swab components were obtained from Medical Packaging Corp. (Camarillo, Calif.). The calcium alginate fiber was attached to an aluminum shaft, and the Dacron fiber was attached to a plastic (polystyrene) shaft. A recent clinical isolate of *B. pertussis* (strain BP5) was cultivated for 72 h on charcoal agar (Oxoid Unipath Ltd., Basingstoke, Hampshire, England) containing 10% defibrinated sheep blood. The bordetellae were harvested, washed three times by centrifugation at 2,000 × *g*, and suspended in sterile saline to provide suspensions with desired concentrations of the bordetellae. Template DNA was obtained from suspensions of bordetellae by digestion with lysozyme and proteinase K, and the chromosomal DNA was purified by extraction with phenol-chloroform-isoamyl alcohol and precipitation with ethanol as described previously (14).

**PCR.** Frozen samples (described below) were thawed and then treated with proteinase K (GIBCO BRL Life Technologies, Inc., Gaithersburg, Md.) as described previously (5) except that samples (60 μl) were digested with 60 μl of 0.4 mg of proteinase K per ml in 25 mM Tris-HCl and 4% glycerol. Oligonucleotides were synthesized with an automated DNA synthesizer (model 391; Applied Biosystems, Inc., Foster City, Calif.) using standard β-cyanoethyl phosphoramidite chemistry. A set of primers (BP13 and BP14) was designed to amplify a 201-bp segment of the previously described repeat element (5). A probe (BP15) was designed to detect the PCR product. The nucleotide sequences of the oligonucleotides (nucleotide positions and 5′→3′ orientation) were as follows: BP13 (785→805), CCGCGCTGTGCCATGAGCTGG; BP14 (987→967), GATGCCTTGGTGGGGTCGATG; and BP15 (875→912), CGGCCTTGCGTGAGTGGGCTTACGCTCACACCTACCAG. The probe was end labeled with $^{32}P$ by using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) (11). DNA was amplified in 100-μl reaction mixtures containing 200 μM (each) dATP, dUTP, dCTP, and dGTP; 2.0 U of Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.); 1.0 U of uracil-*N*-glycosylase (GIBCO BRL Life Technologies, Inc.); 10 pM primers BP13-BP14; 2.5 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.3); and 50 mM KCl. The reaction mixtures also contained 50 μl of either sample digest, positive control template DNA, or negative control. Precycle conditions consisted of incubation at 37°C for 10 min for degradation of amplimeric DNA by uracil-*N*-glycosylase followed by incubation at 94°C for 10 min for inactivation of uracil-*N*-glycosylase and denaturation of target DNA. Amplification (32 cycles) was performed in a Perkin-Elmer Cetus 9600 thermal cycler as follows: denaturation, 94°C for 1 min; primer annealing, 62°C for 30 s; and primer extension, 72°C for 1 min. The 201-bp PCR product was detected in all cases by either or both of the following electrophoretic methods. Agarose gel electrophoresis was performed by electrophoresis of 10-μl aliquots through gels consisting of 3% (wt/vol) Nusieve

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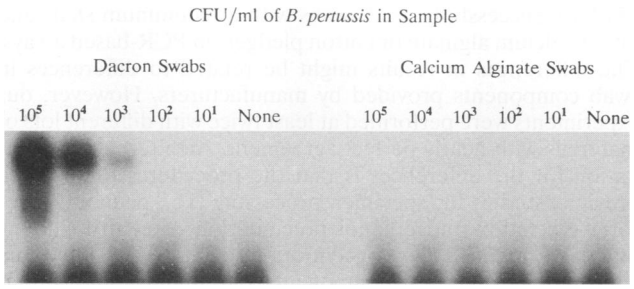


FIG. 1. Autoradiograph comparing Dacron and calcium alginate swabs for detection of *B. pertussis* by PCR-based assay.

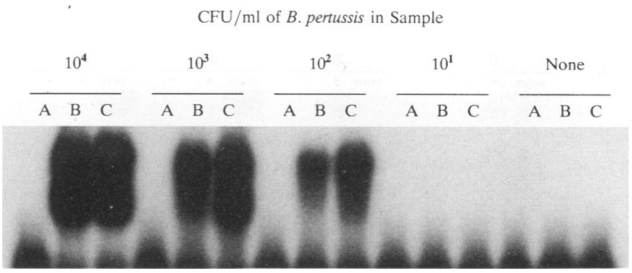


FIG. 2. Autoradiograph comparing effect of brief exposure of suspensions of bordetellae to calcium alginate fiber and aluminum shafts on detection of *B. pertussis* by PCR-based assay. Replicate samples in the following lanes were treated with the following materials: A, calcium alginate fiber; B, aluminum shaft; C, none.

agarose (FMC Bio Products, Rockland, Maine) and 1% (wt/vol) agarose (GIBCO BRL Life Technologies, Inc.) for 90 min at 80 V. Products were visualized by staining with ethidium bromide and then illuminated with UV light and by photography. Liquid hybridization of the $^{32}$P-labeled probe (BP15) with the PCR products, followed by separation of the hybrid molecules by polyacrylamide gel electrophoresis, and detection with autoradiography were performed as previously described (14).

Testing for PCR products from reaction mixtures containing serial dilutions of template DNA revealed a single band corresponding to a 201-bp product in agarose gel. The liquid hybridization format detected this product from reaction mixtures containing 5 fg (approximately 1 copy) of template DNA and was 200 times more sensitive than the agarose gel format. The liquid hybridization format was therefore incorporated into the PCR-based assay for detection of *B. pertussis*.

**Calcium alginate versus Dacron swabs.** The effect of calcium alginate and Dacron swabs on detection of *B. pertussis* by PCR and liquid hybridization was assessed with seeded swabs. Saline suspensions containing approximately $10^1$, $10^2$, $10^3$, $10^4$, and $10^5$ CFU of *B. pertussis* per ml and sterile saline (negative control) were each dispensed (in 0.5-ml portions) into two replicate sterile 13-by-100-mm glass test tubes (Fisher Scientific, Springfield, N.J.). The swabs were dipped into the tubes, immediately removed without being wrung, and placed into transport tubes, 12-by-75-mm polypropylene vials that contained 0.5 ml of sterile saline (Medical Packaging Corp.). Bordetellae were dislodged from the swabs by vortexing the contents of the vial for 10 s. The swabs were wrung against the inside wall of the vial to express moisture and discarded. Aliquots (0.1 ml) of the remaining suspension were stored at −80°C and subsequently tested in the assay. Figure 1 shows that the Dacron swabs that were seeded with the suspensions containing $10^3$ to $10^5$ CFU of the bordetellae per ml yielded the 201-bp product, whereas the calcium alginate swabs that were seeded with the suspensions containing $10^1$ to $10^5$ CFU of the bordetellae per ml were negative for the product.

**Inhibitory components of calcium alginate swabs.** The calcium alginate fiber and aluminum shaft components of the calcium alginate swab were evaluated for inhibition of the PCR-based assay. Suspensions of bordetellae containing approximately $10^1$, $10^2$, $10^3$, and $10^4$ CFU/ml and sterile saline (negative control) were each divided into three 0.5-ml portions in the 13-by-100-mm sterile glass test tubes. The replicate samples received either 12.9 mg of calcium alginate fiber (i.e., the amount per swab), an aluminum shaft, or no treatment. The samples were then immediately vortexed for 10 s. Any visible calcium alginate fiber that remained and the aluminum shafts were removed from the respective samples within 1 min and discarded. Aliquots (0.1 ml) of the samples were frozen at −80°C and subsequently tested by the assay. The 201-bp product was detected from the suspensions of bordetellae ($10^2$, $10^3$, and $10^4$ CFU/ml) exposed to the aluminum shafts or those that received no treatment, but it was not detected from those suspensions that were treated with calcium alginate fiber (Fig. 2). These results indicated that calcium alginate was a strong inhibitor of the PCR-based assay and inhibited at least one of the following steps: the lysis of *B. pertussis* by proteinase K, the amplification of the target sequence, or the hybridization of the probe with the 201-bp product.

Reaction mixtures were then prepared with 10 pg of template DNA and either 20 μg of calcium alginate or no calcium alginate (positive control) to assess the effect of calcium alginate on the PCR amplification independent from its effects on proteinase K activity and the liquid hybridization reaction. Analysis of the amplification products by agarose gel electrophoresis showed a single band corresponding to the 201-bp product from the positive control, whereas no bands were visible from the reaction mixture that contained the calcium alginate (gel not shown). This finding shows that a relatively small amount of calcium alginate inhibits the PCR amplification step of the assay.

Calcium analysis (1) performed on 0.5-ml saline transport samples that were exposed to calcium alginate swabs for less than 1 min showed that the concentration of calcium carried over into PCR mixtures was 1.4 ± 0.2 mM. To determine whether this concentration of calcium inhibits the PCR amplification, various concentrations of calcium chloride were added to PCR mixtures containing 10 pg of template DNA. Analysis of the amplified product by agarose gel electrophoresis showed that a concentration of 3.0 mM calcium did not inhibit the PCR amplification, whereas concentrations of ≥3.5 mM were inhibitory (gel not shown). These results indicated that the concentration of calcium, which was carried over from the swabs into the reaction mixtures, was insufficient to inhibit the PCR amplification and suggest that alginate per se is responsible for the inhibition. The alginate could cause the inhibition by adsorbing a critical component (e.g., $Mg^{2+}$) from the reaction mixture or entrapping the *Taq* DNA polymerase, preventing it from functioning. Entrapment of protein molecules by calcium alginate can occur, as evidenced by the development of a calcium alginate bead delivery system for protein growth factors (3). Since alginate is a crude extract from seaweed (2, 12), it is also possible that other substances from the seaweed are responsible for the inhibition.

The aluminum shaft component was also tested for its effect on the PCR-based assay following prolonged storage at room

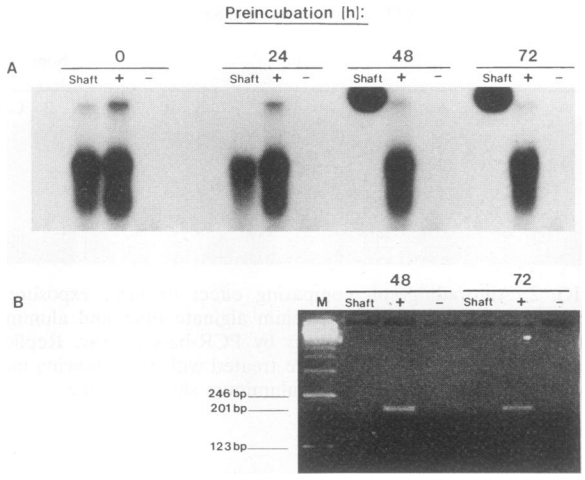


FIG. 3. Autoradiograph (A) and agarose gel stained with ethidium bromide (B) showing effect of prolonged incubation of aluminum shafts with $10^3$ CFU of *B. pertussis* per ml on detection of *B. pertussis* by PCR-based assay. Lanes: +, positive control; −, negative control; M, 123-bp ladder marker; Shaft, aluminum shaft.

temperature in 0.5-ml saline transport samples. Replicate suspensions of *B. pertussis* ($10^3$ CFU/ml) were incubated with and without shafts (positive control), as described above. Sterile saline served as a negative control. Figure 3A shows that incubation of the shafts in the bordetella suspensions for 0 and 24 h did not inhibit the amplification of the 201-bp target in comparison to the positive control samples; however, the $^{32}$P-labeled probe failed to migrate into the polyacrylamide gel following liquid hybridization with the amplified product from the bordetella suspensions that were incubated for 48 and 72 h with the shafts. A nonmigrating band was also observed from the liquid hybridization product of a 72-h, saline shaft eluate and the $^{32}$P-labeled probe (autoradiograph not shown). Analysis of agarose gels did not reveal the 201-bp product from the bordetella suspensions that were preincubated with the shafts for 48 and 72 h, although the product was detected from the respective positive control samples (Fig. 3B). Addition of the 72-h, saline shaft eluate to a PCR mixture containing 10 pg of template DNA inhibited the amplification of the 201-bp target, as determined by agarose gel analysis. These results indicate that a substance from the shaft inhibited the PCR amplification step of the PCR-based assay and adversely affected the migration of the probe through the polyacrylamide gel. The nonmigrating bands present in the polyacrylamide gels may represent probe molecules that are complexed into aggregates by the $Al^{3+}$ ions. The size and structure of the complex may prevent it from migrating through the gel. Repeat experiments indicated that the inhibition from the aluminum shaft was sporadic, usually occurring within 48 to 72 h of storing a shaft in saline but sometimes not occurring after a 72-h storage period. The total dissolved aluminum concentration was measured in six 72-h, saline shaft eluates by atomic adsorption spectrophotometry after the samples were acidified to a pH of 2.0. The range of aluminum concentrations in the eluates was 352 to 270,000 ng/ml, whereas saline controls contained <3 ng/ml, showing that $Al^{3+}$ ions are eluted from shafts upon storage in saline. Although our studies suggest that $Al^{3+}$ ions are responsible for the observed effects of the shaft on the PCR-based assay, the effects may have been caused by some other substance that we did not measure.

In contrast to our findings, the authors of recent reports (6, 13) have successfully used swabs with an aluminum shaft and either calcium alginate or cotton pledgets in PCR-based assays. The differences in results might be related to differences in swab components provided by manufacturers. However, our experiments were performed at least twice with different lots of materials with nearly perfect agreement. An alternative explanation for the differences is that the procedures used in the previous studies for specimen processing (i.e., phenol-chloroform extraction and ethanol precipitation or centrifugation) may have reduced the concentrations of potential inhibitors contributed by the swab components. Our findings suggest that the use of Dacron swabs with plastic shafts may eliminate the need for cumbersome DNA purification steps prior to the PCR and thereby simplify the testing of clinical specimens.

Our studies have led to the development of a collection kit (Medical Packaging Corp., Inc.) for obtaining nasopharyngeal swab specimens for detection of *B. pertussis* by PCR and culture. The kit consists of a Dacron swab with a plastic shaft and a polypropylene tube containing 0.5 ml of sterile saline for collection and transport of the PCR specimen; a calcium alginate swab with an aluminum shaft is provided in the kit for immediate inoculation of a nasopharyngeal specimen onto culture media or, alternatively, for delivery of the specimen in a transport medium. We are presently using this kit in a clinical evaluation of the PCR-based assay.

In conclusion, our studies show that both the calcium alginate and the aluminum shaft components of calcium alginate swabs inhibit the PCR-based assay for *B. pertussis*. We therefore recommend the use of Dacron swabs with plastic shafts over that of the calcium alginate swabs for obtaining specimens that are tested in PCR-based assays.

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