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Preservation of Nasopharyngeal Smears for Fluorescent Antibody Detection of *Bordetella pertussis*

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Smears from throat and nasal washings seeded with *Bordetella pertussis* were either treated with aprotinin (a protease inhibitor) or fixed with 1 or 10% Formalin. These smears were stored at 23, 4, and −70°C. Smears were removed and stained with a fluorescent antibody conjugate for *B. pertussis* at intervals during 22 days of storage. Results indicate that treatment of smears with 4 or 8 U of aprotinin per ml preserved fluorescent antibody staining qualities of *B. pertussis* for 22 days; fixation with either concentration of Formalin was unsatisfactory.

The incidence of pertussis in the United States has decreased from 49,000 reported cases in the late 1950s (2) to 1,200 reported cases in 1979 (3). However, outbreaks and sporadic cases of the pertussis syndrome still occur (1). An accurate diagnosis of pertussis is dependent upon the identification of the causative agent *Bordetella pertussis*, since other agents may cause similar clinical symptoms (5).

Ideally, immediately after nasopharyngeal (NP) specimens are taken, the swabs should be inoculated onto Bordet-Gengou plates for isolation of the organism. However, because of the decreased incidence of the disease, many laboratories do not maintain a fresh supply of Bordet-Gengou agar plates, and NP swabs are often transported to a central laboratory for processing. The viability of the pertussis organism is often lost after 2 h, even in a 1% Casamino Acids solution (7). Transport media which maintain viability for more than 2 h have not been adequately evaluated.

Direct fluorescent antibody (FA) staining of NP smears is a valuable adjunct to culturing, and, with the problems associated with transporting specimens for culture, FA is often the only diagnostic route available. The stability of the FA staining properties of *B. pertussis* in NP smears often varies with the age of the specimen. This presents a diagnostic problem, since many NP smears are transported to a central laboratory, and several days may elapse between the time the specimen is taken and the time it is processed. In those situations where NP smears must be stored before being examined by FA, they must be preserved to stabilize the antigen and thus retain the bright FA staining characteristics of the *B. pertussis* organism.

Unlike heat-fixed smears of pure cultures of *B. pertussis* which retain their FA staining properties for relatively long periods, *B. pertussis* in NP smears that are heat-fixed often lose FA staining properties within 1 to 2 days after fixation. It was suspected that the labile character of the FA staining properties of *B. pertussis* cell in NP smears might be due to degradation of antigens by enzymes associated with polymorphonuclear cells. As a result of the cellular response to the invading organism, these cells are present in the upper respiratory tract during infection with *B. pertussis* (4). For this reason, we investigated the possible effects of the addition of a proteolytic enzyme inhibitor (aprotinin; Sigma Chemical Co., St. Louis, Mo.) to enhance the stability of surface antigens of the *B. pertussis* organism in NP smears and thus retain the bright FA staining characteristic of the *B. pertussis* organism.

In the preliminary study, a MacFarland no. 2 suspension of *B. pertussis* cells (strain 10536) was prepared in an aqueous solution containing 10 U of trypsin. Smears were prepared at 5-min intervals, air dried, heat fixed, and stained with the routine test dilution of the *B. pertussis* FA conjugate prepared by the Biological Products Division of the Center for Disease Control. A second MacFarland no. 2 suspension of *B. pertussis* cells was prepared in a solution of aprotinin containing 4 trypsin inhibitor units (TIU)/ml and allowed to stand for 5 min. Smears were prepared and air dried. These smears were placed in a solution containing 10 U of trypsin and were removed at 10-min intervals. Smears were then air dried, heat fixed, and stained as described above.

In the initial study of the simulated NP smears, throat and nasal swabs were taken from several healthy volunteers and washed in phos-

phate-buffered saline (PBS), pH 7.6. These washings were combined into one pool, and 0.2-ml portions were dispensed into sterile tubes. A 0.3-ml amount of a 1:6 dilution of aprotinin was added to one set of swab washings so that each tube contained 2 TIU/ml (6). A 0.3-ml amount of a 1:3 dilution of aprotinin was added to a second set so that these swab washings contained 4 TIU/ml. A third set of swab washings was diluted with PBS to yield a final volume of 0.5 ml as described above. A suspension of *B. pertussis* cells (strain 10536) was adjusted to a MacFarland no. 2 standard in PBS. A 0.1-ml amount of a 1:4 dilution of this suspension was added to all test tubes so that approximately 10 organisms per oil immersion field were observed on a stained smear. Several smears were prepared from the PBS-diluted swab washings, air dried, and divided into three groups. One group of smears was then fixed with 1% Formalin, another group was fixed with 10% Formalin, and the third group was left unfixed as a control. Smears from all sets were prepared and then were stored at 23, 4, and −70°C. On days 1, 4, 8, 11, and 15, smears from each set were removed from storage, heat fixed, and stained as described above. All smears were stained with the routine test dilution of the *B. pertussis* FA conjugate.

In the second study, throat and nasal swabs were taken from several healthy volunteers and washed in PBS. Each washing was treated as a separate specimen, and 0.2-ml amounts from each washing were dispensed into sterile tubes. A 0.3-ml amount of a 1:3 dilution of aprotinin was added to one set of swab washings so that each tube in that set contained 4 TIU/ml. A 0.3-ml amount of a 1:1.5 dilution of aprotinin was added to a second set so that the swab washings contained 8 TIU/ml. A third set of swab washings was diluted with sterile PBS as a control to yield a final volume of 0.5 ml as described above. *B. pertussis* cells were suspended in PBS and added to all tubes as described above. Several smears were made from all sets of swab washings, and the smears were stored at 23°C. On days 1, 4, 8, 11, 15, and 22, smears from each set were removed from storage, heat fixed, and stained as described above.

All smears were read on the Leitz Dialux microscope with the Ploem illuminating system. The filter system consisted of a BG-38 red absorbing filter, KP490 FITC exciting filter, and a K510 ocular filter.

In the preliminary study, we found that the FA staining of untreated *B. pertussis* cells dropped to a 3^+ staining intensity after 5 min in trypsin and to a 1^+ intensity after 30 min. However, the *B. pertussis* cells that were treated with aprotinin and then placed in trypsin stained with a 4^+ intensity after 30 min of exposure to the enzyme.

The results of the initial study of NP smears are shown in Table 1. All smears stored at 23°C maintained a 4^+ staining intensity through day 4 except the smears fixed in 10% Formalin; these dropped to a 2^+ staining intensity on day 4 (Table 1). The smears treated with 2 and 4 TIU of aprotinin per ml and stored at 23°C maintained the 4^+ staining intensity through day 8, and the smears treated with 4 TIU of aprotinin per ml maintained a 4^+ staining intensity through day 15. The staining intensity of the control smears dropped to 2^+ by day 15. Smears treated with 2 and 4 TIU of aprotinin per ml and stored at 4°C stained at an intensity of 4^+ on day 15, although smears fixed in either 1 or 10% Formalin and stored under the same conditions had decreased to a 2^+ staining intensity by day 15. The staining intensity of all smears stored at −70°C dropped from 4^+ to 2^+ by day 15.

All smears in the secondary study gave a 4^+ staining intensity through day 4 (Table 2). Of the 12 specimens treated with 4 TIU of aprotinin per ml, 11 gave a 4^+ staining intensity through day 22. Of the 12 specimens treated with 8 TIU of aprotinin per ml, 8 maintained the 4^+ staining intensity through day 22. Only 1 of the 12 specimens in the PBS control maintained the 4^+ staining intensity through day 22 (Table 2).

TABLE 1. *Effects of various preservatives on FA detection of B. pertussis stored at three temperatures*

Preservative	Storage temp (°C)	Staining intensity[a] on day			
		1	4	8	15
1% Formalin	23	4^+	4^+	3^+	2^+
	4	4^+	4^+	3^+	2^+
	−70	4^+	3^+	3^+	2^+
10% Formalin	23	4^+	2^+	2^+	2^+
	4	4^+	3^+	3^+	2^+
	−70	4^+	3^+	2^+	2^+
Aprotinin					
2 TIU/ml	23	4^+	4^+	4^+	3^+
	4	4^+	4^+	4^+	4^+
	−70	4^+	4^+	3^+	2^+
4 TIU/ml	23	4^+	4^+	4^+	4^+
	4	4^+	4^+	4^+	4^+
	−70	4^+	4^+	3^+	2^+
None[b]	23	4^+	4^+	3^+	2^+
	4	4^+	4^+	4^+	3^+
	−70	4^+	3^+	3^+	2^+

[a] On a scale of 1^+ to 4^+ (see text).
[b] PBS control.

TABLE 2. *Effect of aprotinin on FA staining of B. pertussis in NP smears stored at room temperature*

Preservative	No. of specimens	No. of specimens staining with 4^+ intensity on day				
		1	4	11	15	22
Aprotinin						
4 TIU/ml	12	12	12	11	11	11
8 TIU/ml	12	12	12	12	10	8
None[a]	12	12	12	12	5	1

[a] PBS control.

These data show that aprotinin (a protease inhibitor) preserves the FA staining characteristics of the *B. pertussis* organism. This preservation is probably due to the inhibition or the decrease of the activity of enzymes that denature the protein antigens of the *B. pertussis* outer membrane. The preliminary study showed that trypsin, a proteolytic enzyme, did destroy those antigens of *B. pertussis* that are associated with FA staining. However, aprotinin preserved these antigens from the enzymatic activity of trypsin. In this study, the use of 4 TIU of aprotinin per ml for the treatment of simulated NP smears preserved the antigens of *B. pertussis*, so that after 22 days of storage at 23°C, the organisms fluoresced at a 4^+ intensity when stained with the FA conjugate. Use of aprotinin on several NP smears from clinical specimens has confirmed the preliminary results. The smears were treated by allowing one drop (0.05 ml) of aprotinin (4 TIU/ml) to dry on the surface of the smear. Aprotinin-treated smears stored at 23°C for 4 weeks fluoresced at a 4^+ intensity when stained with the FA conjugate, whereas the duplicate heat-fixed NP smears from the same clinical specimens had dropped to a 1 to 2^+ staining intensity. The aprotinin solution is stable for 6 to 12 months at 0 to 5°C. Therefore, from these data, we recommend that NP smears from patients with suspected pertussis be treated with 4 TIU of aprotinin per ml before the smears are transported to a reference laboratory.

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