JOURNAL OF CLINICAL MICROBIOLOGY, July 1993, p. 1715–1725
0095-1137/93/071715-11$02.00/0

Vol. 31, No. 7

Major Outbreak of Pertussis in Northern Alberta, Canada: Analysis of Discrepant Direct Fluorescent-Antibody and Culture Results by Using Polymerase Chain Reaction Methodology

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Received 17 July 1992/Accepted 30 March 1993

A major outbreak of 5,683 cases of pertussis occurred in northern Alberta, Canada, from December 1989 to January 1991. The outbreak highlighted a number of problems with current methods of pertussis diagnosis. In particular, an exceptionally high proportion of direct fluorescent-antibody (DFA)-positive, culture-negative specimens (88.4%) was identified. We took this opportunity to use polymerase chain reaction (PCR) methodology to examine whether the low culture rates were due to specimens containing dead organisms or whether the DFA results represented high numbers of false-positive results. A set of primer sequences within a *Bordetella pertussis*-specific repetitive element was used to amplify proteinase K extracts of *B. pertussis* DNA recovered from 279 submitted slides inoculated at the point of collection with nasopharyngeal material obtained from pernasal swabs. The PCR data corroborated the culture results: 84.6% of DFA-positive, culture-negative specimens were similarly PCR negative. At least three different bacterial species that were significantly cross-reactive with the commercial DFA reagent were identified in clinical specimens and in pure culture, providing one possible explanation for the false-positive DFA results. These results and other limitations of current diagnostic techniques underline the urgent need for a new DFA reagent with improved specificity and a standardized means of measuring the patient antibody response for the diagnosis of pertussis.

Whooping cough (pertussis) may be 10 to 40 times more prevalent than passive reporting systems indicate (46). The true incidence of disease could be better determined if the means of diagnosing the disease was improved in North America (15, 17, 23, 39). For one, the case definition of pertussis varies among different state and provincial health departments (8, 15, 27, 42, 46), which complicates reporting. For another, diagnosis itself is dramatically influenced by the time in the course of their disease that patients are seen. The clinical symptoms of pertussis are typically not unique early in the catarrhal stage (21, 38, 45) and the classic paroxysmal coughing that occurs later in the course of the disease may be absent, depending on the age of the patient, the patient's immune status, and whether the patient was treated with antibiotics (2, 4, 29, 35, 44).

Laboratory diagnosis of pertussis is similarly affected by the same factors that influence symptomatology. Culture and fluorescent-antibody detection of organisms in nasopharyngeal swabs or aspirates are more likely to be positive just before or early after the onset of symptoms but are increasingly less likely to be positive as time elapses after the onset of cough (38, 45, 47). Conversely, serological tests, which are dependent on the new synthesis of pertussis-specific antibody in the patient, are more likely to be positive only after symptoms begin (29, 32, 45).

In the province of Alberta, the case definition of pertussis is (i) characteristic paroxysmal cough, cough episodes ending in apnea, vomiting, or inspiratory whoop with no other known cause, or (ii) a cough lasting for at least 2 weeks and epidemiologically linked to a laboratory-confirmed case of pertussis. A confirmed case of pertussis in Alberta is defined as clinically compatible symptoms with positive culture for *Bordetella pertussis*. These definitions are similar to those used by state health departments in the United States (37, 39, 42) and were used during a major outbreak of pertussis that occurred in Alberta from December 1989 to January 1991 in which 5,683 cases were reported. In addition to culture, direct fluorescent-antibody (DFA) testing of nasopharyngeal smears was used to help confirm suspected clinical cases of pertussis. During the outbreak, 4,533 culture- and/or DFA-positive specimens for *B. pertussis* and 78 culture-positive specimens for *Bordetella parapertussis* were reported by the Provincial Laboratory of Public Health for Northern Alberta (PLNA) in Edmonton, Alberta, Canada. Of the 4,533 *B. pertussis*-positive specimens, only 524 (11.6%) were confirmed to be positive by culture, while the remaining 4,009 (88.4%) specimens were positive by the DFA method only. We assumed that a certain percentage of our specimens might be dead after transport, accounting for some of the DFA-positive, culture-negative results, but even this seemed to be an unlikely explanation for so many discordant results.

The specificity and sensitivity of the DFA reagent have been questioned (6, 16, 19, 23, 38, 40). In general, the range of false-positive results obtained by the DFA method varies depending on how DFA test positivity is corroborated. When compatible clinical illness is used as a criterion, false-positive DFA results are found for between 0 and 40% of samples (9, 11, 16, 19, 25, 37). When culture and serology were used as criteria to corroborate DFA, Halperin et al. (23) reported a 44% false-positive rate. This is still half the rate seen in our study on the basis of polymerase chain reaction (PCR) results.

To address this discrepancy, we retrospectively compared culture and DFA results obtained for 279 specimens received

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between December 1990 and May 1991 with results obtained by a PCR method for amplification of *B. pertussis* DNA (20). The results of the assay by the PCR method supported the culture results, suggesting that a high proportion of the DFA-positive, culture-negative results may be false-positive results. To explore how this could occur, we then examined species of normal flora found in the mouth and nasopharynx, and we found that they cross-reacted with the commercially available fluorescence-labeled pertussis-specific antiserum used in the study. These organisms or other, yet unidentified bacteria could possibly account for at least a portion of the false-positive DFA results.

MATERIALS AND METHODS

Specimen collection, transport, and processing. Medical doctors and local health unit nurses used calcium alginate nasopharyngeal swabs (Calgiswab; Spectrum Scientific) to obtain pernasal specimens from individuals suspected of having whooping cough. Two swabs were collected for *B. pertussis* detection. After removal from the nasopharynx, the first swab was placed directly into a glass Bijoux vial containing 5 ml of Regan-Lowe transport medium supplemented with 40 μg of cephalexin (Sigma Chemical Co., St. Louis, Mo.) per ml (41), while the second swab was first rotated onto two glass slides within a pre-etched circle approximately 1 cm in diameter and was then placed into the transport medium described by Amies (1). Swabs in transport medium were mailed directly to PLNA at ambient temperature; the majority of specimens were plated within 24 to 48 h of collection. If the specimen could not be mailed immediately to PLNA, the health units were advised to refrigerate the swabs at 4°C in the interim.

Culture of nasopharyngeal swabs. Once received by PLNA, each swab was used to inoculate two charcoal agar CM 119 plates (Oxoid Ltd., London, England) (41) supplemented with 5% defibrinated sheep blood, one containing 40 μg of cephalexin (Sigma) per ml and one without antibiotic. The four plates used to culture samples from each patient were incubated at 35°C in a humidified incubator and were examined daily for 7 days for colonies with morphologies typical of those of *B. pertussis* or *B. parapertussis*. Representative colonies from positive culture plates with typical gram-negative morphology were confirmed by DFA staining with chicken anti-*B. pertussis* or anti-*B. parapertussis* antiserum (Bacto FA *B. pertussis* and Bacto FA *B. parapertussis*; Difco Laboratories, Detroit, Mich.). From December 1990 to May 1991, the numbers of *B. pertussis* and *B. parapertussis* colonies on primary plates supplemented with cephalexin were quantitated for the purposes of comparison with DFA and PCR data. The numbers of colonies on plates with 1 to 100 colonies were counted; for plates with more than 100 colonies, colony numbers were estimated as closely as possible.

Direct immunofluorescence. Smeared slides were fixed gently with heat or methanol and were stained with fluorescein isothiocyanate (FITC)-conjugated *B. pertussis* or *B. parapertussis* antiserum (Difco) according to the manufacturer's instructions. This included twofold dilution titration of the reagent antibody to obtain a 4+ fluorescence. The working dilution of antiserum was one dilution lower than the dilution that gave 4+ fluorescence, as directed by the manufacturer's package insert. Each group of 20 slides containing samples from patients was accompanied by a positive and a negative control. These were graded for degree of fluorescence; the positive control was not allowed to fall below a fluorescence of 3+ and the negative control was not allowed to increase above a fluorescence of 1+. Slides were examined for the presence of small oval-shaped cells with a yellow-green fluorescence surrounding a dark cell center by using a 100× oil immersion objective on fluorescence microscopes. A slide was considered positive if five or more cells with the characteristic morphology and bright fluorescence were observed. Up to six technologists with various degrees of experience reading *Bordetella* DFA slides participated in reporting the results during the height of the epidemic, when more than 117 specimens were received daily for detection of bordetellae. For the portion of the study following the peak of the epidemic, in which DFA results were compared with PCR data (December 1990 to May 1991), a maximum of three experienced technologists read the DFA slides. During this period, the numbers of fluorescent bacteria per slide were also quantitated for the purposes of comparison; if there were fewer than 100 bacteria per slide, they were counted directly; when there were 100 to 1,000 bacteria per slide, they were estimated to the nearest 100 bacteria; when there were more than 1,000 bacteria per slide, their numbers were estimated to the nearest 1,000.

Amplification of *B. pertussis* DNA by PCR and product detection. *B. pertussis* DNA was recovered from the bacteria smeared onto glass slides following nasopharyngeal swab collection. The material on the slide was suspended in 50 μl of 12 mM Tris hydrochloride buffer (pH 7.6) by using the edge of a plugged micropipet tip (USA/Scientific Products, Ocala, Fla.) affixed to a micropipet. The suspended material was aspirated into the tip and was then placed into a 500-μl plastic conical centrifuge tube. This process was repeated with 15 μl of Tris buffer in order to remove any remaining material. Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to a final concentration of 0.2 mg/ml, and the samples were incubated at 65°C for 90 min to digest the nasopharyngeal material and were then incubated at 100°C for 20 min to inactivate the added enzyme.

The two oligonucleotide primers used for amplification of *B. pertussis* DNA by PCR were based on sequences kindly provided by Martha McLafferty, Department of Pathology, University of Virginia School of Medicine, Charlottesville. Their sequences were as follows: 5′-GACTTCGTCTTC GTGGCCAT-3′ and 5′-TCGTCCAGGTTGAGTCTGGA-3′. These sequences are located within a 1,053-bp insertion sequence element, tentatively designated IS*481* (30) which is present in multiple (50 to 100) copies within the *B. pertussis* chromosome (30, 31). Twenty-five microliters of the digested material was used for amplification of *B. pertussis* DNA by PCR in a reaction volume of 100 μl containing a final concentration of 20 pM each primer, 50 μM deoxynucleotide triphosphates (Pharmacia Biotechnology, Baie D'Urfé, Quebec, Canada), 1 U of *Taq* polymerase (Bio/Can Scientific, Mississauga, Ontario, Canada), and 1× reaction buffer (Bio/Can Scientific). Two positive controls consisting of proteinase K-extracted *B. pertussis* DNA corresponding to approximately 5,000 and 50 CFU were included with each PCR run; in addition, one negative control consisting of proteinase K-extracted 12 mM Tris-HCl buffer and a second negative control with 25 μl sterile distilled water substituted for template DNA in the PCR mixture were included with each PCR run. Amplification of *B. pertussis* DNA was performed in a DNA thermal cycler (Perkin-Elmer Cetus Corporation, Norwalk, Conn.) during 30 thermocycles each consisting of a 2-min denaturation step at 94°C, a 2-min annealing step at

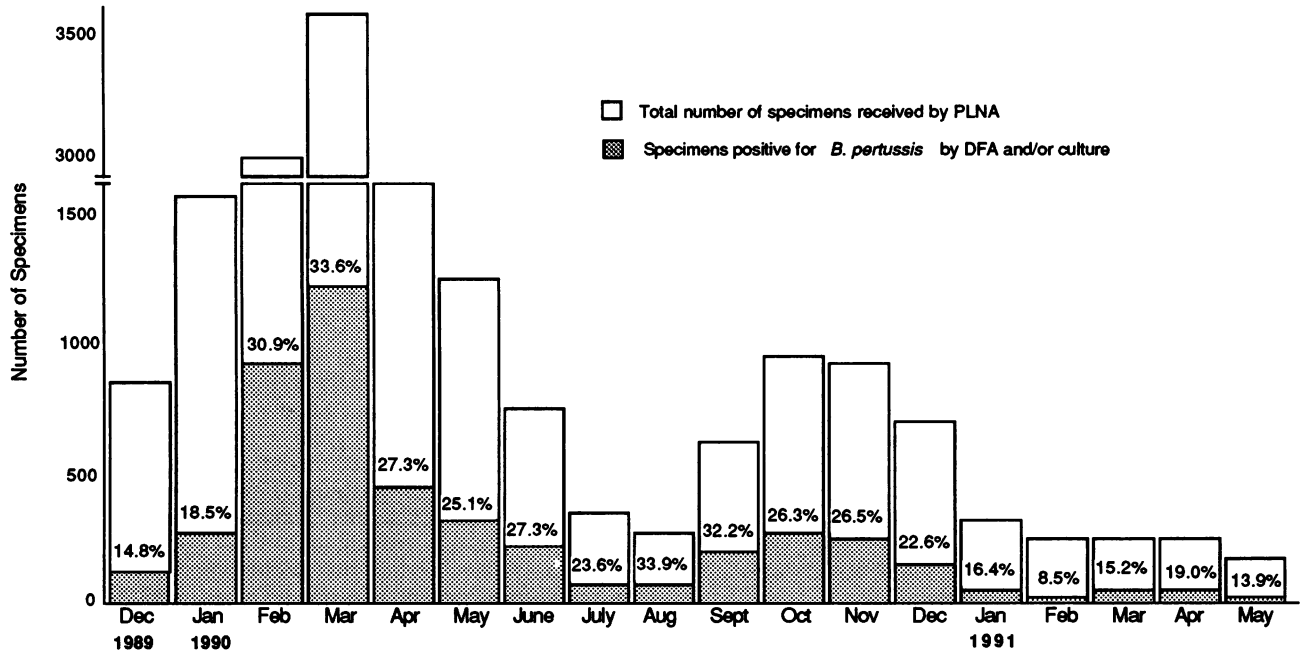

FIG. 1. Specimens received by PLNA for detection of *Bordetella* spp. by culture and DFA testing between the months of December 1989 and May 1991. Values within vertical bars indicate the numbers of specimens positive by the DFA method and/or culture methods expressed as a percentage of the total number of specimens received for detection of *B. pertussis*.

60°C, and a 2-min elongation step at 72°C. The amplified product was visualized by UV illumination following electrophoresis for 90 min at 160 V through a 1.0% agarose gel containing 0.05 μg of ethidium bromide per ml. Photographs taken with UV illumination provided a permanent record of the results.

The sensitivities of the primers were determined by first suspending the virulent phase of *B. pertussis* Tohama 1, which was grown for 24 h on Regan-Lowe medium (41) at 35°C in a humidified atmosphere, in 12 mM Tris hydrochloride (pH 7.6) to an optical density at 540 nm of 0.12. The suspension was serially diluted 10-fold to a final dilution of 10^{-7}. Duplicate 50-μl aliquots from each dilution between 10^{-2} and 10^{-7} were removed; one of the two aliquots was placed directly into a 500-μl plastic centrifuge tube (the tube dilution series), while the other was dried onto a glass slide within a circle of approximately 1 cm in diameter. The dried aliquots corresponding to the 10^{-2} to 10^{-7} dilution series were removed from the slides by emulsification in 65 μl of Tris buffer as outlined above and were then placed in a 500-μl plastic tube. The latter slide dilution series was performed in order to determine the efficiency of the suspension procedure used in the present study to remove specimen material from slides. The *B. pertussis* DNA within the tubes was crudely extracted by the proteinase K procedure described above and was then subjected to the PCR amplification and detection protocol outlined above. Viable counts for determination of numbers of CFU within each dilution were performed by plating aliquots onto Regan-Lowe agar for later enumeration.

A potential limitation of the PCR technique could be its susceptibility to inhibitors in the secretions collected on the nasopharyngeal swab specimens. To test this, auger suction material was collected from patients suspected of having respiratory diseases other than pertussis. The material was plated to ensure that it was free of endogenous *B. pertussis*. A 3-day-old Bordet-Gengou agar-grown culture of a fresh clinical isolate of *B. pertussis* was suspended and serially diluted 10-fold in phosphate-buffered saline. On a clean microscope slide, 50 μl of bacterial suspension was mixed with 50 μl of the auger suction material and was allowed to dry. The slides were processed as described above and tested by PCR.

Cross-reactivity of DFA antiserum. Two approaches were used to determine whether the commercial preparation of FITC-conjugated *B. pertussis*-specific antiserum used by PLNA was cross-reactive with normal flora or potential pathogens found in the human oral cavity and/or nasopharynx. (i) Stock strains of various aerobic and anaerobic bacteria were stained directly with the commercial preparation. (ii) Auger suctions, nasopharyngeal aspirates, and nasopharyngeal swabs submitted to the University of Alberta Hospitals Microbiology Laboratory for detection of pathogens other than *B. pertussis* and *B. parapertussis* were cultured anaerobically in stoppered tubes supplemented with commercial peptone broth (Becton-Dickinson and Co., Lincoln Park, N.J.) for 48 h at 37°C and were then smeared and stained with DFA for detection of fluorescent organisms as outlined above.

RESULTS

Laboratory data for December 1989 to May 1991. Figure 1 charts the total number of specimens received monthly by PLNA for detection of *Bordetella* spp. and shows the percentage of specimens positive by at least one of the detection methods (DFA method and culture) between the months of December 1989 and May 1991. On the basis of the total number of specimens received and the total number of specimens positive by the DFA method and/or culture, the pertussis outbreak in northern Alberta appears to have occurred in two waves, the peaks of which were March 1990

and October 1990. At the peak of the epidemic in March 1990, 3,612 specimens, corresponding to approximately 117 specimens per day, were received by PLNA. In general, the proportion of specimens positive for *B. pertussis* paralleled the total number of specimens received. Thus, the two main bar graphs illustrated in Fig. 1 possess the same basic shape.

Figure 2A provides a closer look at the subdivisions of "positive" specimens of *B. pertussis* represented by the shaded bars in Fig. 1. The following four categories are shown in Fig. 2A: (i) DFA positive, culture negative, (ii) DFA positive, culture positive, (iii) DFA negative, culture positive, and (iv) the sum of categories i to iii or the total number of positive specimens. For reference, the shaded bars in Fig. 2A are the identical data shown by the shaded bars in Fig. 1.

Two features of Fig. 2A stand out. The first is the exceptionally high number of specimens which fell into the DFA-positive, culture-negative category, and the second is how this category follows the same two-wave shape outlined by the total positive specimen category. Overall, 88.4% of all positive specimens received between December 1989 and May 1991 were from patients suspected of having whooping cough, as confirmed by DFA criteria alone.

In contrast, specimens that were culture positive (DFA positive or negative) showed a different curve profile. In Fig. 2A, the profiles of culture-positive samples (either culture-positive and DFA-positive samples or culture-positive and DFA-negative samples) are dwarfed by the DFA-positive, culture-negative and total-positive-specimen categories. The percentages in Fig. 2A show that the percentage of culture-positive (DFA-positive or -negative) specimens dropped markedly, from 41% in December 1989 to a low of 7% during the highest peak of specimen acquisition in March 1990. Likewise, of the second wave of specimens, the percentage that was culture positive (DFA positive or negative) dropped from a high of 42% in August 1990 to 11% in October 1990. Thus, the culture positivity rate did not increase proportionally to the DFA positivity rate.

This is shown in more detail by the monthly culture-positive rate in Fig. 2B. The proportion of all culture-positive specimens of *B. pertussis* and *B. parapertussis* are shown. Notable is how the shape of the *B. pertussis* culture-positive curve in Fig. 2B differs from the shape of the total-positive-number curve and DFA-positive, culture-negative curve in Fig. 2A. The culture-positive curve in Fig. 2B less clearly shows two peaks of activity. In addition, the slope leading to the March 1990 peak of positivity is less steep and the second culture-positive peak in Fig. 2B occurred in August 1990, preceding the second DFA-positive, culture-negative peak in Fig. 2A by 2 to 3 months. The relatively poor agreement between DFA-positive, culture-negative specimens and culture-positive specimens is supported by their correlation coefficient of 0.591. Moreover, only 242 (43.9%) of the 551 culture-positive specimens were positive by DFA, indicating that the DFA test has poor sensitivity.

On the basis of these data, we felt that the most likely explanation for the discrepancy between DFA positivity and culture positivity was a problem with the reading and/or interpretation of DFA slides.

Use of PCR to amplify *B. pertussis* DNA. *B. pertussis* DNA was amplified and detected by PCR (20) to determine the nature of the discrepancy between the DFA results and those obtained by culture. The region of DNA amplified by the set of primers used in the present study lies within an insertion element (30, 31), designated IS*481* (30), found in multiple copies within the *B. pertussis* chromosome (20, 30, 31). The amplified product produced by these primers is 424 bp in length.

Initial attempts to perform PCR on extracts from nasopharyngeal swabs after they had been used to plate viable counts met with limited success. We therefore used extracts from nasopharyngeal swab specimens which had been smeared onto a glass microscope slide to detect *B. pertussis* by PCR.

The sensitivity of the primers, determined as described above with both the tube and the slide dilution series, indicated a limit of detection of approximately 1 CFU for both series. The bands corresponding to 14 CFU for both the tube and the slide dilution series are visible in the ethidium bromide-stained agarose gel pictured in Fig. 3, lanes D; the bands corresponding to 1.4 CFU (Fig. 3, lanes E) are difficult to discern in the reprinted photograph, although they are clearly visible on the original negative from which the specimen results were read. In addition, the amplified product had a mobility of between 517 and 396 bp when compared with those of known standards (Fig. 3), which is consistent with its expected size of 424 bp.

The possibility existed that secretions collected with the nasopharyngeal swab specimens might inhibit the PCR, affecting its sensitivity or even causing false-negative results. On the basis of viable counts done in parallel, the sensitivity of the PCR in the presence of mucus obtained by auger suction was between 3 and 30 CFU. Thus, respiratory tract secretions did not cause a notable inhibition of PCR.

The specificities of the primers for *B. pertussis* DNA were ascertained by using the proteinase K procedure outlined above to crudely extract DNA from related organisms (*B. parapertussis* and *Bordetella bronchiseptica*) and unrelated bacteria resident in the oral cavities and nasopharynxes of both healthy and sick individuals. In total, 32 species representing 23 genera, including *Legionella*, *Neisseria*, *Proteus*, *Haemophilus*, *Klebsiella*, *Escherichia*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Pseudomonas*, *Pasteurella*, and *Micrococcus*, were subject to amplification by PCR under the conditions outlined above. No cross-reactivity was observed with any of the species tested, including *B. parapertussis* and *B. bronchiseptica* (data not shown).

Having confirmed the sensitivity and specificity of the primers, we subjected a total of 279 specimens received by PLNA between 1 December 1990 and 31 May 1991 to PCR amplification and detection by agarose gel electrophoresis. One of each set of two slides submitted to PLNA for detection of *B. pertussis* spp. was used for PCR amplification of *B. pertussis* DNA, while the other was used for detection by the DFA method. The slide with the greater amount of material was routinely selected for DFA testing, introducing a potential negative bias into the study because the slides used for PCR analysis generally carried less material. In order to determine whether the amount of material per slide correlated with the numbers of *B. pertussis* organisms, we compared the numbers of bacteria stained by immunofluorescence on 10 pairs of submitted slides with positive DFA results. Of the 10 pairs of slides examined, 4 pairs had approximately equal numbers of fluorescent bacteria, 2 pairs had no fluorescent bacteria on the slides used for PCR analysis and 100 to 200 bacteria on the slide used for DFA testing, 2 pairs of slides had 4- and 50-fold more bacteria on the slides used for PCR testing than the slides used for DFA testing, and 2 pairs of slides had 50 and 75% fewer fluorescent bacteria on the slides used for PCR testing than on those used for DFA testing. These data indicate that the numbers of *B. pertussis* on pairs of submitted slides were significantly

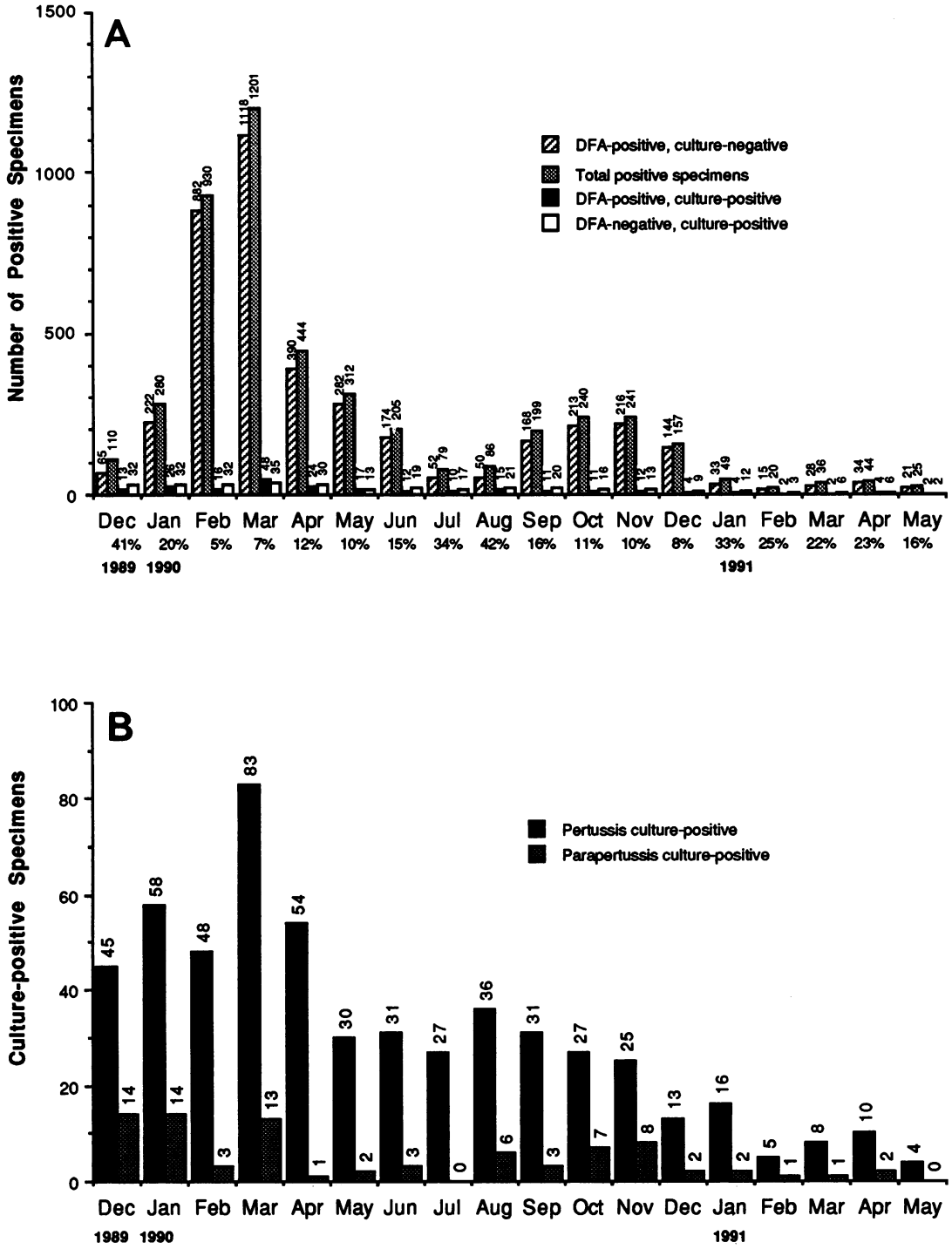

FIG. 2. (A) Total number of specimens positive for *B. pertussis* by the DFA method and/or culture, as expressed by month from December 1989 to May 1991. These values are further divided into three groups on the basis of the method of detection: DFA positive, culture negative, DFA positive, culture positive, and DFA negative, culture positive. The number of specimens in each category is given above each bar. Below each month is shown the percentage of all positive specimens that were culture positive. (B) Specimens received by PLNA each month from December 1989 to May 1991 that were culture positive (DFA positive or negative) for *B. pertussis* and *B. parapertussis*. The numbers of specimens in each category are given above each bar.

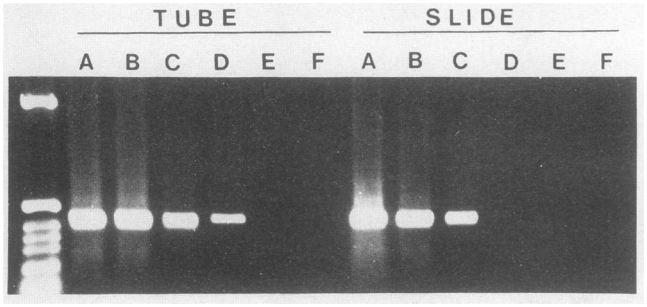

FIG. 3. Visualization of PCR-amplified *B. pertussis* DNA on an agarose gel by ethidium bromide staining following electrophoresis. Duplicate 50-μl aliquots from a suspension of serially diluted *B. pertussis* were amplified; one of the two aliquots was placed directly into a 500-μl plastic centrifuge tube (the tube dilution series), while the other was dried onto a glass slide, emulsified in buffer, and then placed in a 500-μl plastic tube (the slide dilution series). The far left, unmarked lane contains DNA size markers (pBR322 DNA digested with *Hin*fI restriction enzyme): 1,632, 517 and 506 (unresolved), 396, 344, 298, 221 and 220 (unresolved), and 154 bp, respectively, from top to bottom. Lanes labeled A through F contain amplified DNA corresponding to 14,000, 1,400, 140, 14, 1.4, and 0.14 CFU of *B. pertussis*, respectively (as determined by viable counts).

variable. In addition, the amount of material on the slide did not correlate with the numbers of *B. pertussis*.

The results presented in Table 1 compare the results obtained by the PCR procedure with those obtained by the culture and DFA methods. Twenty-nine (72.5%) of the 40 culture-positive specimens were also PCR positive. Three (23.1%) of the 13 specimens positive by both DFA and culture methods were PCR negative. As indicated in Table 2, low numbers of bacteria were generally found on both culture plates for these specimens (specimens 1 to 3). Eight (29.6%) of the 27 DFA-negative, culture-positive specimens were PCR negative. Table 2 also indicates the numbers of colonies from these specimens (specimens 4 to 11) on culture plates. Interestingly, 16 (11.8%) of the 135 DFA- and culture-negative specimens were positive by PCR analysis. Not indicated in Table 1 are the results for three specimens from which *B. parapertussis* was cultured, which were likewise PCR negative.

One of the most important values listed in Table 1 is the number of DFA-positive, culture-negative specimens which were also negative by PCR analysis; of the 104 specimens in this category, 88 (84.6%) were negative according to the PCR data. This finding strengthened our suspicion that a significant proportion of the previously reported DFA-positive, culture-negative results were, in fact, false-positive results.

TABLE 1. Culture, DFA, and PCR results for 279 specimens received by PLNA for detection of *Bordetella* spp. between December 1990 and May 1991

DFA result	Culture result	PCR result	No. of specimens
+	+	+	10
+	+	−	3
+	−	+	16
+	−	−	88
−	+	+	19
−	+	−	8
−	−	+	16
−	−	−	119

TABLE 2. Quantitative comparison of the two swabs used for culture alone or DFA, PCR, and culture of *B. pertussis* organisms in specimens that were PCR negative

Specimen no.	No. of colonies or fluorescent bacteria		
	Swab 1 (CFU)[a]	Swab 2[b]	
		CFU	DFA
1	0	2	90
2	0	27	100
3	≥20,000[c]	7	50
4	0	2	0
5	1	NS[d]	0
6	10	2	0
7	0	4	0
8	6	0	0
9	0	8	0
10	2	≥10,000	0
11	4	44	0

[a] Swab 1 was placed directly into Regan-Lowe transport medium (41) following collection and was streaked onto Regan-Lowe agar (41) for quantitation upon receipt.

[b] Swab 2 was first used to make two slides, one for DFA testing and one for PCR testing, and was then placed in the transport medium described by Amies (1) for later culture.

[c] Values were estimated for comparison of total counts.

[d] NS, not submitted.

Cross-reactivity of DFA antiserum. Since PCR analysis indicated that a significant number of DFA-positive, culture-negative results were false-positive results, we examined whether various species of bacteria (normal flora and potential pathogens) found in the mouth and nasopharynx would cross-react with the commercial preparation of FITC-conjugated anti-*B. pertussis* antiserum used by PLNA. Initially, stock strains of various aerobic and anaerobic bacteria were stained directly with the commercial preparation and were examined for fluorescence under UV illumination. Of the aerobic or facultative organisms tested (including various species within the genera *Staphylococcus*, *Streptococcus*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Proteus*, *Klebsiella*, *Pasteurella*, and *Pseudomonas*, among others), all were uniformly negative for fluorescence except for unencapsulated *Haemophilus influenzae*, *Proteus mirabilis*, *Staphylococcus epidermidis*, and an unidentified "diphtheroid" species, which were weakly fluorescent. When pure cultures of 12 species of anaerobic bacteria native to the oral cavity and/or nasopharynx (43) were tested, however, we found that two were significantly cross-reactive. As shown in Fig. 4C, a sporulating culture of *Clostridium sporogenes* was extremely cross-reactive. In experienced hands, it was virtually indistinguishable from *B. pertussis* when viewed directly by UV illumination. A *Lactobacillus* sp. was also significantly cross-reactive, as shown in Fig. 4B, although its distinct morphology and comparatively less brilliant fluorescence would not likely be confused with *B. pertussis* by an experienced slide reader.

Although *C. sporogenes* and *Lactobacillus* spp. are native to the human mouth (43), we wanted to determine whether they or other cross-reactive species would be sufficiently prevalent to account for at least a portion of the false-positive DFA results. To this end, we stained and read 77 48-h anaerobic broth cultures of auger suctions, nasopharyngeal aspirates, and nasopharyngeal swabs that had been submitted to the University of Alberta Hospitals for the

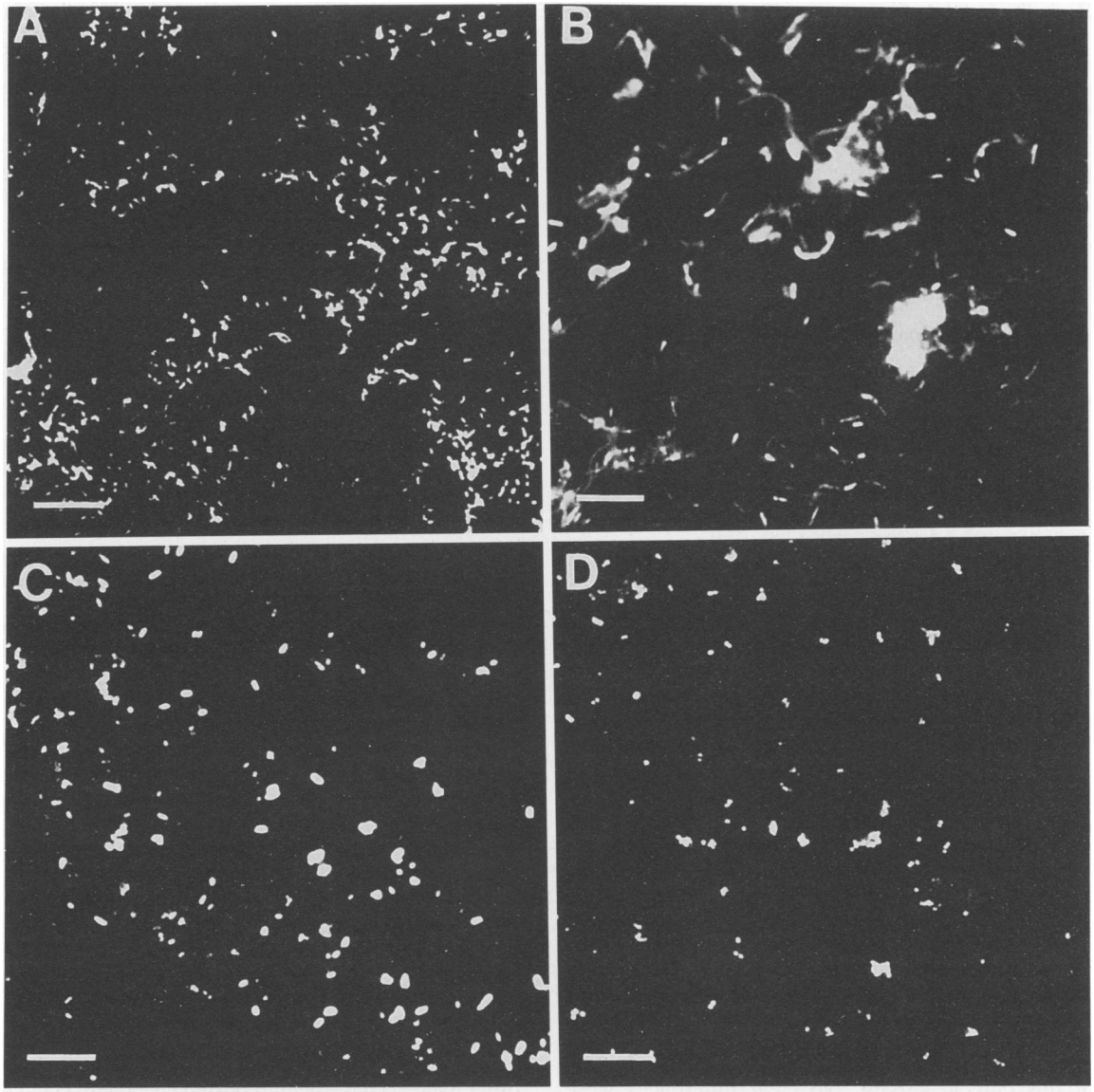

FIG. 4. DFA staining with a commercial FITC-conjugated antiserum, of pure bacterial cultures (A to C) and an anaerobic culture (D) of an auger suction. (A) *B. pertussis*; (B) *Lactobacillus* sp.; (C) sporulating culture of *C. sporogenes*; (D) anaerobic broth culture of auger suction material. Bars, 10 μm.

detection of respiratory pathogens other than *B. pertussis*. The use of cultured clinical specimens also allowed us to survey many species of normal flora for which pure cultures were not available.

Anaerobic or facultatively anaerobic bacterial species which displayed significant cross-reactivity with the commercial antiserum were visualized in 4 (5%) of the 77 specimens examined. The obligate oxygen requirement of bordetellae rules out the possibility that the fluorescent bacteria were *B. pertussis* because the specimens were cultured anaerobically. Since the cross-reactive bacteria in these specimens shared a similar size and shape, it is likely that they belong to the same genus and/or species. Figure 4D illustrates a representative example of this cross-reactivity, in which highly fluorescent bacteria are seen among normal respiratory flora from a sample obtained by auger suction cultured under anaerobic conditions.

Clinical data. The high rate of DFA-positive, culture-negative, PCR-negative specimens obtained during the pertussis outbreak in Alberta from December 1990 to May 1991 suggested that many of the reported cases were not actually caused by *B. pertussis*. Clinical data were therefore collected and compared to determine whether there was a clinically defined difference between the two main groups of patients: those whose nasopharyngeal swabs were DFA positive, culture negative, and PCR negative and those patients whose nasopharyngeal swabs were positive by one of the following six other combinations of criteria: (i) DFA positive, culture positive, and PCR positive; (ii) DFA positive, culture positive, and PCR negative; (iii) DFA positive, culture negative, and PCR positive; (iv) DFA negative, culture positive, and PCR positive; (v) DFA negative, culture positive, and PCR negative; and (vi) DFA negative,

TABLE 3. Clinical data for patients positive for *B. pertussis* by DFA, culture, and/or PCR between December 1990 and May 1991

Clinical parameter	DFA-positive, culture-negative, PCR-negative specimens (n = 68)	All other specimens (n = 51)[a]
Percent return of symptomatology reports	77	71
Age (yr)		
Median	5	7.5
Range	0.2–63	0.1–40
Sex (no. [%])		
Male	37 (54)	22 (43)
Female	31 (46)	29 (57)
Age-appropriate immunization (no. [%])[b]		
Yes	42 (62)	31 (61)
No	15 (22)	11 (21.5)
Unknown	11 (16)	9 (17.5)
Fits case definition (no. [%])[c]		
Yes	39 (57)	33 (65)
No	25 (37)	13 (25)
Unknown	4 (6)	5 (10)
Duration (wk) of cough prior to sampling (no. [%])		
<1	23 (34)	12 (23.5)
1–2	19 (28)	16 (31)
2–4	16 (23)	11 (21.5)
>4	8 (12)	7 (14)
Unknown	2 (3)	5 (10)
Use of antibiotics prior to sampling (no. [%])	2 (3)	4 (8)
Hospitalization (no. [%])	9 (13)	15 (29)
Contacts (no. [%])		
Suspected or confirmed	23 (34)	21 (41)
Unknown	45 (66)	30 (59)

[a] Includes DFA-positive, culture-positive, PCR-positive; DFA-positive, culture-positive, PCR-negative; DFA-positive, culture-negative, PCR-positive; DFA-negative, culture-positive, PCR-positive; DFA-negative, culture-positive, PCR-negative; and DFA-negative, culture-negative, PCR-positive specimens.

[b] Determined by following the recommendations of the National Advisory Committee on Immunization (36), which advises that the vaccine be given at ages 2, 4, 6, and 18 months, in addition to a fifth dose at between 4 and 6 years of age. In the present study, the recommended ages for vaccination were extended to allow for a reasonable delay in vaccination, as follows: 4, 6, 8, and 22 months for the first four vaccine doses and 7 years for the fifth vaccine dose.

[c] On the basis of previous information (13), which states that characteristic paroxysmal cough, cough episodes ending in apnea or vomiting, or an inspiratory "whoop" without other known cause or cough lasting for at least 2 weeks and epidemiologically linked to a laboratory-confirmed case of pertussis.

culture negative, and PCR positive. Table 3 summarizes the results of this clinical survey. No clear differences were discernible between the two major groups with regard to age, sex, age-appropriate immunization, agreement with case definition, duration of cough prior to sampling, use of antibiotics prior to sampling, hospitalization, or contact with an individual with pertussis.

DISCUSSION

Our results emphasize some of the problems that still plague pertussis diagnosis today. What criteria can be reliably used to confirm a case of pertussis and what is the basis for that reliability?

Culture is indisputable proof. If *B. pertussis* is isolated from someone who is symptomatic or not, it is significant. The problem with culture is its lack of sensitivity. A positive culture result is most likely to be obtained in the early stages of infection. In a study by Steketee et al. (45), culture only identified 41 (27%) of 152 cases of pertussis in an outbreak in an institution. The remaining 111 (73%) cases were determined by serology (45).

Fluorescent-antibody assays suffer from the same temporal constraints as culture, but they are further suspect because of their lack of specificity. Cross-reactions with other bacteria have been reported elsewhere (11, 17), and we have extended those observations here. The nature and the extent of cross-reactions have never been fully studied, nor are such studies required for a pertussis-diagnostic fluorescent antibody to be in compliance with the Code of Federal Regulations in the United States (12).

Comparison of our results with those of others is complicated by the variety of reagents and conditions used in various studies. Antisera made in rabbits (7, 11, 16, 47) or chickens (25, 29, 45) have been used, in many instances without fractionation of immunoglobulins by coupling a fluorescein dye to whole serum. Rarely have the polyclonal sera been adsorbed with other organisms to increase the specificity of an assay (11). In one report, indirect immunofluorescence was used (7), and numerous studies of pertussis incidence have altogether forgone the DFA method as a means of diagnosis (2, 4, 5, 22, 33, 39, 44).

Despite this variety of methodologies, our data bear similarities to those obtained from other large outbreaks. Definitions play a critical role in ascertaining these similarities. For example, our results suggest 82% false-positive results by the DFA method on the basis of PCR results. Yet,

our clinical data (Table 3) show that 57% of patients with DFA-positive, culture-negative, PCR-negative specimens had clinical symptoms compatible with the case definition. If we were to use clinical data as the confirmation of DFA, as have others (9, 11, 16, 19, 25, 37), then our "false-positive" rate for DFA drops to 43% of 87% or 35.3%, which is within the range previously reported by others (23). Even at a false-positive rate of 35.3%, the DFA result does not instill much confidence in an accurate diagnosis. Justifying the DFA result by clinical case criteria ignores the PCR results stated above. If these individuals do not really have pertussis on the basis of negative culture and PCR test results, their reported symptoms suggest some form of respiratory disease. The specter of "pertussis syndrome" (3) raises its head. However, in the face of a culture-documented outbreak, unless serological or other confirmatory evidence could be obtained, an alternate etiology remains speculative.

As a test of the reliability of PCR, culture can be used as a "gold standard" of a positive pertussis specimen with which DFA- and PCR-positive rates can be compared. Forty specimens in the survey were culture positive. Of these, 29 (72.5%) were PCR positive, whereas only 13 (32.5%) were DFA positive. Thus, when it was known, by culture, that a specimen was positive for pertussis, PCR was positive more than twice as often as the DFA test was. While it can be argued that the 72.5% PCR positivity rate is still a poor correlation to culture, it has already been stated that, in general, low colony counts were found in our samples that were culture positive but PCR negative (Table 2). Inconsistencies in bacterial counts were also observed between the matched slides made for DFA testing and PCR from the same swab, but these inconsistencies do not appear to account for the differences between PCR and DFA test results. No bias was introduced in the selection of slides for PCR or DFA testing that would explain the markedly different correlation between DFA testing and PCR with culture. It is possible that the correlation between DFA testing and culture could have been improved by treating the slides with aprotinin (23, 24) to preserve the antigens recognized by the DFA method. This was not done in our study and is not specified by the manufacturer in their product insert. It is a paradox that while so many samples were DFA positive, so few that were culture proven were DFA positive.

The poor correlation that we saw between culture and the DFA method is not unprecedented (10, 18, 19, 23). An explanation is that culture is more sensitive; one organism potentially yields one colony, whereas a minimum of five clearly fluorescening organisms is needed to call a slide positive by the DFA method. Depending on the collection method used, the volume of sample for culture can be larger, enhancing the sensitivity of the culture method. Rarely, however, have the actual numbers of positive colonies been compared with the number of fluorescent organisms to test the impact of this possibility.

PCR was used in the present study to corroborate the results obtained by the DFA method. It suggested that DFA-positive, culture-negative specimens were, in fact, false-positive specimens. PCR itself is not infallible, and we did not include a positive internal control in the PCR runs. Such a control could monitor for false-negative results caused by inhibition of PCR by specimen material. However, we found no inhibition of PCR when auger suction material was admixed with cultured organisms and tested by our slide specimen procedure. In addition, PCR agreed with culture 72.5% of the time. As stated above, this percentage would probably have improved had the specimens been identical (i.e., obtained from one, not two swabs). If we ignore this known variability between slide and culture specimens and assume that all 27.5% of PCR-negative, culture-positive results are false-negative results, we can use this percentage to make a rough estimate of the number of false-negative specimens by PCR among the DFA-positive, culture-negative specimens. We stated above that PCR identified 82% of DFA-positive, culture-negative specimens as PCR negative. If we assume that 27.5% of these PCR-negative specimens are false-negatives specimens, this still leaves 73.5% of 82%, or 60.9%, of DFA-positive, culture-negative specimens as potentially false-positive specimens.

Viewed in the proper context, our results are not atypical. It is important to emphasize that the outbreak in northern Alberta consisted of over 5,700 cases, one of the largest ever recorded in North America since the introduction of pertussis vaccine in 1943. In this respect, the size of the workload for PLNA has few precedents. The outbreak of 1,030 cases of pertussis in Kansas in 1986 is the closest in number of cases for which laboratory data are published (10). Only 4% of the cases in Kansas were confirmed by culture, whereas 87% of the cases were positive by the DFA method without culture confirmation. The results of Broome et al. (6) would predict that in larger outbreaks, the correlation of the DFA method with culture would falter. The use of multiple readers to handle the high numbers of DFA slides in a large outbreak could lead to multiple interpretations of DFA test results (6). In the outbreak in Alberta, up to six slide readers were employed, and at the height of the outbreak 117 specimens were received each day. This emphasizes an important consideration about the DFA method: the larger the number of specimens, the more the diagnostic laboratory is stressed and the less reliable the DFA method becomes, despite fastidious attention to detail. In short, when it is needed the most, the DFA method fails. This claim is substantiated within our own outbreak. Culture-positive specimens decreased during the high point of specimen activity in March and October of 1990 (Fig. 2A). Similarly, the peak of the culture-positive curve (Fig. 2B) preceded the peak of the DFA-positive, culture-negative curve (Fig. 2A). The culture-positive data show that there was a significant outbreak of pertussis. It appears, however, that the numbers were inflated by the DFA test results.

We have explored a number of possibilities to explain the dynamics between the DFA method, culture, and PCR. (i) Repeated freeze-thaw cycles during the winter months in Alberta reduced the viabilities of the organisms, leading to large numbers of DFA-positive, culture-negative samples. Over 80% of our specimens were received within 24 h of being sent, and the great majority were shipped in heated vehicles. During the coldest days of the winter, a specimen occasionally freezes. This is apparent because the agar medium collapses and is markedly different from normal agar medium. This happens so infrequently that it cannot be a factor in the recovery rate. (ii) The percentage of culture-positive specimens decreased in the winter months. This was true only for February and March 1990. In December 1989, January 1990, and January and February 1991, we had some of our highest recovery rates by culture. The low culture-positive rate in February and March 1990, when word of the outbreak first began to spread, was probably due to indiscriminate swabbing of every contact and every child of concerned parents. This was especially true for outlying areas. While this may explain the low positive culture rates, it does not explain why the DFA test result would be positive. (iii) Specimens from the Edmonton, Alberta, area

(local) may have a higher culture-positive rate than specimens shipped in from outlying areas (PLNA serves an area of 505,978 km^2, with a population of approximately 1,412,300). Our data indicate that the culture rate for local specimens was not higher. From December 1990 to April 1991, a total of 1,748 specimens were received, of which 415 were from the Edmonton area. For specimens from Edmonton, there were 13 (3%) culture-positive specimens and 61 (14%) DFA-positive specimens; these results are comparable to those for all other localities, for which there were 31 (2.3%) culture-positive specimens and 193 (14%) DFA-positive specimens.

The results of our study dramatically illustrate the problems of diagnosing pertussis (6, 17, 23, 26, 38). The problems arise at two levels. One reflects the biological variability of the disease itself, which influences the clinical diagnosis and the time a given sample is most likely to be positive. The second level is in the reliabilities of the diagnostic methods themselves. Culture, for example, is of undisputed specificity for diagnosis, but it can be enhanced by careful attention to medium preparation and the method of sample collection (6, 16, 23). PCR has the potential of being a means of checking culture-negative specimens, especially if future studies include a positive PCR control to effectively monitor false-negative results because of the presence of inhibitory substances in clinical samples. The DFA method can also have a place in the diagnosis of pertussis if its specificity can be demonstrated.

Our data suggest that the polyclonal antibody preparations used in our study are capable of cross-reacting with several non-*B. pertussis* organisms. These included aerobic and anaerobic bacterial pathogens and normal flora which can reside in the human oral cavity and/or nasopharynx. In addition, anaerobic cultures of nasopharyngeal aspirates and auger suctions were tested. We identified at least three different bacterial species which were significantly cross-reactive with the commercial DFA test; a more exhaustive search would undoubtedly reveal others. Further study is needed to determine whether cross-reactive organisms are responsible for the reported lack of specificity of the DFA test for *B. pertussis* (6, 11, 14, 23, 28) and whether they may have influenced the interpretation of DFA slides at PLNA.

The detection of pertussis-specific antibody in patients has been shown to be a powerful adjunct to the diagnosis of whooping cough (7, 22, 23, 29, 33, 34, 39, 45). These newer enzyme-linked immunosorbent assays to defined pertussis antigens provide a technique that does not rely on the presence of organisms or their components, as does the DFA method, culture, or PCR. Since organisms occur less frequently as the disease progresses, while antibody levels increase during the same time frame, the two techniques are complementary. The potential exists for early detection of pertussis in patients without severe symptoms (e.g., case contacts) by culture, PCR, or a more specific DFA method, whereas patients with more clinically advanced disease could be identified by detection of appropriate antibody production in response to pertussis infection. Improved diagnosis and epidemiology of pertussis depend on the development and standardization of combinations of such techniques.

ACKNOWLEDGMENTS

We thank Martha McLafferty of the Department of Pathology, University of Virginia School of Medicine, Charlottesville, for the sequence of PCR primers used in the present study; Pamela Kibsey, Terry Chelich, Loreen Hodge, and Betty Lui of the University of Alberta Hospitals Microbiology Laboratory for provision of anaerobic strains and anaerobic specimens; Marguerite Lovgren of the Provincial Laboratory of Public Health for Northern Alberta for provision of monthly statistics; San Vinh and Richard Sherburne of the Department of Medical Microbiology and Infectious Diseases for photographic assistance; John Waters and Anita Hanrahan of the Province of Alberta Social Services and Community Health (Communicable Disease Control and Epidemiology) and numerous local physicians for provision of clinical symptomatology reports; Sharee Kuny for technical assistance; and the Canadian Bacterial Diseases Network for financial support.

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