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DNA Probe Technology for Rapid Detection of *Haemophilus influenzae* in Clinical Specimens

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In a previous study, we reported that a 5-kilobase *Haemophilus influenzae* DNA fragment involved in penicillin-binding protein expression could be used as a probe for specific detection of *H. influenzae* strains (F. Malouin and L. E. Bryan, Mol. Cell. Probes 1:221–232, 1987). Here, we report the ability of this probe to detect *H. influenzae* in clinical specimens. In a bacterial dot experiment, there was strong hybridization of the ^{32}P-labeled probe to nonencapsulated and serotype a through f *H. influenzae* strains. The detection of *H. influenzae* in body fluids was then evaluated by using pooled human serum, urine, cerebrospinal fluid, and sputum as dilution media for *H. influenzae*, *Haemophilus aegyptius*, *Haemophilus parainfluenzae*, and *Escherichia coli* cells. At 65°C, the probe hybridized to *H. influenzae* and *H. aegyptius* ($\geq 10^5$ cells) in all fluids. There was no hybridization with the *E. coli* negative control, and *H. parainfluenzae* hybridized when $\geq 10^7$ cells were used. Experiments performed at 73 and 80°C permitted elimination of *H. parainfluenzae* hybridization. The detection of *H. influenzae* in 232 sputa from patients with respiratory tract infections was very specific (96 to 97%) and sensitive (74 to 100%) when the total time of the procedure was sufficient (6 to 24 h) and when the experiments were performed at 80°C. In addition, the probe detected three of three and four of four *H. influenzae*-infected cerebrospinal fluids and blood cultures, respectively, and did not react with pneumococcus- or streptococcus-infected cerebrospinal fluids. Finally, by using a small-scale procedure, the probe rapidly detected *H. influenzae* in cerebrospinal fluid and sputum specimens (4 and 8 h, respectively). These results imply prompt diagnosis of *H. influenzae* infections caused by nonencapsulated and serotype a through f strains.

Haemophilus influenzae type b is an important meningeal pathogen for young children. This bacterium is also one of the leading causes of respiratory tract infections and causes serious problems in immunocompromised adults (9, 10). In addition, it is now well established that nontypable *H. influenzae* strains play an important etiologic role in infections of both children and adults (14). Consequently, the need for a prompt and reliable diagnostic tool for all *H. influenzae* strains, from all types of body fluids, remains of primary importance.

Actual methods used for rapid diagnosis of *H. influenzae* infections include counterimmunoelectrophoresis, latex agglutination, staphylococcal coagglutination, and enzyme-linked immunosorbent assay (5, 6, 11, 20). All of these methods are based upon detection of type b capsular polysaccharide antigen and are not suitable for nontypable strains.

In a previous publication (12), we demonstrated the specificity and the potential use of an *H. influenzae* DNA fragment coding for clinical beta-lactam resistance through alteration of penicillin-binding proteins, as a probe for detection of *H. influenzae*. We report here an approach for rapid detection of *H. influenzae* (typable and unencapsulated strains) on the basis of a limited clinical evaluation of this DNA probe in clinical specimens (sputa and cerebrospinal fluids [CSFs]) and after growth amplification in blood cultures.

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MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study are shown in Table 1.

Media. The medium, brain heart infusion agar or broth (GIBCO Diagnostics, Madison, Wis.), supplemented as previously described (15), was used for the growth of all *Haemophilus* strains. Agar plates were incubated in an atmosphere containing 5% CO_2. Tryptic soy agar or broth (Difco Laboratories, Detroit, Mich.) was used for the cultivation of *Escherichia coli*.

Preparation of the labeled DNA probe. (i) Specific *H. influenzae* DNA fragment. The preparation and purification of an *H. influenzae* DNA fragment involved in penicillin-binding protein expression was previously described (12). The 5-kilobase (kb) *H. influenzae* *Hin*dIII-*Bgl*II DNA fragment purified from the recombinant plasmid pLB185 was used as the specific DNA probe.The 5-kb restriction fragment was purified from the agarose gel by electrophoretic transfer onto DEAE-cellulose paper (Bio-Rad Laboratories, Mississauga, Ontario, Canada) by the method of Winberg and Hammarskjöld (21).

(ii) Radioactive labeling of the DNA fragment. The radioactive labeling of the DNA fragment was performed by a nick translation reaction as previously described (12). We used *E. coli* DNA polymerase I and DNase I from International Biotechnologies, Inc. (New Haven, Conn.), labeled with [α-^{32}P]dATP at 400 Ci/mmol (Amersham Canada Ltd., Oakville, Ontario, Canada) or at 800 Ci/mmol (Dupont, NEN Research Products, Lachine, Quebec, Canada). The DNA-specific activity varied from 2×10^5 to 3×10^7 cpm/μg.

(iii) Biotin labeling of the DNA fragment. The biotinylation of the DNA fragment was also performed by a nick transla-

tion reaction. To 1 μg of the purified DNA fragment suspended in 70 μg of distilled water, the following solutions were added: 10 μl of a buffer containing 25 mM Tris hydrochloride (pH 7.8), 50 mM sodium chloride, 10 mM magnesium chloride, 100 μg of bovine serum albumin, and 1 mM dithiothreitol; 10 μl of a cold nucleotide mix containing 0.5 mM dTTP, 0.5 mM dGTP, and 0.5 mM dCTP; 12 μl of biotin-7-dATP (GIBCO/Bethesda Research Laboratories Canada, Burlington, Ontario, Canada) and 2 U of *E. coli* polymerase I with 10 ng of DNase I (International Biotechnologies, Inc.). The mixture was incubated at 15°C for 2 h. The reaction was stopped by the addition of 5 μl of 0.25 M EDTA (pH 8.0) and 55 μl of 7.5 M ammonium acetate. The mixture was heated to 65°C for 15 min before ethanol precipitation. The heating and precipitation steps were repeated.

Preparation of samples before hybridization. (i) Bacterial dots. Cells from 1-ml cultures grown to an A_{600} of 0.5 were spun down and suspended in 50 μl of 50 mM Tris hydrochloride (pH 7.5). For each bacterial strain used, one sample of 10 μl was spotted on side A of a GeneScreenPlus hybridization transfer membrane (Dupont). To study the effect of body fluids on the *H. influenzae* probe detection technique, known amounts of bacterial cells (CFUs) were diluted in sterile body fluids (pooled human serum, urine, CSF, or sputum) and filtered through side A of the hybridization membrane with a 96-well Minifold I filtration unit (Schleicher & Schuell, Inc., Keene, N.H.). Cells were lysed in situ by placing the membrane on 0.5 M sodium hydroxide drops aligned on a stretched plastic wrap. After 2 min, the membrane was removed and placed on a filter paper for 2 min before repeating the lysis step. The membrane was then treated the same way with 1 M Tris hydrochloride (pH 7.5) and dried over a filter paper on a hot plate.

(ii) Electroblot procedure for chromosomal DNA. The cells from 1-liter cultures grown overnight were collected by centrifugation, and the chromosomal DNA was extracted by the method of Silhavy et al. (16). The extracted chromosomal DNA from several bacterial strains was digested with *Bgl*II or with *Hin*dIII and *Bgl*II at 37°C for 2 h. Restriction fragments were electrophoresed on duplicate 0.8% agarose gels. One gel was stained with ethidium bromide (0.5 μg/ml), and the other one was soaked for 30 min in 0.4 M sodium hydroxide before neutralization in 0.04 M Tris acetate (pH 8.0) and 0.002 M EDTA for another 30 min. The treated gel was placed on side B of a GeneScreenPlus membrane, and the DNA was electrophoresed by using a Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.) at 25 V for 1 h and at 40 V for 2 h. The membrane was dried overnight at room temperature, and the gel was stained with ethidium bromide to evaluate the transfer efficacy.

(iii) Sputum specimens. Sputum specimens (232 samples) were obtained from the microbiology laboratory of Foothills Hospital. All samples were obtained from patients with clinical findings of respiratory infection, based on history, physical examination, and in most cases radiological evidence of the disease. Diagnoses were either pneumonia or purulent bronchitis. In our clinical laboratory, sputum specimens were examined microscopically for cell types and nature and approximate number of bacteria prior to processing for culture. The only specimens cultured routinely are those which have 25 or more leukocytes per field at 100× magnification (low power) and 0 to 25 epithelial cells per low-power field. The only exception is when a larger number of epithelial cells is detected and one morphological form of bacteria is the only form observed or is in much greater quantity (usually approximately 50-fold or more excess) in five oil immersion microscopy fields. Specimens are reported here by naming the predominant bacterium detected. A predominant species was defined as the only bacterial morphological type seen by oil immersion microscopy or by counting bacteria over five oil immersion fields and indicating those forms as predominant which were present at a minimum of 50-fold excess over other morphotypes. Culture results were correlated with the probable predominant pathogen from smear evaluation, and if the organism was detected by culture, it was reported as the predominant isolate. For the purpose of this study, specimens with more than a single predominant species or in which there was disagreement between the culture and the smear were excluded.

Upon arrival at the laboratory, sputa were plated onto chocolate agar, 5% sheep blood agar, and MacConkey agar plates. The specimens were immediately thereafter frozen at −20°C for the probe analysis. Plates were examined for significant pathogens after 18 to 24 h of incubation in a 5% CO_2 or O_2 (MacConkey plates) incubator and were reincubated for a further 24 to 48 h. A decision was made on the predominant bacterial species (pathogen) by comparing culture results at both times with smear results. All bacterial identifications were by standard methods (10).

For DNA hybridization, specimens were diluted 1:1 with sputolysin (Calbiochem-Behring, La Jolla, Calif.). A volume of 10 μl of the samples was spotted on side A of a GeneScreenPlus membrane and lysed in situ as described above.

(iv) Blood cultures. Microorganisms pelleted from positive blood cultures (75 samples) obtained after growth amplification were from Foothills Hospital and were kept frozen at −20°C until used. In the clinical laboratory, the BACTEC 460 blood culture system (Johnston Laboratories, Inc., Cockeysville, Md.) was used to detect, within 48 h, any positive cultures (aerobic and anaerobic) unless apparent growth was seen. Organisms were identified from a pellet obtained from positive cultures (10-ml aliquot) after differential centrifugations; the culture was centrifuged for 10 min at 150 × *g* to remove cellular components from blood, and the supernatant was centrifuged for 2 min at 1,500 × *g* to collect the microorganisms. *H. influenzae* was identified by standard methods (10).

For DNA hybridization, the microorganisms collected after growth amplification as mentioned above were suspended in the residual supernatant and were spotted (5 μl) on side A of a GeneScreenPlus membrane. The membrane was then treated as described above.

(v) CSFs. Five CSF specimens were obtained from the Hôpital Sainte-Justine and five others (negative growth controls) from the Foothills Hospital. In the clinical laboratory, an aliquot of the CSF was spun in a microfuge. The sediment was plated onto 5% sheep blood agar and chocolate agar plates and incubated in a 5% CO_2 incubator. Smears from the sediments were also gram stained and examined for the presence of organisms, and residual CSF was thereafter kept frozen at −20°C until used in the probe detection study. *H. influenzae* isolates recovered from the culture were identified by standard methods (10). The specimens were tested for the presence of pathogen (antigens) by the Directigen Meningitis Combo test (Becton Dickinson, Cockeysville, Md.) at the Foothills Hospital and by counterimmunoelectrophoresis at Hôpital Sainte-Justine.

For DNA hybridization, the remaining specimens (0.3 to 0.7 ml) were spun in a microfuge at 14,000 rpm for 10 min. The supernatant was removed and concentrated to a volume

of 100 μl by using a Minicon-B15 concentrator (Amicon Div., Danvers, Mass.). The cell pellets were resuspended in the residual volume (about 20 μl). A volume of 10 μl of the samples was spotted on side A of a GeneScreenPlus membrane and lysed in situ as described above.

Hybridization method. All hybridizations were performed as recommended by the GeneScreenPlus supplier (Dupont). The time and the temperature parameters of the incubation periods varied from one experiment to another and are specified in the figure legends. Most hybridizations were done in a 20-ml PR800 hybridization chamber (Hoefer Scientific Instruments). For hybridization to DNA digests, transfer membranes (45 by 85 mm) were rolled in 15-ml Falcon polystyrene conical screw-cap tubes (17 by 20 mm; Becton Dickinson and Co., Oxnard, Calif.) with DNA facing in. A rapid small-scale procedure was used for hybridization to some clinical specimens, and the membranes (20 by 30 mm) were then placed in 1.8-ml Nunc Cryotubes (Nunc InterMed, Roskilde, Denmark). The tubes were protected by two plastic bags sealed under vacuum.

The membranes were prehybridized for 1 to 6 h at 65, 73, or 80°C with constant agitation in a preheated solution containing 1% sodium dodecyl sulfate, 1 M sodium chloride, and 10% dextran sulfate (Sigma Chemical Co., St. Louis, Mo.). Hybridization was performed by adding to the prehybridization solution 100 μg of denatured salmon sperm DNA (Sigma) and a specified amount of the denatured DNA probe per ml. Denaturation of DNA was done by heating to 100°C for 10 min. Membranes were incubated for 1 to 12 h at the specified temperature with constant agitation.

The membranes were washed as follows: twice with 100 ml of 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) for 2 to 5 min at room temperature with shaking; twice with 200 ml of 2× SSC and 1% sodium dodecyl sulfate at 65, 73, or 80°C for 10 to 30 min with shaking; and twice with 100 ml of 0.1× SSC for 5 to 30 min at room temperature with constant agitation. Membranes were dried rapidly over a filter paper on a hot plate or overnight at room temperature.

Hybridization with the ^{32}P-labeled probe was detected by autoradiography with X-Omat AR films (Eastman Kodak Co., Rochester, N.Y.) exposed for 0.5 to 48 h at room temperature before development. Alternatively, the spotted samples hybridized with the probe were cut out of the GeneScreenPlus membrane and beta-particle emissions were counted by using LS 6800 liquid scintillation system and Ready Safe liquid scintillation cocktail (Beckman Instruments [Canada] Inc., Mississauga, Ontario, Canada). Hybridization with the biotinylated probe was detected by a colorimetric reaction using the Blugene detection system (GIBCO/Bethesda Research Laboratories), in which biotin-streptavidin-alkaline phosphatase complexes are visualized by adding the following reagents: Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Sensitivity and specificity of the probe. In a previous study (12), the probe specificity was assessed by dot blot hybridization with 75 bacterial strains encompassing 41 species. Here, additional strains were tested (Table 1) and showed that all of the *H. influenzae* and *Haemophilus aegyptius* strains hybridized very strongly with the probe. Overall, we demonstrated that the probe reacted very well with all *H. influenzae* serotypes: 14 nontypable, 4 type b, and 1 of each of type a and c through f strains (data not shown).

TABLE 1. Bacterial strains used in hybridization experiments

Species, strain, and serotype	Source (reference)
Haemophilus influenzae	
Rd (unencapsulated)	12
T-1, 3 (unencapsulated)	12
35039 (unencapsulated)	ATCC[a] (12)
9006 (type a)	ATCC (12)
9795 (type b)	ATCC
UC-1 (type b)	University of Calgary (12)
UC-2 (type b)	University of Calgary (12)
UC-3 (type b)	University of Calgary (12)
9007 (type c)	ATCC
9008 (type d)	ATCC
8142 (type e)	ATCC
9833 (type f)	ATCC
Haemophilus aegyptius	
FH45-5	University of Calgary
KC200	W. L. Albritton[b] (12)
Haemophilus parainfluenzae KC269	W. L. Albritton (12)
Escherichia coli ATCC 33849	ATCC (12)

[a] American Type Culture Collection, Rockville, Md.
[b] University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

The detection of *H. influenzae* and *H. aegyptius* in body fluids was then evaluated. Sterile, pooled human serum, urine, CSF, and sputum were used as dilution media for *H. influenzae* Rd, *H. aegyptius* KC200, *Haemophilus parainfluenzae* KC269, and *E. coli* ATCC 33849 cells. Samples were filtered through the nylon membrane, lysed, and hybridized to the probe at 65°C (Fig. 1). The probe hybridized to *H. influenzae* and *H. aegyptius* ($\geq 10^5$ cells) in all body fluids, and there was no hybridization with the *E. coli* negative control. The biotinylated probe gave a weaker positive signal but was as sensitive (Fig. 1C). In this experiment, *H. parainfluenzae* hybridized when $\geq 10^7$ cells were used.

We examined the effect of temperature on the specificity of the probe (Fig. 2). We found that the probe was still sensitive enough to detect 10^5 *H. influenzae* cells at either 65, 73, or 80°C, and that the specificity of the probe was much greater at 80°C, when even 10^8 *H. parainfluenzae* cells could not be detected.

Hybridization to chromosomal DNA demonstrated DNA homology between *H. influenzae* and *H. aegyptius* (Fig. 3). Chromosomal DNA from *H. influenzae* strains showed one or two *Bgl*II restriction fragments hybridizing with the probe. However, the probe hybridized distinctively to a 15-kb *Bgl*II DNA fragment in all the *H. influenzae* strains tested and in *H. aegyptius* FH45-5 (Fig. 3, lanes 1 through 7). The *Hin*dIII-*Bgl*II digest of the DNA from the two *H. aegyptius* strains showed hybridization to a 5-kb DNA fragment having an electrophoretic mobility identical to the *Hin*dIII-*Bgl*II DNA probe used in the present study (Fig. 3, lanes 8 and 9).

Detection of *H. influenzae* in clinical specimens. The detection method for *H. influenzae* in 232 sputum specimens from patients with respiratory tract infections was very specific and sensitive when the total time of the procedure was sufficient (Table 2). At 6, 16, and 24 h, the specificities were 96.7, 96.7, and 96.2%, respectively; the sensitivities were 73.9, 95.7, and 100%; the positive predictive values were 70.8, 75.9, and 74.2%; and the negative predictive values were 97.1, 99.5, and 100%. The X-ray film exposure time (2, 12, or 20 h) determined the total duration of the detection procedure (6, 16, or 24 h).

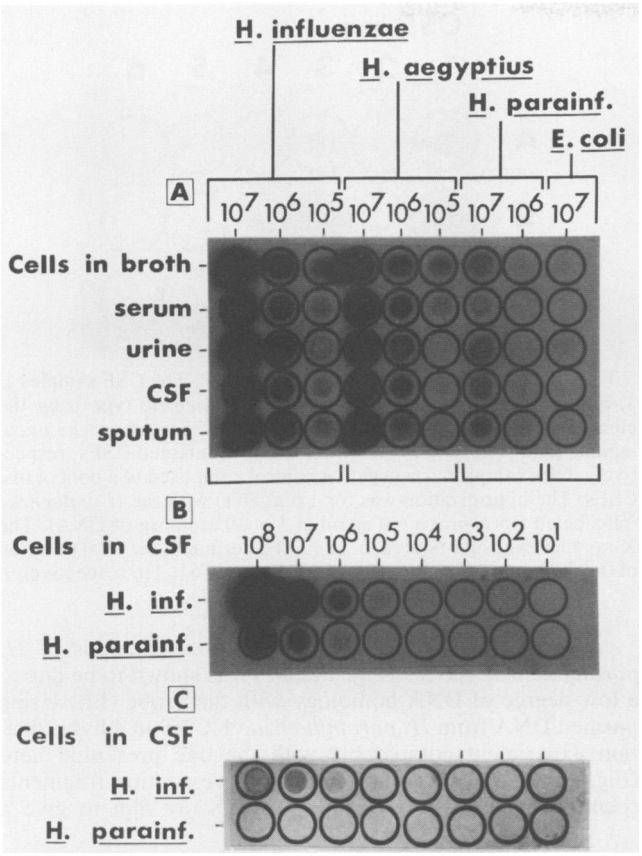

FIG. 1. Effect of body fluids on *H. influenzae* detection. (A) Bacterial strains of known CFUs were diluted in broth (BHI medium) or in pooled human serum, urine, CSF, or sputum. (B and C) Cells were diluted in pooled human CSF. The hybridization was for 12 h at 65°C either with the *H. influenzae* ^{32}P-labeled DNA probe (20 ng/ml at 5×10^6 cpm/μg of DNA) (A and B) or with the biotinylated probe (15 ng/ml) (C). The X-ray films were exposed for 48 h. H. inf., *H. influenzae*; H. parainf., *H. parainfluenzae*.

A total of 75 growth-amplified blood cultures were tested with the probe. The detection of *H. influenzae* in four out of four blood cultures was sensitive and rapid (4.5 h), because the bacteria were spun and spotted directly onto the hybridization membrane (data not shown). In addition, all *H. influenzae*-infected CSF samples (~10^6 bacteria per ml) reacted strongly with the probe (Fig. 4). *H. influenzae* could be detected in a time period as short as 5 h (Fig. 4A). The detection method for *H. influenzae* in CSF was even faster (approximately 4 h) when a Beckman liquid scintillation system was used. Indeed, when the samples were cut out of the nylon membrane and counted, the cpm values of CSF samples 1, 3, and 4 (Fig. 4) were 1,236, 1,711, and 280, respectively, and represented 82, 114, and 19 times the value of CSF sample 6, the negative control (15 cpm). CSF sample 2 (*Streptococcus pneumoniae*) and CSF sample 5 (*Neisseria meningitidis* group C) showed no reaction with the probe (each 11 cpm). The biotinylated probe could also detect the three *H. influenzae*-infected CSFs but, again, the positive signal was less evident than the reaction observed with the ^{32}P-labeled probe (data not shown). Finally, as expected, the spotted CSF supernatants did not react at all with the DNA probe (data not shown).

Finally, by using a small-scale procedure, we were able to identify a *H. influenzae* colony in 4.5 h and *H. influenzae*

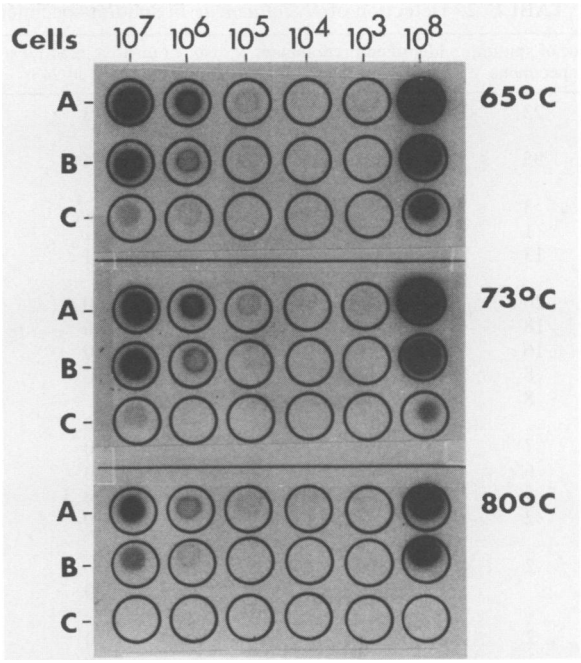

FIG. 2. Effect of temperature on *H. influenzae* detection. Bacterial strains of known CFUs were diluted in pooled human CSF and spotted onto a GeneScreenPlus membrane. Row A, *H. influenzae* Rd; row B, *H. aegyptius* KC200; row C, *H. parainfluenzae* KC269. The hybridization was for 12 h with the ^{32}P-labeled *H. influenzae* DNA probe (5 ng/ml at 5×10^6 cpm/μg of DNA) at either 65, 73, or 80°C. The X-ray films were exposed for 48 h.

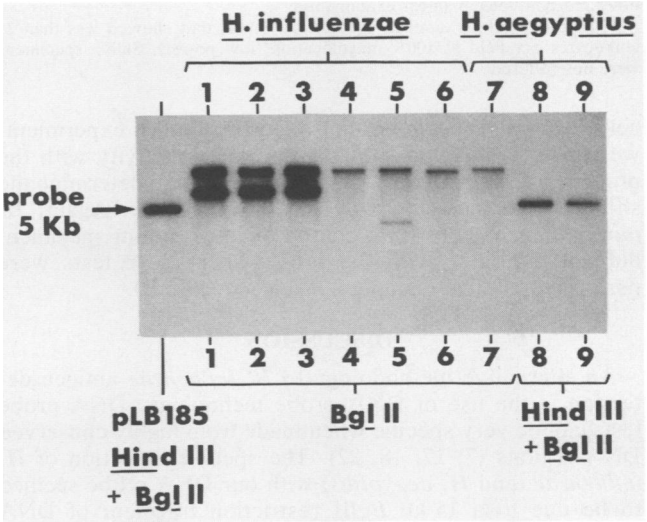

FIG. 3. Autoradiogram of a hybridization experiment with chromosomal DNA. Chromosomal DNAs were digested with *Bgl*II (lanes 1 through 7) or with *Bgl*II and *Hin*dIII (lanes 8 and 9), electrophoresed on a 0.8% agarose gel, denatured with 0.4 N sodium hydroxide, electroblotted onto a nylon membrane, and hybridized for 12 h at 65°C with the *H. influenzae* ^{32}P-labeled DNA probe (30 ng/ml at 2×10^5 cpm/μg of DNA). The X-ray film was exposed for 12 h. Lanes 1 through 6, *H. influenzae* Rd, T-1,3, ATCC 35039, UC-2, UC-1, and UC-3, respectively; lanes 7 and 8, *H. aegyptius* FH45-5; lane 9, *H. aegyptius* KC200.

TABLE 2. Detection of *H. influenzae* in sputum specimens

No. of sputum specimens	Identified predominant species[a]	No. of positive reactions with the probe at 24 h[b]
23	*Haemophilus influenzae*	23
95	None[c] (normal flora)	4
3	*Proteus mirabilis*	2
1	*Klebsiella oxytoca*	1
13	*Pseudomonas aeruginosa*	1
20	*Staphylococcus* sp.	0
18	*Streptococcus* sp.	0
16	None[d]	0
8	None (no growth)	0
8	*Candida* sp.	0
7	*Escherichia coli*	0
6	*Neisseria* sp.	0
4	*Enterobacter* sp.	0
2	*Haemophilus parainfluenzae*	0
2	*Klebsiella pneumoniae*	0
2	*Citrobacter* sp.	0
1	*Morganella morganii*	0
1	*Serratia liquefaciens*	0
1	*Acinetobacter* sp.	0
1	*Branhamella* sp.	0

[a] The determination of the predominant species isolated from the sputum specimens was as described in Materials and Methods.

[b] The preparation of the sputum specimens before hybridization is described in Materials and Methods. The hybridization was done with 30 ng of probe per ml (at 10^7 cpm/μg of DNA) for 1 h at 80°C. The X-ray film exposure time (2, 12, or 20 h) determined the total duration of the detection procedure (6, 16, or 24 h).

[c] No predominant species was found at a minimum of 50-fold excess over other morphotypes in smear examination.

[d] Microscopically examined clinical specimens that showed less than 25 leukocytes per field at 100× magnification (low power). Saliva specimens were not cultured.

cells in sputum specimens in 8 h (Fig. 5). In this experiment, we chose three sputa showing different reactivity with the probe (Fig. 5B, sputum samples 3, 4, and 5) to determine the shortest time needed to detect *H. influenzae*. Again, *H. parainfluenzae* cells (in a colony or in a sputum specimen) did not hybridize with the probe when these tests were performed at 80°C (sample 2 in Fig. 5A and B).

DISCUSSION

An alternative methodology to *H. influenzae* antigen detection is the use of DNA probe technology. DNA probe tests can be very specific when made from highly conserved DNA regions (7, 17, 18, 22). The specific detection of *H. influenzae* (and *H. aegyptius*) with our DNA probe seemed to be due to a 15-kb *Bgl*II restriction fragment of DNA present in all strains tested (Fig. 3). The DNA homology with *H. aegyptius* (42 to 91%) has been previously reported in the literature (2, 13). The *H. influenzae* strain-to-strain DNA homology for the specific region corresponding to the probe was present in all strains tested by dot blot hybridization. Along with a previous study (12), we demonstrated that the probe reacted strongly with all the 14 *H. influenzae* nontypable strains and with all the *H. influenzae* serotypes (4 type b, and 1 of each of type a and c through f strains) tested.

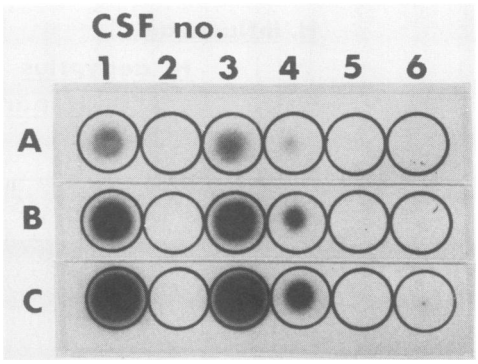

FIG. 4. Detection of *H. influenzae* in CSFs. The CSF samples 1, 3, and 4 were identified positive for *H. influenzae* type b by the clinical laboratory, and samples 2 and 5 were *Streptococcus pneumoniae*- and *Neisseria meningitidis* group C-infected CSFs, respectively. CSF sample 6 is a negative control composed of a pool of five CSFs. The hybridization was for 1 h at 80°C with the *H. influenzae* ^{32}P-labeled DNA probe (50 ng/ml at 3×10^7 cpm/μg of DNA). The X-ray film exposure time (1, 6, or 12 h) determined the total duration of the detection procedure (5 [A], 10 [B], and 16 [C] h, respectively).

At 65°C, the hybridization seen with $\geq 10^7$ cells of *H. parainfluenzae* KC269 (Fig. 1 and 2) was shown to be due to a low degree of DNA homology with the probe (12). Using purified DNA from *H. parainfluenzae* KC269 in a hybridization experiment comparable with the one presented here (Fig. 3), we showed that homologous restriction fragments required 14 days of exposure of an X-ray film to give a

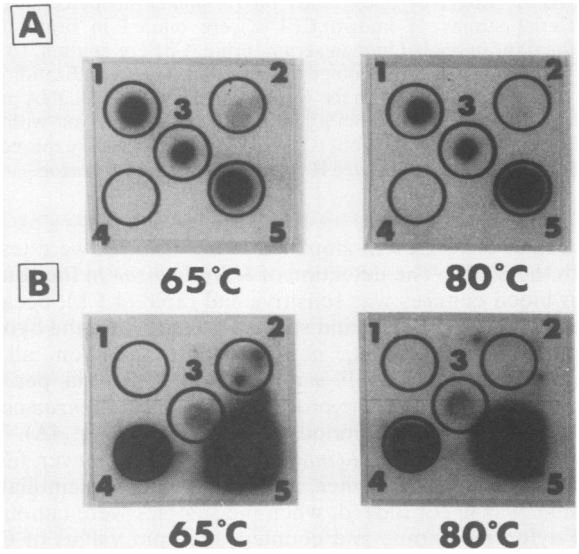

FIG. 5. Rapid detection method for *H. influenzae* colonies (A) and *H. influenzae*-containing sputum specimens (B). The hybridization technique was scaled-down to allow shorter incubation time. A single colony or 5 μl of sputum was spotted on a 2 by 3 cm GeneScreenPlus membrane. The hybridization was for 1 h at 65 or 80°C with the *H. influenzae* ^{32}P-labeled DNA probe (70 ng/ml at 10^7 cpm/μg). The X-ray film exposure time (0.75 and 4.25 h for panels A and B, respectively) determined the total duration of the detection procedure, 4.5 (A) and 8.0 h (B). (A) Colony 1, *H. influenzae* Rd; colony 2, *H. parainfluenzae* KC269; colony 3, *H. aegyptius* KC200; colony 4, *E. coli* ATCC 33849; and colony 5, *H. influenzae* ATCC 9795. Predominant species in panel B, sputum sample 1, none (normal flora); sputum sample 2, *H. parainfluenzae*; sputum samples 3, 4, and 5, *H. influenzae*.

detectable reaction and that the strongest signal represented no more than 1/50 of the signal recorded for *H. influenzae* and *H. aegyptius* DNAs by laser densitometry analysis (12). Therefore, as expected, hybridization experiments performed at high stringency (80°C) permitted an increase of the specificity of the probe (no hybridization to 10^8 *H. parainfluenzae* KC269 cells) while retaining its sensitivity for $\geq 10^5$ *H. influenzae* or *H. aegyptius* cells (Fig. 2).

To improve the specificity, we could have used a smaller DNA fragment to construct the probe. However, we felt that this would be unnecessary if hybridizations were performed at 80°C. Also, a smaller probe would likely reduce the sensitivity. This would be unfavorable, in our view, as the risk of missing a potential signal from sputa of patients with *H. influenzae* bronchopulmonary infection would increase. We based this view on studies quantitating bacteria in clinical disease which have shown that potential pathogens are present in sputa at a mean concentration of $10^{6.6}$ CFU/ml (3), which is close to the apparent sensitivity limit of our probe (10^5 *H. influenzae* cells per 10 μl of sputum).

Our DNA probe showed a useful sensitivity range for *H. influenzae* in all kinds of body fluids (Fig. 1). This characteristic is essential for clinically useful probes. In our limited clinical evaluation, the probe was able to detect *H. influenzae* directly from all positive specimens: sputa (Table 2) and CSFs (Fig. 4) without the need for subcultivation and in four of four growth-amplified blood cultures. Interestingly, no hybridization was observed with the two *H. parainfluenzae*-containing sputa, but we observed hybridization to eight sputa (at 24 h) in which *H. influenzae* was not identified by the clinical laboratory (Table 2). Unfortunately, we could not trace back these particular isolates in our study, but since none of the predominant bacterial species identified hybridized with the probe in a dot assay performed at 65°C (12), it is possible that such positive reactions were due to the presence of *H. influenzae* cells in these clinical specimens which were tested under even higher stringency conditions at 80°C. Thus, our probe may detect *H. influenzae* in sputum specimens not found by common clinical laboratory procedures. If so, the positive predicted values (70 to 76%) would be higher. However, no definitive evidence of this was possible in our study.

The interpretation of a positive probe result for *H. influenzae* in a sputum sample must be cautious. Part of this caution is similar to that needed to evaluate growth of *H. influenzae* by cultures. The significance of a positive signal or culture is enhanced by assurance of proper sputum collection, transport, and evaluation. The use of numbers of leukocytes and epithelial cells per low-power microscopy field of each sputum specimen as criteria to indicate the probability that the sample represents the infectious process similarly improves the diagnostic value of culture as well as the probe. Special problems that may present for use of the probe relate to the minor cross-reactivity with *H. parainfluenzae*. Use of the 80°C hybridization procedure means that more than 10^{10} organisms per ml (10^8 cells for a 10-μl spot) would need to be present. This is highly unlikely for a commensal organism in properly collected and transported sputa. A second problem may be the sensitivity of the probe. Our data showed that all clinically significant positive cultures could be detected by 24 h. At 6 h, the sensitivity was clearly limiting. We feel the current role of our probe should be to enhance identification of *H. influenzae* and not replace conventional culture. The value of this approach, for example, would be that about 74% of the positive sputum results could be detected as early as 6 h. In some circumstances, particularly in the era of acquired immunodeficiency syndrome, the identification of *H. influenzae* would allow early specific antimicrobial therapy. This is particularly so because the range of pathogens causing lung infection is now large in these patients. Another potential area of utilization for *H. influenzae* DNA probes was recently suggested by Terpstra et al. (19). They used in situ DNA hybridization to detect *Haemophilus* species in sputa from patients with cystic fibrosis. The main advantage of the probe was its ability to detect *Haemophilus* species directly in sputa where overgrowth by other mucoid bacteria interfered with diagnosis by culture.

In the clinical laboratory setting, DNA probe technology must be easily applicable to be of interest. Therefore, we looked at a small-scale procedure to address this problem (Fig. 5). Fortunately, the probe showed even better sensitivity with these conditions. In addition, we were able to eliminate the X-ray film exposure and development steps (about 1 h), simply by counting the samples spotted onto the membrane after hybridization by a liquid scintillation system. The prehybridization time (about 1 h) can also be reduced or may be completely eliminated. Based on the results shown in Fig. 4, the detection time of *H. influenzae* in CSFs would have been about 3 h in these conditions. These modifications would render this DNA probe technology as attractive as current antigen detection tests on a time-based comparison.

Another concern for the clinical laboratory is the use of radiolabeled probe as opposed to nonradioactive materials. As shown in other studies (18, 22), we felt that the radiolabeled probe was superior to the biotinylated probe for the clarity of its positive signal, although the sensitivity seemed equivalent (Fig. 1B and C). An alternative to these detection methods which we have not yet tried is the use of alkaline phosphatase-conjugated probe which can replace biotin-avidin systems (8).

Because of the recognition of the importance of *H. aegyptius* strains in purulent conjunctivitis and in Brazilian purpuric fever (1, 4) and of the importance of nontypable as well as *H. influenzae* type b strains in human infections (14) and because reliance of methods for prompt diagnosis are exclusively based upon detection of type b capsular polysaccharide antigen (5, 6, 11, 20), we believe that a nonsegregative *H. influenzae* DNA probe similar to ours may be useful in clinical microbiology.

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