Journal of Clinical Microbiology, July 1995, p. 1835–1838 Vol. 33, No. 7
0095-1137/95/$04.00+0
Copyright © 1995, American Society for Microbiology

High Incidence of *Haemophilus influenzae* in Nasopharyngeal Secretions and Middle Ear Effusions as Detected by PCR

TOMOYO UEYAMA,[1] YUICHI KURONO,[1] KOMEI SHIRABE,[2] MASAZUMI TAKESHITA,[2] AND GORO MOGI[1]*

Departments of Otolaryngology[1] and Biochemistry,[2] Oita Medical University, Oita, Japan

Received 3 January 1995/Returned for modification 6 March 1995/Accepted 24 April 1995

PCR was used to detect *Haemophilus influenzae* in samples of nasopharyngeal secretion and middle ear effusion (MEE). Nasopharyngeal secretions were collected from 102 patients with otitis media with effusion and from 111 healthy subjects. Eighty samples of MEE were collected from patients with otitis media with effusion. A pair of primers was designed to amplify a DNA segment of the gene encoding P6 outer membrane protein of *H. influenzae*. The amplified PCR product was detected with an internal probe that hybridized specifically to the P6 DNA of *H. influenzae*. Samples of MEE and nasopharyngeal secretion were also examined by a conventional culture method. The incidence of P6 gene DNA in nasopharyngeal secretions detected by PCR was about two times higher than that of *H. influenzae* detected by the conventional culture. Culture-positive samples were all positive in the PCR test. In MEEs, the rate of detection of the P6 gene DNA target was about five times higher than that of *H. influenzae* detected by the culture method. All patients who had P6 gene DNA in MEEs were found to have the DNA in nasopharyngeal secretions. These findings suggest that the presence of *H. influenzae* in MEEs and in nasopharyngeal secretions is more common than previously reported.

Otitis media with effusion (OME) is characterized by persistent middle ear effusion (MEE) without any symptoms of infection such as otalgia and fever which are commonly observed in acute otitis media (3). MEE was thought to be sterile until Senturia et al. (14) first reported the presence of bacteria. Recent studies have shown that many cases of OME are preceded by an episode of acute otitis media (15) and that *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* are frequently cultured from MEEs. Moreover, many of the isolates are identical to those found in the nasopharynx (7). Although *H. influenzae* is commonly cultured from the nasopharynges of healthy children, the carriage rate is higher in patients with OME than in healthy children (4). It is well acknowledged that microorganisms can reach the middle ear cavity via the eustachian tube from the nasopharynx and cause middle ear infection (8). Those findings suggest that *H. influenzae* in the nasopharynx, as well as that in MEEs, plays an important role in the persistent presence of MEEs and in the pathogenesis of OME.

The incidence of bacteria in MEEs detected by conventional culture methods was only 23.7% (7). Previous treatment with antibiotics may explain the low detection rate. Failure to detect bacteria in the majority of patients with OME raises a question about whether bacterial infection is truly a prerequisite for diagnosis of the condition. DeMaria et al. (2) detected endotoxin in 80% of MEEs, including many culture-negative samples. They also reported that experimental OME was induced by middle ear inoculation with endotoxin of nontypeable *H. influenzae* (3). Although the existence of endotoxins in MEEs indicates that gram-negative bacteria were present in MEEs, the bacterial species cannot be identified, because endotoxin is ubiquitous for gram-negative bacteria. Thus, it is necessary to develop a sensitive and specific method to detect bacteria in MEEs in order to examine the relationship between bacterial infection and the pathogenesis of OME. The P6 protein, a 16.6-kDa outer membrane protein of *H. influenzae*, is a highly conserved antigen among most strains of *H. influenzae* (9) and has been demonstrated to be a potential vaccine component in preventing infections caused by *H. influenzae* (10).

Recently, Hotomi et al. (6) applied PCR to detect the P6 gene DNA of *H. influenzae* in MEEs by use of a primer set reported by van Ketel et al. (16). However, this primer set also amplifies DNA of other genera of *Haemophilus*, such as *Haemophilus parainfluenzae*, which is also frequently cultured from MEEs and from the nasopharynx (16). In the present study, newly designed oligonucleotide probes were used in dot blot hybridization tests to differentiate the P6 gene DNA of *H. influenzae* from that of *H. parainfluenzae.*

MATERIALS AND METHODS

Nasopharyngeal secretions and MEEs. Nasopharyngeal secretions were collected from 102 patients with OME and 111 healthy control subjects. Eighty samples of MEE were collected from 65 patients with OME, and bacterial concordance between MEEs and nasopharyngeal secretions was investigated. A Juhn Tym-Tap (Xomed, Jacksonville, Fla.) was inserted into the nasopharynx through the nose, and nasopharyngeal secretions were collected by aspiration. MEEs were collected as follows. The external ear canal was cleansed of ear wax, and the canal was disinfected with saline containing 0.01% iodine after iontophoretic anesthesia which was applied with an equal mixture of 0.01% epinephrine and 4% lidocaine hydrochloride. Following myringotomy, MEEs were collected by aspiration with a Juhn Tym-Tap (Xomed) through the eardrum. All samples were collected from November 1991 to September 1992 at the Department of Otolaryngology of Oita Medical University (Oita, Japan).

Bacterial cultivation. Samples of nasopharyngeal secretion and MEE were inoculated on blood agar and chocolate agar with sterile cotton-tipped swabs and were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. *H. influenzae* was identified by its V (NAD) and X (hemin) growth factor requirements. Differentiation of *H. influenzae* and *H. parainfluenzae* was comfirmed by porphyrin tests. Bacterial identifications were carried out in the microbiological laboratory at the Oita Medical University Hospital.

Preparation of DNA. DNA was prepared from nasopharyngeal secretion and MEE specimens by the method reported by van Ketel et al. (16) with a slight modification, as follows. Specimens (100 μl) were diluted with 100 μl of 50 mM Tris-HCl (pH 8.5)–50 mM EDTA–2.5% sodium dodecyl sulfate (SDS) and digested overnight at 50°C with proteinase K (20 mg/ml). Sodium perchlorate was then added to a final concentration of 1 M, and the mixture was incubated at 50°C for 1 h. After addition of 200 μl of STE (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH 8.5]), the DNA was extracted with phenol-chloroform (1:1)

* Corresponding author. Mailing address: Department of Otolaryngology, Oita Medical University, Hasama-machi, Oita 879-55, Japan. Phone: (975) 49-4411. Fax: (975) 49-0762.

twice and precipitated with ethanol and glycogen (20 mg/ml). SepaGene (Sanko Junyaku Co., Ltd., Tokyo, Japan) was used to extract DNA from bacterial cells by following the manufacturer's instructions. Precipitated DNA was washed with 70% ethanol and dissolved with 200 μl of H_2O. The DNA was stored at −20°C for later use.

PCR amplification of P6 DNA. The primer set, F1 and R1, was designed based on the published DNA sequence for the P6 gene, using DNAsis software (Hitachi, Tokyo, Japan) to preclude hairpin loops. The length of the amplified products was expected to be 351 bp. The sequences of oligonucleotides for the primer set and probes were as follows: primer F1, 5′-AAC TTT TGG CGG TTA CTC TG-3′ (melting temperature, 56°C); and primer R1, 5′-CTA ACA CTG CAC GAC GGT TT-3′ (melting temperature, 59°C). The oligonucleotides were custom synthesized by Kurabo (Osaka, Japan). PCR was performed as described by Saiki et al. (11) in a final volume of 100 μl. The standard reaction for amplification contained 2.5 U of *Thermus aquaticus* DNA polymerase (*Taq* polymerase; Perkin-Elmer Cetus, Norwalk, Conn.), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM (each) deoxynucleoside triphosphate (dNTP; dATP, dCTP, dGTP, and dUTP), 0.2 μM (each) primer, 5.0 U of Uracil N-Glycosylase (Perkin-Elmer Cetus), and 10 μl of the obtained DNA as a template. The reaction mixture was incubated at room temperature for 10 min to allow digestion with Uracil N-Glycosylase prior to incubation at 95°C for 10 min to degrade possible carry-over products before the start of PCR. For PCR, a programmable DNA thermal cycler, model PJ480 (Perkin-Elmer Cetus Instrument, Norwalk, Conn.), was used. Conditions for PCR consisted of 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min (5 min for the last cycle).

Discrimination between *H. influenzae* and *H. parainfluenzae* P6 gene targets by probe hybridization. Oligonucleotide probes, HI and HP, were designed as follows to distinguish the P6 gene of *H. influenzae* from that of *H. parainfluenzae*: probe HI, 5′-TAA ATA TGA CAT TAC TGG TG-3′; probe HP, 5′-CAA ATT CAA CAT CGA AGG-3′. (HI and HP represent *H. influenzae* and *H. parainfluenzae*, respectively.) PCR products were extracted with chloroform to remove mineral oil. The amplified samples (6 μl) were fractionated on 3% agarose gels by electrophoresis (12). pHY marker (Takara Shuzo Co., Kyoto, Japan) was used as a molecular weight marker. Southern blot hybridization was carried out to detect *H. influenzae* DNA. The DNA was transferred onto a nylon filter (Amersham, Buckinghamshire, United Kingdom) at room temperature overnight in 20× SSC (3 M NaCl, and 0.3 M trisodium citrate). The DNA was fixed by UV irradiation (0.3 J/cm^2). DNA labeling, hybridization, and detection were carried out by using ECL rate-enhanced hybridization buffer and ECL 3′-oligonucleotide labeling and detection systems (Amersham) according to the manufacturer's instructions. The filters were washed twice with 5× SSC–0.1% SDS at room temperature for 5 min and twice with 1× SSC–0.1% SDS at 42°C for 15 min. Filters were exposed to radiographic films for 10 min in cassettes. For the detection by dot blot hybridization, 1/50 of the amplified DNA was blotted onto a nylon filter and fixed by UV irradiation (0.3 J/cm^2).

Sequence analysis of PCR-amplified P6 DNA. For cloning of PCR-amplified fragments on M13 vector, primers were phosphorylated at the 5′ end before PCR (12). The primers used for the cloning of the P6 gene, previously reported by Deich et al. (1), were as follows: HI-IV, 5′-ACT TTT GGC GGT TAC TCT GT-3′; and HI-V, 5′-TGT GCC TAA TTT ACCC AGC AT-3′. The PCR products were extracted with chloroform and separated by 4% agarose gel electrophoresis. After addition of 4 volumes of 10 mM Tris-HCl–1 mM EDTA (pH 8.0), the gel slice containing the DNA fragment was melted and phenol extracted once. After ethanol precipitation, the ends of the DNA fragment were filled in with the Klenow fragment (Takara Shuzo Co.) in the presence of 0.2 mM dNTPs (12). The resulting DNA fragments were cloned at the *Sma*I site of M13 mp8, and the nucleotide sequence was determined by the dideoxy-chain termination method (13).

Statistical analysis. The carriage rates of *H. influenzae* in nasopharyngeal secretions of patients with OME and of control subjects were compared by use of the chi-square test. Comparisons of the positive percentages by the culture method and those by PCR for patients with OME or for control subjects were analyzed by use of the McNemar test. Concordance between the incidence of P6 gene DNA in nasopharyngeal secretions and that in MEEs was analyzed by use of the chi-square test.

RESULTS

PCR analysis. The oligonucleotide primers were designed to amplify the P6 gene target of *H. influenzae* on the basis of the previously reported DNA sequence (1). Gel electrophoresis was used to analyze the specificity of the primers. In the preliminary study, DNA was prepared from 60 bacterial isolates cultured from nasopharyngeal secretions in this study, as shown in Table 1. Distinct bands of the expected size (approximately 351 bp) were observed only in the samples with *H. influenzae* and *H. parainfluenzae* (Fig. 1). For sequence analysis, shorter DNA fragments were amplified by PCR with the HI-IV and HI-V primers (16) to avoid false-positive results caused by carry-over products of amplified DNA into clinical samples. The P6 gene target sequence from the *H. influenzae* samples was almost identical to that of the P6 gene DNA of *H. influenzae* previously reported (1). In contrast, the sequence from the *H. parainfluenzae* samples differed in 30 of 274 bases that resulted in eight amino acid substitutions. This result indicates that the amplified fragment may be derived from the P6 gene homolog of *H. parainfluenzae*. To distinguish the amplified DNA of *H. influenzae* from that of *H. parainfluenzae*, we designed two oligonucleotide probes, HI and HP, to exploit the sequence differences. Southern blotting analysis showed that probe HI hybridized only with the PCR-positive sample of *H. influenzae* and did not react with any other products isolated from nasopharyngeal secretions and MEEs (Fig. 2A). These results indicate that probe HI can be used to detect the P6 gene DNA of *H. influenzae* specifically. Next, we tested the specificity of dot blot hybridization. Probe HI hybridized with the PCR-amplified sample of *H. influenzae* (Fig. 2B), while probe HP, designed to detect the P6 gene of *H. parainfluenzae*, reacted only with the control sample of *H. parainfluenzae* (Ta-

TABLE 1. Results of PCR DNA amplification and hybridization of *H. influenzae* and other bacterial species

Bacterial species	No. of samples	No. of samples identified by:		
		PCR	Hybridization	
		Primer set (+)	HI (+)	HP (+)
H. influenzae	28	28	28	0
H. parainfluenzae	4	4	0	4
Type α *Streptococcus* sp.	4	0	0	0
Type β *Streptococcus* sp.	4	0	0	0
S. aureus	4	0	0	0
Corynebacterium sp.	4	0	0	0
S. pneumoniae	4	0	0	0
M. catarrhalis	4	0	0	0
Coagulase-negative *Staphylococcus* sp.	4	0	0	0
Total	60	32	28	4

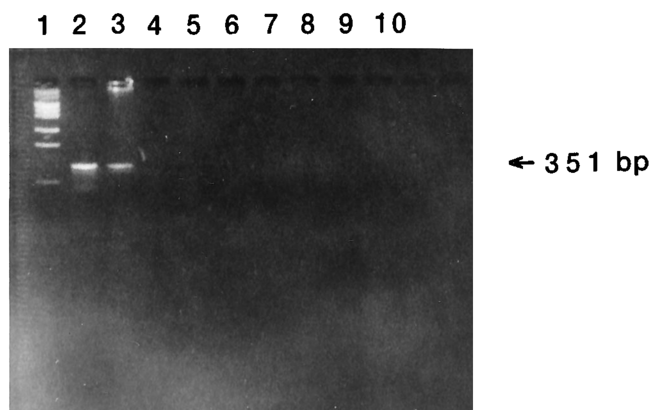

FIG. 1. Specificity of oligonucleotides shown by agarose gel (3%) electrophoresis of PCR-amplified DNA from various bacterial isolates. Lanes: 1, pHY molecular size markers (4,870, 2,016, 1,360, 1,107, 926, 658, 489, 267, and 80 bp); 2, *H. influenzae*; 3, *H. parainfluenzae*; 4, type α *Streptococcus* sp.; 5, type β *Streptococcus* sp.; 6, *Staphylococcus aureus*; 7, *Corynebacterium* sp.; 8, *S. pneumoniae*; 9, *M. catarrhalis*; 10, coagulase-negative *Staphylococcus* sp. The amplified PCR product is indicated by the arrow.

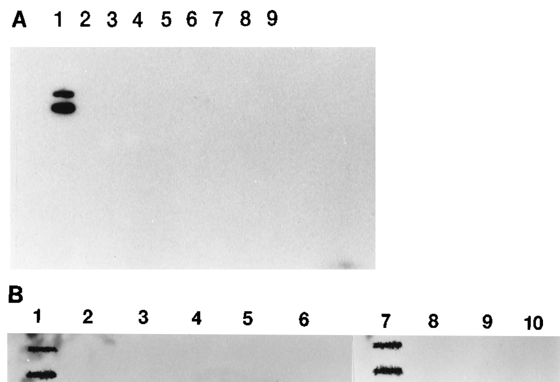

FIG. 2. Hybridization analysis of amplified DNA of *H. influenzae* and *H. parainfluenzae* with probe HI. (A) Southern blot analysis to examine the specificity of probe HI. Lanes 1 to 9 correspond to lanes 2 to 10 of Fig. 1. The lower major band was assigned a length of 351 bp corresponding to the P6 gene DNA. The upper minor band may be attributed to single-stranded DNA of the P6 gene generated by heat denaturation during PCR that migrated more slowly than double-stranded DNA under nondenaturing conditions. (B) Dot blot analysis to test the specificity of probe HI. Lanes: 1, *H. influenzae*; 2, *H. parainfluenzae*; 3, type α *Streptococcus* sp.; 4, type β *Streptococcus* sp.; 5, *S. aureus*; 6, *Corynebacterium* sp.; 7, *H. influenzae*; 8, *S. pneumoniae*; 9, *M. catarrhalis*; 10, coagulase-negative *Staphylococcus* sp.

ble 1). Thus, the dot blot hybridization of PCR-amplified samples (PCR-DB) with probe HI was shown to be a useful method to detect the P6 gene target of *H. influenzae* in a large number of clinical samples. To examine the sensitivity of dot blot hybridization, we serially diluted PCR samples of DNA prepared from *H. influenzae*. The smallest amount of DNA that generated a signal in the dot blot experiment was 10^{-2} ng (Fig. 3). This corresponds to approximately 3,000 *H. influenzae* cells.

Examination of nasopharyngeal secretions and MEEs by PCR-DB and culture method. Two hundred thirteen nasopharyngeal secretion samples and 80 MEE samples were subjected to both conventional culture and PCR-DB. The list of isolates cultured from those samples is shown in Table 2. In nasopharyngeal secretions, *H. influenzae* was detected by culture in 30% (64 of 213) of the samples, as shown in Table 3. In contrast, the P6 gene DNA was detected by PCR-DB in 64% (139 of 213) of the samples. The positive rate was significantly greater by PCR-DB than by the culture method ($\alpha < 0.001$). All samples in which *H. influenzae* was detected by culture were also positive by the PCR-DB, and all PCR-DB-negative samples did not contain any culturable *H. influenzae*. The positive rates were significantly higher for patients with OME than for control subjects by both PCR-DB and culture methods ($P < 0.005$ and $P < 0.005$, respectively). In MEE samples, the positive rate by PCR-DB was significantly higher than that by the culture method, as shown in Table 4 ($\alpha < 0.001$). All

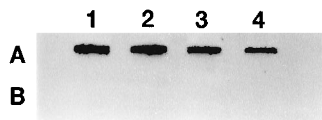

FIG. 3. Sensitivity of dot blot analysis. PCR was performed on serially diluted DNA isolated from *H. influenzae* under the conditions described in Materials and Methods. Blots A1 to A4 contained 10, 1, 10^{-1}, and 10^{-2} ng of DNA, respectively. Blots B1 to B4 contained 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} ng of DNA, respectively.

TABLE 2. Bacterial species cultured from 213 nasopharyngeal secretions and 80 MEEs

Bacterial species	No. of isolates cultured from:		
	Nasopharyngeal secretions		MEEs
	OME+	OME−	
H. influenzae	43	21	9
H. parainfluenzae	2	2	0
S. aureus	26	14	3
Corynebacterium sp.	20	15	1
S. pneumoniae	31	21	2
Coagulase-negative *Staphylococcus* sp.	24	28	25
Neisseria sp.	4	12	0
Others	20	18	0
Total	186	145	42

culture-positive MEE samples were PCR-DB positive. There was no false-negative sample detected by PCR-DB.

Concordance between P6 DNA targets in nasopharyngeal secretions and MEEs. Eighty pairs of nasopharyngeal secretion samples and MEE samples were applied to study the concordance between the presence of the P6 DNA target in nasopharyngeal secretions and that in MEEs (Table 5). In all 46 patients who had P6 gene DNA in MEEs, P6 gene DNA was also detected in nasopharyngeal secretions, whereas significantly fewer ($P < 0.05$) patients had P6 gene DNA only in nasopharyngeal secretions.

DISCUSSION

van Ketel et al. (16) succeeded in detecting the P6 gene DNA of *H. influenzae* in cerebrospinal fluids of patients with meningitis by PCR. They suggested that PCR is a highly sensitive method for the diagnosis of infection caused by *H. influenzae*. However, P6 gene DNAs not only from *H. influenzae* but also from other bacteria of the genus *Haemophilus* were detected. In order to increase the specificity of PCR in detecting *H. influenzae* from nasopharyngeal secretions and MEEs, PCR-DB with a probe that reacts with *H. influenzae* specifically was employed. Since all the bacterial isolates from nasopharyngeal secretions and MEEs other than *H. influenzae* were negative by PCR-DB with the *H. influenzae*-specific probe, the P6 gene DNA detected in the clinical samples was considered

TABLE 3. Incidence of P6 gene DNA and *H. influenzae* in nasopharyngeal secretions as determined by PCR-DB and conventional culture method

Source group	No. of samples	No. (%) of samples detected by:			
		P6 gene DNA PCR		Culture	
		+	−	+	−
Subjects with OME	102	88 (86)[a]	14 (14)	43 (42)[b]	59 (58)
Controls	111	51 (46)[a]	60 (54)	21 (19)[b]	90 (81)
Total	213	139[c]	74	64[c]	149

[a] Detection of 88 of 102 versus 51 of 111 samples ($P < 0.005$, by chi-square test with Yates correction).

[b] Detection of 43 of 102 versus 21 of 111 samples ($P < 0.005$, by chi-square test with Yates correction).

[c] Detection of 139 of 213 versus 64 of 213 samples ($\alpha < 0.001$, by McNemar test).

TABLE 4. Incidence of *H. influenzae* detected by PCR-DB and conventional culture method in 80 MEEs

Culture result	No. (%) of samples identified by culture	No. of samples identified by P6 gene DNA PCR/culture identified	
		+	−
Positive	9 (11)[a]	9/9	0/9
Negative	71 (89)	37/71	34/71
Total	80	46/80[a]	34/80

[a] Detection of 9 of 80 versus 46 of 80 samples ($\alpha < 0.001$, by McNemar test). P6 gene DNA testing identified 58 and 42% of the samples as positive and negative, respectively.

to originate from *H. influenzae*. The sensitivity of PCR was examined by hybridization of serially diluted DNA isolated from *H. influenzae*. PCR performed with samples containing 3,000 copies of the P6 gene DNA showed an intense signal in the hybridization experiment. The results indicate that the sample contained the equivalent of over 60,000 cells of *H. influenzae* when the signal was observed after PCR, since 1/20 of the amount was used for PCR.

According to the previous reports based on the conventional culture method, the positive rates of *H. influenzae* in the nasopharynges of healthy children varied in a low range between 9 and 20% (4). In the present study, the positive rate of P6 gene DNA in nasopharyngeal secretions of healthy subjects was 46%, significantly higher than the positive rate of *H. influenzae* detected by the conventional culture method (19%). Previous reports also showed that the carriage rates of *H. influenzae* in the nasopharynges of otitis-prone children or patients with OME were higher than that of healthy subjects (4). We detected P6 gene DNA from the nasopharynx in 86% of patients with OME. These findings indicate that PCR is more sensitive than the conventional culture method in detecting *H. influenzae* and that the proliferation of *H. influenzae* in the nasopharynx is closely related to the pathogenesis of OME.

Although the previous reports have shown the presence of *H. influenzae* in MEEs, the positive rate was not high enough (from 6 to 20%) to conclude that *H. influenzae* is one of the chief pathogens of OME (5, 7). In the present study, *H. influenzae* was cultured from 9 (11%) of 80 MEEs. Coagulase-negative *Staphylococcus* sp., which was most frequently detected in MEEs, was considered contamination from the external ear canal, as previously reported (7). It can be speculated that the low incidence of pathogens in MEEs is due to inactivation of bacteria by the administration of antibiotics or by immunoglobulins. On the other hand, PCR can detect those inactivated bacteria. The present study showed that 58% of MEEs contained P6 gene DNA of *H. influenzae*. Moreover, the positive rate in nasopharyngeal secretions exceeded 90% in the group of children with OME aged 1 to 10 years, who are known to be most susceptible to this disease (data not shown). Previously, we reported that the same strain of *H. influenzae* was frequently isolated from MEEs and from the nasopharynx simultaneously (7). The present study showed that more than half of the patients with P6 gene DNA in the nasopharynx had the DNA in paired MEEs. All patients with the P6 gene DNA target in MEE had the DNA target in nasopharyngeal secretions. These results suggest that OME may be caused by bacterial invasion from the nasopharynx through the eustachian tube, and that *H. influenzae* may be one of the major pathogens associated with this disease.

TABLE 5. Incidence of P6 gene DNA in nasopharyngeal secretions and MEEs determined by PCR

PCR result for MEEs	No. of samples	No. (%) of nasopharyngeal secretion samples with P6 gene DNA	
		+	−
Positive	46	46 (100)[a]	0 (0)
Negative	34	26 (76)[a]	8 (24)
Total	80	72	8

[a] Detection of 46 of 46 versus 26 of 34 samples ($P < 0.05$, by chi-square test with Yates correction).

In summary, PCR was more sensitive than the conventional culture method in detecting *H. influenzae* from the nasopharynx and from MEEs of patients with OME. The specificity was confirmed by PCR-DB. The results suggest that the presence of *H. influenzae* in the nasopharynx and in MEEs is more dominant than previously reported and may play an important role in the pathogenesis of OME.

ACKNOWLEDGMENT

This work was supported in part by Grant-in-Aid for General Scientific Research (C) 06671724 from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Deich, R. A., B. J. Metcalf, C. W. Finn, J. E. Farley, and B. A. Green.** 1988. Cloning of genes encoding a 15,000-dalton protein from *Haemophilus influenzae*. J. Bacteriol. **170:**489–498.
2. **DeMaria, T. F., R. B. Prior, B. R. Briggs, and D. J. Lim.** 1983. Endotoxin in middle ear effusions from patients with chronic otitis media with effusion, p. 123–125. *In* D. J. Lim (ed.), Recent advances in otitis media with effusion. BC Decker Inc., Burlington, Ontario, Canada.
3. **DeMaria, T. F., T. Yamaguchi, and D. J. Lim.** 1989. Quantitative cytologic and histologic changes in the middle ear after the injection of nontypable *Haemophilus influenzae* endotoxin. Am. J. Otolaryngol. **10:**261–266.
4. **Freijd, A., S. Bygdeman, and B. Rynnel-Dagöö.** 1984. The nasopharyngeal microflora of otitis-prone children, with emphasis on *H. influenzae*. Acta Otolaryngol. **97:**117–126.
5. **Giebink, G. S., S. K. Juhn, M. L. Weber, and C. T. Le.** 1982. The bacteriology and cytology of chronic otitis media with effusion. Pediatr. Infect. Dis. J. **1:**98–103.
6. **Hotomi, M., T. Tabata, H. Kakiuchi, M. Kunimoto.** 1993. Detection of *Haemophilus influenzae* in middle ear of otitis media with effusion by polymerase chain reaction. Int. J. Pediatr. Otorhinolaryngol. **27:**119–126.
7. **Kurono, Y., K. Tomonaga, and G. Mogi.** 1988. *Staphylococcus epidermidis* and *Staphylococcus aureus* in otitis media with effusion. Arch. Otolaryngol. Head Neck Surg. **114:**1262–1265.
8. **Lim, D. J., T. F. DeMaria, and L. O. Bakaletz.** 1988. Current concepts of pathogenesis of otitis media: a review. Acta Oto-Laryngol. **458:**174–180.
9. **Murphy, T. F., L. C. Bartos, A. A. Campagnari, M. B. Nelson, and M. A. Apicella.** 1986. Antigenic characterization of the P6 protein of nontypable *Haemophilus influenzae*. Infect. Immun. **54:**774–779.
10. **Murphy, T. F., M. B. Nelson, and M. A. Apicella.** 1992. The P6 outer membrane protein of nontypeable *Haemophilus influenzae* as a vaccine antigen. J. Infect. Dis. **165**(Suppl. 1)**:**203–205.
11. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
12. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **76:**5463–5467.
14. **Senturia, B. H., C. F. Gessert, C. D. Carr, and E. S. Baumann.** 1958. Studies concerned with tubotympanitis. Ann. Otol. Rhinol. Laryngol. **67:**440–467.
15. **Teele, D. W., J. O. Klein, and B. A. Rosner.** 1980. Epidemiology of otitis media in children. Ann. Otol. Rhinol. Laryngol. **89**(Suppl. 68)**:**5–6.
16. **van Ketel, R. J., B. De Wever, and L. Van Alphen.** 1990. Detection of *Haemophilus influenzae* in cerebrospinal fluids by polymerase chain reaction DNA amplification. J. Med. Microbiol. **33:**271–276.