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Identification of *Streptococcus pneumoniae* and *Haemophilus influenzae* in culture-negative middle ear fluids from children with acute otitis media by combination of multiplex PCR and multi-locus sequencing typing

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Abstract

Objective—*Streptococcus pneumoniae* (*Spn*) and *Haemophilus influenzae* (*Hflu*) are major etiologic pathogens for acute otitis media (AOM). However, when *Spn* and *Hflu* strains are not identified by traditional culture methods, use of alternative PCR-based diagnosis becomes critical. This study aimed to develop a combined molecular method to accurately detect these otopathogens.

Methods—Middle ear fluid (MEF) samples were collected by tympanocentesis from children with AOM to isolate *Spn* and *Hflu* by standard culture procedures. Multiplex PCR (mPCR) and multi-locus sequence typing (MLST) techniques were used to detect *Spn* and *Hflu* in culture-negative MEF samples.

Results—We found 20 *Spn* or *Hflu* culture-positive MEF samples that were mPCR-positive and typeable by MLST. The sequences of the housekeeping genes and the MLST allelic profiles obtained from *Spn* or *Hflu* culture isolates matched exactly MEF samples that were tested directly without culture isolation. Of 63 MEF samples that were culture-negative for *Spn*, 38% (24/63) were mPCR-positive for *Spn*. Of 50 MEF samples that were culture-negative for *Hflu*, 24% (12/50) were mPCR-positive for *Hflu*. Among these culture-negative but mPCR-positive MEF samples, 25% (6/24) and 25% (3/12) were typeable by MLST for *Spn* and *Hflu*, respectively.

Conclusions—MEF samples may be analyzed with mPCR and MLST directly without culture isolation and the addition of mPCR and MLST may accurately identify *Spn* and *Hflu* in MEF of children with AOM when bacterial culture is negative.

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Conflict of Interest Statement:

The authors have no conflict of interest to declare.

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1. Introduction

Acute otitis media (AOM) accounts for 33% of all visits to pediatricians and 40% of all antibiotic use in young children. By three years of age, most children have experienced at least one episode of AOM, and approximately 15% to 20% of children suffer from two or more incidents of AOM [1; 2]. *Streptococcus pneumoniae* (*Spn*) and *Haemophilus influenzae* (*Hflu*) are two of the major bacterial pathogens that cause AOM in young children [1]. Our laboratories are studying countermeasures against *Spn* and *Hflu* infection that might be used to reduce or prevent AOM. Accurate identification of *Spn* and *Hflu* in middle ear fluid therefore becomes critical before an effective intervention can be evaluated.

Middle ear fluid (MEF) is the sample recommended for standard bacterial culture for etiologic diagnosis of AOM [1; 3]. For a variety of reasons, however, only approximately 60% of MEF samples from children with AOM are culture-positive for the major bacterial pathogens [3][25]. It is known that host defense mechanisms and antibiotic treatment prior to collection of MEF specimens may effect pathogen survival and detection [1; 2; 3; 4]. In addition, some bacterial strains, for example optochin-resistant, bile-insoluble, and non-encapsulated *Spn* strains, cannot be identified by conventional culture methods [5]. Biofilm mode of growth on the middle ear mucosa may be another possible reason for negative cultures of organisms from MEF [26,27]. During the past decade, polymerase chain reaction (PCR)-based assay methods, including conventional PCR, reverse transcript PCR (RT-PCR), real time PCR, multiplex PCR (mPCR), and others, have been employed to assist etiologic diagnosis of AOM by detecting DNA or RNA of suspected organisms. Although these PCR-based methods have been shown to significantly improve the sensitivity of bacterial detection in AOM [6; 7; 8; 9], false positive results by these methods may occasionally happen. Therefore, the results from PCR-based analysis should be reconfirmed by a secondary method which will provide further detailed information about pathogens before a diagnostic conclusion can be made. Multi-locus sequence typing (MLST) can provide detailed information regarding particular molecular types of the pathogens. The aim of the present study was to improve laboratory methods to accurately detect and identify *Spn* and *Hflu* in culture-negative MEF samples from children with AOM using multiplex PCR, followed by MLST to determine molecular types directly from MEF samples.

2. Materials and methods

2.1. Patient population, clinical specimens, and bacterial isolation

The children at age of 6 to 36 months enrolled in this study were recruited from a private pediatric practice, Legacy Pediatrics, in a suburban area of Rochester, New York, from 2003 to 2007. All of the children received standard vaccinations including the 7-Valent Pneumococcal Conjugate Vaccine (Prevnar, Wyeth Pharmaceuticals, Collegeville, PA) at the age appropriate times. Diagnosis of AOM was made by criteria endorsed by the American Academy of Pediatrics (AAP) as previously described [10]. The study was approved by the Institutional Review Board (IRB) and written informed consent was obtained from parents or guardians for the study and for all tympanocentesis procedures. MEF was collected by tympanocentesis as previously described [28]. MEF samples varied in quantity of material obtained from 50-250 μ l and entire sample was added to one ml of PBS (pH 7.4). The bacterial isolation and identification from MEF were performed according to instructions described in the 8th edition of Manual of Clinic Microbiology [29]. MEF samples were stored at -80°C for subsequent PCR and MLST analyses after using for bacterial isolation. Serotypes of pneumococci were determined by latex agglutination (Pneumotest-Latex, Statens Serum Institute, Copenhagen, Denmark) according to the manufacturer's instructions and confirmed by Quellung reaction.

2.2. Multiplex PCR (mPCR)

Bacterial genomic DNA was extracted from pure cultures of *Spn* or *Hflu*, and / or MEF samples from children with AOM as described previously [18]. An mPCR procedure was used for the simultaneous detection of *Spn* and *Hflu* using primers as previously described and shown in Table 1 [6]. The 16S rRNA gene of bacteria was chosen as the marker gene. The procedure used one common lower primer and two species-specific upper primers to generate PCR products of different sizes (484 bps for *Spn*, and 525 bps for *Hflu*). For each reaction, 2×PCR Master Mix (Promega) containing 0.05 unit/μl *Taq* DNA polymerase in reaction buffer with 4 mM MgCl₂ and 0.4 mM of each dNTP was used in a 50-μl reaction volume. The reaction profile was 3 min of initial denaturation and 38 cycles of 94°C for 30 s, 62°C for 45 s, and 72°C for 1 min, followed by a 5-min final extension at 72°C. The amplification products were separated in a 3% agarose gel containing ethidium bromide for 1.5-2.0 h and were visualized by UV light illumination.

2.3. Multi-locus sequence typing (MLST)

The multiplex PCR positive samples were further used to determine molecular types of *Spn* and *Hflu* by MLST as described in the MLST database (<http://Spneumoniae.mlst.net> & <http://haemophilus.mlst.net>) and previous publications [11; 12; 13]. Briefly, the internal fragments of seven housekeeping genes of *Spn* and *Hflu* were amplified by PCR, using a PCR Master Mix (Promega) and primers shown in Table 1. PCR conditions were as follows: initial denaturation at 95°C for 4 min, followed by 30 cycles of 95°C for 30 seconds, 50-55°C annealing for 30 seconds and 72°C extension for 30 seconds. Sizes of PCR products were checked by running 1.5% agarose gel electrophoresis stained with ethidium bromide. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and identified by DNA sequencing. Sequencing reactions were performed on an Applied Biosystems Prism 377 automated sequencer using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the same primers used for PCR in Table 1. The sequences of each gene were submitted to the international databases for the respective bacteria and compared with the sequences of all known alleles of corresponding genes in the *Spn* MLST database (<http://Spneumoniae.mlst.net>) and the *Hflu* MLST database (<http://haemophilus.mlst.net>) [11; 12; 13]. Sequences of genes amplified from MEF samples were also used for a blast analysis in the GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to reconfirm that the sequences of PCR products matched with the genes of *Spn* or *Hflu*.

When all of the seven house-keeping genes were able to be amplified, the sequences at each of loci were then compared with the sequences of all of the known alleles at those loci in the database at the *Spn* MLST database (<http://Spneumoniae.mlst.net>) and *Hflu* MLST database (<http://haemophilus.mlst.net>). The allele at each of the seven loci defines the allelic profile of each sample as well as their sequence type (ST). A MLST number represents an allelic profile or sequence type. When appropriate, new allelic numbers and new MLST sequence types were assigned by a curator of the international database. The identical sequences to a known sequence were assigned the same allele number, whereas non-identity to any known allele sequence was sent to the database manager and subsequently assigned new allele numbers.

3. Results

3.1. Confirmation of *Spn* and *Hflu* by mPCR and MLST in culture-positive MEF from children with AOM

In order to test the sensitivity and efficiency of mPCR and MLST, 10 MEF samples that were culture-positive respectively for *Spn* or *Hflu* were randomly selected to detect *Spn* or *Hflu* by mPCR, and then to determine the sequence types by MLST directly from the MEF

without an intermediate culture step. The sequence type data obtained from the MEF samples and their corresponding bacterial culture isolates are shown in Tables 2, 3, and 4. The *Spn* culture-positive MEF samples were all mPCR-positive for *Spn* (Table 2), when the MEF fluid was tested directly and all the *Spn* could be typed by MLST (Table 3). The sequences of each gene and their allelic profiles (MLST types) obtained from *Spn* culture isolates and directly from MEF samples were exactly the same. Interestingly, when MLST data obtained directly from MEF were used to predict the serotypes according to the allelic profiles (MLST) and archives of the database, the predicted serotypes of *Spn* matched very well with the results from the Quellung reaction assay (Table 3).

Similarly, the MEF samples that were culture-positive for *Hflu* were all mPCR-positive for *Hflu*, and were typeable by MLST. The sequences of PCR products amplified from MEF samples were all the same as those from *Hflu* culture isolates. The allele profile and MLST types obtained from *Hflu* culture isolates and MEF samples were all the same. Based on serotype information of strains that had the same MLST in the database, we predicted that the serotype of *Hflu* in the eight samples were all nontypeable (NT), while another two samples could not be predicted due to lack of existing information in the database (Table 4). We did not analyze the serotype of *Hflu* isolates since we anticipated that all were non-typeable.

3.2. Identification of *Spn* by mPCR and MLST in culture-negative MEF from children with AOM

63 MEF samples that were culture-negative for *Spn* were randomly chosen to detect *Spn* by mPCR, followed by MLST to determine the corresponding MLSTs when mPCR was positive. Of these 63 MEF samples, 38% (24/63) were mPCR-positive for *Spn*, while 62% (39/63) mPCR-negative (Table 2). Among these culture-negative but mPCR-positive samples, we found 25% (6/24) of the MEF samples could be used to type the *Spn* with MLST (Table 2 and 5). All of the allelic profiles (MLST types) obtained for the six MEF samples that were culture-negative but mPCR-positive for *Spn* matched one or more strains in the MLST database. Possible serotypes of the *Spn* in MEF were predicted according to existing data in the MLST database (Table 5). Using the MLST database information we could predict the serotypes of detected *Spn* characterized directly from culture-negative MEF (Table 3 and 5). For example, the allelic profiles from MEF sample S64 matched 27 isolates in the MLST database and all 27 were of serotypes 19, 19A or 19F. Therefore, we could predict that the *Spn* in MEF S64 would be a serotype 19, 19A or 19F (Table 5). Similarly, we could predict serotypes of *Spn* from other culture negative MEF samples (Table 5).

Our results improve characterization of *Spn* in culture-negative MEF, though they are not fully optimal. 62% of MEF samples (39/63) that were culture-negative but mPCR-positive for *Spn* could not be typed by MLST, as all seven genes could not be amplified (Table 2). Although some of the genes were amplified and corresponding allele numbers were assigned from the database, so we can be sure the organisms were *Spn*, because all seven genes could not be amplified the allelic profiles, namely MLST could not be assigned. The proportion of genes which were not successfully amplified was randomly (data not shown).

3.3. Detection of *Hflu* by mPCR and MLST in culture-negative MEF from children with AOM

Of 50 MEF samples that were culture-negative for *Hflu*, 24% (12/50) were mPCR-positive for *Hflu*, while 76% (38/50) were mPCR-negative (Table 2). Among the 12 samples that were culture-negative but mPCR-positive, 25% (3/12) were typeable by MLST (Table 2 and 6). All of the three allelic profiles (MLST types) obtained from the MEF samples that were culture-negative but mPCR-positive for *Hflu* matched one or more strains in the MLST

database. As occurred with *Spn*, a high proportion of the MEF samples (8/12) that were culture-negative but mPCR-positive for *Hfla* were non-typeable by MLST because all seven genes could not be amplified (Table 2). The proportion of genes which were successfully amplified was randomly distributed (data not shown).

We also observed that some of the culture negative samples have both *Hfla* and *Spn* detected by mPCR. Out of 79 culture negative samples, 12.5% samples were mPCR positive for both *Hfla* and *Spn*.

4. Discussion

In this study, we have shown that mPCR and MLST results directly from MEF samples matched perfectly (100%) with that of bacterial culture method when MEF samples were culture positive (Table 2). This indicated that the cultured bacterial strains are representative of the *in vivo* strains when the MEF is culture positive. More importantly, this is the first study, as far as we are aware, to use mPCR and MLST together to detect *Spn* and *Hfla* in culture-negative MEF samples from children with AOM. Our results demonstrated that the combination of mPCR and MLST assays improves accurate detection of *Spn* and *Hfla* in MEF in children with AOM when bacterial culture results are negative. Furthermore, the addition of these molecular detection techniques allows identification of these important otopathogens and in about 25% of cases, prediction of capsular serotype (for *Spn*) and other relevant virulence features (for both *Spn* and *Hfla*).

There has been some debate about the use of PCR based assay for molecular detection of otopathogens, because nucleic acid can be amplified from both viable and nonviable bacteria by PCR [14; 15]. Studies in a chinchilla animal model of AOM found that bacterial DNA in MEF samples disintegrates within two days of bacterial cell death [16], thereby suggesting that it originates from bacteria involved in active disease and PCR detection is reliable as a detection methodology to identify causative otopathogens. In children, Virolainen et al (1994) reported that 80% of *Spn* culture-negative MEF samples were PCR-positive for *Spn* and they suggested that PCR was diagnostic [8]. Rayner et al (1998) developed a RT-PCR based assay system to detect the presence of bacterial *Hfla* mRNA in middle ear effusions of children with chronic OME, thereby establishing the presence of viable, metabolically active, intact organisms in culture-negative OME when biofilms were suspected [17]. Our group pursued this question specifically among children with AOM and recently published our results demonstrating that detection of *Spn*, *Hfla* and *Mcat* in MEF by mPCR clears in 1 to 3 weeks from the MEF as proven by a second follow-up tympanocentesis [18].

MLST was developed to molecularly type organisms based on the sequence data of five to seven housekeeping genes [19]. In 1998, Maiden et al first proposed MLST as a nucleotide sequence-based portable method to characterize the human bacterial pathogen *Neisseria meningitidis* (*Nmg*) [20]. In the same year, Enright et al developed MLST for pneumococci and established a web-accessible database that allows the identification of pneumococci clones and clonal complexes over the internet [12]. Platonov et al (2003) and Meats et al (2003) developed MLST for *Hfla* using 5 loci and 7 loci, respectively [13; 21]. During the past decade, MLST has become a widely accepted approach to the characterization of microorganisms. At this time, there are at least 51 MLST schemes established, covering a wide range of bacteria with many of the most important pathogens; most of them have web-accessible databases (<http://ukmirror2.pubmlst.org/databases.shtml>) [19].

Previous studies have demonstrated that MLST can be used for molecular typing of microorganisms directly from clinical samples of cerebrospinal fluid (CSF), blood, and throat swabs from which bacteria could not be cultured [14; 22; 23; 24]. For example, Enright et al. found that 12 of 12 *Spn* culture-positive CSF samples, and 2 of 16 *Spn* culture-

negative samples from patients with meningitis (that were culture-positive in blood) could be identified and typed with MLST, and the penicillin susceptibility and serotype could be predicted [22]. Diggle et al. (2003) and Kriz et al. (2002) also reported that MLST-based methods could be used for molecular typing of meningococci directly from CSF [23; 24]. In our study, MLST was used to molecularly type otopathogens when the culture was negative but mPCR was positive. We found that MEF samples could be analyzed without an intermediate step of bacterial culture and in about one-quarter of cases when MEF cultures were negative but mPCR was positive MLST could be used to gather important information about the likely *Spn* serotype and antibiotic resistance pattern and the likely beta lactamase production among *Hflu*. Only 25% of mPCR-positive specimen in culture-negative samples was MLST typeable in this study due to all the genes could not to be amplified in *Spn* and *Hflu*. The proportion of genes that were not successfully amplified was random. We therefore suspect that the detection of *Spn* and *Hflu* in MEF by mPCR in culture negative children and then the failure to obtain the sequences of all seven housekeeping genes of the organisms among our studied children was due to partial degradation of bacterial DNA before the MEF sample was obtained. In addition, compared with previous results which directly determine MLST from other clinical samples, our MLST typeable rate are much lower. The reason might be that the PCR of previous study was mostly nested-PCR which is much more sensitive than conventional PCR [22].

When compared with bacterial culture methods and conventional PCR-based assays, MLST is time-consuming and costly. It is not practical to apply MLST to all culture-negative MEF samples in standard laboratory practice. However, we found that if we first used mPCR to detect whether there were specific DNA of *Spn* or *Hflu* in MEF samples, and undertook MLST for further bacterial characterization, then we could minimize sample numbers to be analyzed by MLST.

In conclusion, the combination of mPCR and MLST can be used to ensure accurate etiologic diagnosis of AOM by identifying otopathogens *Spn* and *Hflu* when bacterial culture are negative in MEF. They can be used to detect the presence of otopathogens *Spn* and *Hflu*, and in some cases to molecularly type the organisms to predict serotype and antibiotic susceptibility.

In conclusion, the combination of mPCR and MLST can be used to ensure accurate etiologic diagnosis of AOM by identifying otopathogens *Spn* and *Hflu* when bacterial cultures are negative in MEF. Although it is not suggested to bypass bacterial culture for only performing molecular diagnostic studies, the methods can be used to detect the presence of otopathogens *Spn* and *Hflu*, and in some cases to molecularly type the organisms to predict serotype and antibiotic susceptibility.

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Table 1PCR primers for *S. pneumoniae* and *H. influenzae*

Genes	Typing Size	Forwards primers (5' to 3')	Reverse primers (5' to 3')
<i>S. pneumoniae</i> MLST			
<i>aroE</i>	405 bps	GCCTTTGAGGCGACAGC	GCAGTTCA(G/A)AAACAT(A/T)TTCTAA
<i>gdh</i>	460 bps	ATGGACAAACCAGC(G/A/T/C)AG(C/T)TT	GCTTGAGGTCCCAT(G/A)CT(G/A/T/C)CC
<i>gki</i>	483 bps	GGCATTGGAATGGGATCACC	TCTCCCGCAGCTGACAC
<i>recP</i>	450 bps	GCCAACTCAGGTCATCCAGG	TGCAACCGTAGCATTGTAAC
<i>spi</i>	474 bps	TTATTCCTCCTGATTCTGTC	GTGATTGGCCAGAAGCGG AA
<i>xpt</i>	486 bps	TTATTAGAAGAGCGCATCCT	AGATCTGCCTCCTTAAATAC
<i>ddl</i>	441 bps	TGC(C/T)CAAGTTCCTTATGTGG	CACTGGGT(G/A)AAACC(A/T)GGCAT
<i>H. influenzae</i> MLST			
<i>ack</i>	477 bps	GGTGACCGGGTGCAGGTAA	CCTAAGATTTTATCTAACTC
<i>atp</i>	447 bps	ATGGCAGGTGCAAAAGAGAT	TTGTACAACAGGCTTTTGCG
<i>frdB</i>	489 bps	CTTATCGTTGGTCTTGCCGT	TTGGCACTTCCACTTTTCC
<i>fucK</i>	345 bps	ACCACTTTCGGCGTGATGG	AAGATTTCACAGGTGCCAGA
<i>mdh</i>	405 bps	TCATTGTATGATATTGCCCC	ACTTCTGTACCTGCATTTTG
<i>pgi</i>	468 bps	GGTGAAAAAATCAATCGTAC	ATTGAAAGACCAATAGCTGA
<i>recA</i>	426 bps	ATGGCAACTCAAGAAGAAAA	TTACCAAACATCACGCCTAT
Multiplex PCR			
<i>Spn 16S</i>	484	AAGGTGCACTTGCATCACTACC	CTACGCATTTCACCGCTACAC
<i>Hflu 16S</i>	525	CGTATTATCGGAAGATGA AAGTGC	

Table 2
Detection and identification of *S. pneumoniae* and *H. influenzae* in MEF samples from children with AOM

ME samples	N	mPCR		MLST (mPCR positive)	
		Positive	Negative	Typeable	Nontypeable
For <i>Spn</i>					
culture-positive	10	100% (10/10)	100% (0/10)	100% (10/10)	100% (0/10)
culture-negative	63	38% (24/63)	62% (39/63)	25% (6/24)	75% (18/24)
For <i>Hflu</i>					
culture-positive	10	100% (10/10)	100% (0/10)	100% (10/10)	100% (0/10)
culture-negative	50	24% (12/50)	76% (38/50)	25% (4/12)	75% (8/12)

Table 3
Comparison of MLST obtained from *S. pneumoniae* isolates of culture and directly from culture-positive MEF

Sample	Serotype	Allelic profile							MLST	mPCR	
		<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>			
0601003V1R	isolate	19A ^{**}	8	13	64	4	17	4	14	1673	N/A
	MEF	19A [*]	8	13	64	4	17	4	14	1673	+
0601007V1R	isolate	19A	7	2	1	1	9	1	14	2265	N/A
	MEF	19A [*]	7	2	1	1	9	1	14	2265	+
0601003V1L	isolate	19A ^{**}	8	13	64	4	17	4	14	1673	N/A
	MEF	19A [*]	8	13	64	4	17	4	14	1673	+
0601004V1R	isolate	19A	4	16	19	15	6	20	1	320	N/A
	MEF	19/19A/19F [*]	4	16	19	15	6	20	1	320	+
0601027V1L	isolate	6A ^{**}	1	5	9	12	94	28	20	1379	N/A
	MEF	6A [*]	1	5	9	12	94	28	20	1379	+
0601032V1L	isolate	19A ^{**}	8	13	14	4	17	4	14	199	N/A
	MEF	19/19A/19F/15/15B/15C/6 [*]	8	13	14	4	17	4	14	199	+
0601004V1L	isolate	19A ^{**}	4	16	19	15	6	20	1	320	N/A
	MEF	19/19A/19F [*]	4	16	19	15	6	20	1	320	+
0701063V1L	isolate	19A	7	1	10	1	6	8	1	156	N/A
	MEF	9A/9V/11/13/14/19F/19A	7	1	10	1	6	8	1	156	+
0602004SV1R	isolate	23A ^{**}	7	13	8	6	1	6	8	338	N/A
	MEF	23/23A/23F [*]	7	13	8	6	1	6	8	338	+
0602024SV1R	isolate	19A ^{**}	8	13	2	4	17	4	14	2816	N/A
	MEF	19A [*]	8	13	2	4	17	4	14	2816	+

* Predicted serotypes, which were the serotypes of the strains in the *Spn* MLST database with same ST;

** Determined serotypes of the isolates.

Note:

Table 4

Comparison of MLST obtained from *H. influenzae* isolates of culture and directly from culture-positive MEF

Samples	Serotype	Allelic profile							MLST	mPCR	
		<i>adh</i>	<i>atpG</i>	<i>frdB</i>	<i>fucK</i>	<i>mdh</i>	<i>pgi</i>	<i>recA</i>			
0601004SV1R	isolate	NA	5	33	7	15	47	51	29	176	N/A
	MEF	No record	5	33	7	15	47	51	29	176	+
0601020V1L	isolate	NA	14	7	13	30	1	21	1	411	N/A
	MEF	NT*	14	7	13	30	1	21	1	411	+
0601011V1L	isolate	NA	14	7	13	7	17	13	17	57	N/A
	MEF	NT*	14	7	13	7	17	13	17	57	+
0601002V1R	isolate	NA	11	2	15	8	71	61	3	257	N/A
	MEF	No record	11	2	15	8	71	61	3	257	+
0601006V1 L	isolate	NA	93	41	41	8	69	48	29	566	N/A
	MEF	NT*	93	41	41	8	69	48	29	566	+
0601020V1R	isolate	NA	14	7	13	30	1	21	1	411	N/A
	MEF	NT*	14	7	13	30	1	21	1	411	+
0601022V1L	isolate	NA	14	8	18	11	17	2	3	196	N/A
	MEF	NT*	14	8	18	11	17	2	3	196	+
0601013V1L	isolate	NA	10	2	15	8	26	61	3	396	N/A
	MEF	NT*	10	2	15	8	26	61	3	396	+
00601025V1R	isolate	NA	1	8	1	14	22	14	13	145	N/A
	MEF	NT*	1	8	1	14	22	14	13	145	+
00601031V1R	isolate	NA	44	2	16	37	17	2	3	165	N/A
	MEF	NT*	44	2	16	37	17	2	3	165	+

Note:

* Predicted serotypes, which were the serotypes of the strains in the *Hflr* MLST database with same ST.

Table 5

Properties of the *S. pneumoniae* characterized directly from culture-negative MEF from children with AOM

Samples	Allelic profile								MLST	Number of matching isolates	Predicted serotype	mPCR
	<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>					
S56	1	5	9	12	94	28	20	1379	7	6A	+	
S51	7	13	8	6	1	1	8	2777	2	6C	+	
S64	4	16	19	15	6	20	1	320	27	19/19A/19F	+	
S78L	7	13	8	6	1	6	8	338	15	23A/F	+	
S63	8	13	14	4	17	4	14	199	100	19/19A/3/6/15B/15C	+	
S66	18	12	4	44	14	77	97	558	21	35/35B/29/NT	+	

Table 6

Properties of the *H. influenzae* characterized directly from culture-negative MEF from children with AOM

Samples	Allelic profile							MLST	Number of matching isolates	mPCR
	<i>adh</i>	<i>atpG</i>	<i>frdB</i>	<i>fucX</i>	<i>mdh</i>	<i>pgi</i>	<i>recA</i>			
S68	7	33	42	8	66	1	29	573	0	+
S103	93	41	41	8	69	48	29	566	1	+
H48	3	43	52	7	78	28	99	new	Not match	+