

Simultaneous Detection of Pathogens in Clinical Samples from Patients with Community-Acquired Pneumonia by Real-Time PCR with Pathogen-Specific Molecular Beacon Probes

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In this study, real-time PCR with pathogen-specific molecular beacons (MB) and primers was evaluated for prediction of community-acquired pneumonia (CAP) causative agents, detecting six main CAP agents, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, *Legionella pneumophila*, and *Streptococcus pyogenes*, simultaneously. The PCR assay was evaluated for fresh clinical specimens from infants and children ($n = 389$) and from adults ($n = 40$). The MB probes and primers are both pathogen specific, namely, the *lytA* gene for *S. pneumoniae*, the *mip* gene for *L. pneumophila*, and 16S rRNA genes for the remaining four organisms. DNA extraction of clinical specimens was performed with a commercially available EXTRAGEN II kit, and amplification was performed with Stratagene Mx3000P. The limit of detection for these pathogens ranged from 2 copies to 18 copies. The whole process from DNA extraction to the analysis was finished in less than 2 h. The obtained sensitivity and specificity of this real-time PCR study relative to those of conventional cultures were as follows: 96.2% and 93.2% for *S. pneumoniae*, 95.8% and 95.4% for *H. influenzae*, 100% and 100% for *S. pyogenes*, and 100% and 95.4% for *M. pneumoniae*, respectively. The sensitivity and specificity for *M. pneumoniae* relative to those of a serologic assay were 90.2% and 97.9%, respectively. In six clinical samples of *C. pneumoniae*, the real-time PCR gave positive predictable values, and in those cases, elevation of the titer value was also observed. In conclusion, we demonstrated that a real-time PCR assay with pathogen-specific MB is useful in identifying CAP causative agents rapidly and in examining the clinical course of empirical chemotherapy in a timely manner, supporting conventional culture methods.

Community-acquired pneumonia (CAP) is still a major threat to individuals, especially children and compromised hosts, such as senior citizens and people with underlying chronic diseases. The main causative pathogens in CAP are *Streptococcus pneumoniae* (30% to 35%), *Haemophilus influenzae* (4% to 20%), and *Mycoplasma pneumoniae* (15% to 20%), and the rates change according to age and the underlying disease (5, 7, 16). Recently, *Chlamydomphila pneumoniae* and *Legionella pneumophila* have frequently been identified as causative pathogens of CAP (9, 30).

In clinical practice, empirical chemotherapy with broad-spectrum antibiotics must be started based on clinical symptoms, radiograph findings, and clinical examinations, such as those for white blood cell (WBC) counts and C-reactive protein (CRP), considering the severity of the symptoms.

On the other hand, the increase of resistant bacteria, including CAP pathogens, is a worldwide health problem (13, 22, 34). One effective measure to prevent new emergence of resistant bacteria is to use only the sufficient quantity of antibiotics to eliminate the target pathogens. For the measure, the rapid and

precise determination of causative pathogens is critical, and a number of studies have been reported on such methods as conventional PCR (2, 24, 25) and multiplex PCR (8, 11, 39). Hybridization assays (14, 17, 18) have been developed to identify *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila* rapidly, a great benefit, since identification by culture techniques requires several days. Recently, real-time PCR (4, 8, 29, 31), iCycler real-time PCR (12, 36, 37, 40), and nucleic acid sequence-based amplification (19, 31) with high sensitivity and specificity have been developed, improving analysis time and cost performance. The performance of one of these, real-time PCR, has been improving recently thanks to the high accuracy and the easy availability of molecular probes of original design on demand and fluorescent oligonucleotides in clinical laboratories. Thus, the introductions of a molecular beacon (MB) probe (15, 31) and a TaqMan probe (2, 8, 9, 33) have allowed more rapid and specific identification of pathogens than does PCR with SYBR green.

In the present study, we report on a simultaneous identification system using a real-time PCR assay with pathogen-specific MB probes and primers for six CAP pathogens, *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, and *Streptococcus pyogenes*. We describe our system design and the results of the clinical specimen assay together with feasibility tests, comparing them with those of conventional cultures.

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TABLE 1. Primers and probes for real-time PCR

Species, primer, and probe	Primer ^a or probe ^b sequence	Amplicon size (bp)
<i>S. pneumoniae</i>		
Sense primer	5'-CAACCGTACAGAATGAAGCGG-3'	319
Reverse primer	5'-TTATTCGTGCAATACTCGTGCG-3'	
Probe	FAM- <u>CGCGATCAGGTCTCAGCATTCCAACCGCCGATCGCG</u> -BHQ1	
<i>H. influenzae</i>		
Sense primer	5'-TTGACATCCTAAGAAGAGCTC-3'	167
Reverse primer	5'-TCTCCTTTGAGTCCCGACCG-3'	
Probe	FAM- <u>CGCGATCCTGACGACAGCCATGCAGCACGATCGCG</u> -BHQ1	
<i>S. pyogenes</i>		
Sense primer	5'-GAGAGACTAACGCATGTTAGTA-3'	317
Reverse primer	5'-TAGTTACCGTCACTTGGTGG-3'	
Probe	FAM- <u>CGCGATCGCGACGATACATAGCCGACCTGGATCGCG</u> -BHQ1	
<i>M. pneumoniae</i>		
Sense primer	5'-GTAATACTTTAGAGGCGAACG-3'	225
Reverse primer	5'-TACTTCTCAGCATAGCTACAC-3'	
Probe	FAM- <u>CGCGATACCAACTAGCTGATATGGCGCAATCGCG</u> -BHQ1	
<i>C. pneumoniae</i>		
Sense primer	5'-TGACAACTGTAGAAATACAGC-3'	248
Reverse primer	5'-CTGTACTAACCAATTGTAGCAC-3'	
Probe	FAM- <u>CGCGATCTCATCTCGCCTTCTCTGATCGCG</u> -BHQ1	
<i>L. pneumophila</i>		
Sense primer	5'-ACCGAACAGCAAATGAAAGA-3'	144
Reverse primer	5'-AACGCCTGGCTTGTITTTGT-3'	
Probe	FAM- <u>CGCGATCAGTACGCTTGGCATCAAATCATCGCG</u> -BHQ1	

^a *S. pneumoniae*, *lytA* gene; *L. pneumophila*, *mip* gene (Hayden et al. [14]); *H. influenzae*, *S. pyogenes*, *M. pneumoniae*, and *C. pneumoniae*, 16S rRNA gene.

^b Stem oligonucleotides are underlined.

MATERIALS AND METHODS

Patients and samples. Clinical samples from pediatric patients were sent to our laboratory from pediatricians belonging to 10 institutions participating in the "Acute Respiratory Diseases (ARD) Study Group" between June 2004 and March 2005. Adult samples also were sent to us from physicians belonging to seven institutions between October 2004 and March 2005. All clinical samples of pediatric and adult patients were collected from patients who were diagnosed with pneumonia based on clinical symptoms, radiography, WBC counts, and CRP at their first visit to a hospital. The samples were collected after obtaining informed consent from patients themselves or from their family members in the case of infants and children, and they were collected from patients with as little previous antibiotic therapy as possible.

A total of 429 samples were acquired from the following sources: nasopharyngeal sections ($n = 375$) from infants and children, throat swabs ($n = 14$) from children, and sputum ($n = 40$) from adults. The sputum samples were homogenized routinely with 50 μ l of SPUTAZYME solution (Kyokuto Pharmaceutical Industries Co., Ltd., Tokyo, Japan) prior to DNA preparation.

Bacterial culture. Clinical samples were suspended in 1.5 ml of PPLO broth (pleuropneumonia-like organisms broth) (Difco, Inc., Detroit Mich.), but in the case of sputum samples, the suspensions with SPUTAZYME were diluted to 1.5 ml with PPLO just after their receipt, and 5- μ l aliquots were inoculated onto sheep blood agar plates and chocolate agar II plates (Nippon Becton Dickinson, Tokyo, Japan) and cultured at 37°C in an atmosphere containing 5% CO₂. After 20 h of incubation, the colonies on the plates were counted and bacteria were identified by standard methods (21, 27). In the case of *M. pneumoniae*, the colonies were determined based on glucose utilization as indicated by the broth color change and by the hemadsorption test of colonies with a 5% suspension of guinea pig erythrocytes (38).

DNA extraction. A 1.0-ml aliquot of the PPLO broth in an Eppendorf tube was centrifuged at 2,000 \times g for 5 min at 4°C to collect bacterial cells, together with epithelial and polymorphonuclear leukocyte cells derived from clinical specimens. The supernatant was discarded, and the harvested pellet was resuspended in 150 μ l of DNase- and RNase-free H₂O (Nacalai Tesque, Inc., Kyoto, Japan). All DNA samples were extracted using EXTRAGEN II (TOSOH Co., Tokyo,

Japan) according to the manufacture's protocol as follows: First, a 100- μ l aliquot of the suspension was transferred to an Eppendorf tube, which contained 8 μ l of detergent for DNA coprecipitation, and vortexed for 10 s. To the mixture, 500 μ l of 60% (vol/vol) isopropanol-containing protein-denaturing detergent was added, and the resulting mixture was vortexed for 10 s and centrifuged at 13,500 \times g for 3 min at 4°C. The supernatant was discarded, and the residue was treated with 200 μ l of 40% (vol/vol) isopropanol again, as described above. Finally, the harvested DNA pellet was resuspended in DNase- and RNase-free H₂O to provide 40 μ l of DNA sample. The DNA extraction process was finished in 20 min.

Real-time PCR. The six sets of bacterium-specific primers and MB probes used in this real-time PCR study are shown in Table 1. To show specificity for CAP pathogens, these primers and MB probes were designed based on appropriate genes for each pathogen. For *S. pneumoniae*, the *lytA* gene sequence (GenBank accession no. M13812), which codes for an autolysin enzyme, was selected, and for *L. pneumophila*, the *mip* gene sequence (GenBank accession no. S72442), which codes for macrophage infectivity potentiator, was used. The 16S rRNA genes of the other four CAP pathogens, *H. influenzae* (GenBank accession no. Z22806), *M. pneumoniae* (GenBank accession no. NC_000912), *C. pneumoniae* (GenBank accession no. Z49874), and *S. pyogenes* (GenBank accession no. NC_002737), which were conserved in all bacteria, were used. In *S. pneumoniae*, *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila*, the sequences of primers and MB probes were conserved in a single gene copy, and in *H. influenzae* and *S. pyogenes*, they were conserved in six copies.

The target genes for the six pathogens were selected for the following reasons. The *lytA* gene encoding autolysin enzyme as well as the pneumolysin gene are specific to *S. pneumoniae* (26, 29); therefore, the *lytA* gene can differentiate *S. pneumoniae* from the genotypically similar *Streptococcus* species (20, 35). Similarly, the *mip* gene of *L. pneumophila* was selected as the MB sequence because the gene is an encoding macrophage infectivity potentiator and is identified only in *L. pneumophila*. All of the MB probes were labeled with a fluorescent reporter, 6-carboxyfluorescein (FAM), at the 5' end and labeled with black hole quencher 1 (BHQ-1) at the 3' end. The reporters and quenchers were connected to stem oligonucleotides with short oligonucleotides (shown in Table 1) accord-

TABLE 2. Sensitivities for six pathogens identified by real-time PCR

No. of DNA copies/50 μ l of reaction tube	Threshold cycle (C_T) ^{a,d}					
	<i>S. pneumoniae</i> (ARD-2743)	<i>H. influenzae</i> (ARD-3185)	<i>S. pyogenes</i> (ARD-2957)	<i>M. pneumoniae</i> (ARD-2700)	<i>C. pneumoniae</i> (AR-39) ^b	<i>L. pneumophila</i> (TD-7)
10 ⁶	17.5	17.2	22.5	18.9	ND ^c	20.5
10 ⁵	20.3	20.3	25.2	21.6	ND	22.4
10 ⁴	24.2	24.7	28.3	24.5	ND	25.6
10 ³	27.5	28.1	32.1	28.0	27.6	29.1
10 ²	30.9	32.3	35.5	31.6	31.3	32.0
10 ¹	33.5	35.9	38	33.4	34.4	35.5

^a The strain no. is given in parentheses. Each sample was analyzed in triplicate.

^b The AR-39 strain was provided by T. Kishimoto (National Institute for Infectious Diseases, Tokyo, Japan).

^c ND, not determined.

^d Correlation coefficients are as follows: for *S. pneumoniae*, 0.9987; for *H. influenzae*, 0.9992; for *S. pyogenes*, 0.9984; for *M. pneumoniae*, 0.9970; for *C. pneumoniae*, 0.9987; and for *L. pneumophila*, 0.9971.

ing to a design made using Beacon Designer 2.0 software (Premier Biosoft International, Palo Alto, CA).

Real-time PCR was performed using a Stratagene Mx3000P (Stratagene, La Jolla, CA) as follows: the reaction mixture consisted of 25 μ l of real-time PCR Master Mix (TOYOBO Co., Osaka, Japan) and 0.15 μ l of primer and MB probe solution in which their final concentrations were both 300 nM, and the final volume of the mixture was adjusted to 50 μ l with the addition of DNase- and RNase-free H₂O. Six reaction mixtures, corresponding to six CAP pathogens, were employed in six wells, which were arranged among the eight wells in one strip. Finally, 2 μ l of sample DNA was added to the six wells while they were subjected to ice cooling, and the remaining two wells were used as positive and negative controls, respectively. Amplification was started at 95°C for 30 s as the first step, followed by 40 cycles of PCRs: at 95°C for 15 s, at 50°C for 30 s, and at 75°C for 30 s, successively. The real-time PCR of this study was finished in 1.5 h.

Analytical sensitivities of the cultures. The analytical sensitivities for *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *L. pneumophila*, and *S. pyogenes*, the strains of which were selected from the stock in our laboratories, were determined by 10-fold serial dilutions of bacterial cells from 10⁹ to 10⁵ per ml, and that for *C. pneumoniae* was determined similarly, but starting from 6.2 \times 10⁵ per ml of the *C. pneumoniae* AR-39 strain, which was kindly provided by T. Kishimoto (National Institute for Infectious Diseases, Tokyo, Japan). Threshold cycle (C_T) values of every dilution of each pathogen were determined from triplicate PCR tests.

Serologic assay. Antibody titers for *M. pneumoniae* in acute- and convalescent-phase sera of patients were determined by a passive agglutination (PA) test using a commercially available kit (Serodia-MycII kit; Fujirebio, Tokyo, Japan). A positive result indicating infection was defined as a fourfold titer rise in the paired sera, and in single-titer cases, a positive result was defined as a 1:320 titer rise. The antibody titer for *C. pneumoniae* was determined by enzyme-linked immunosorbent assay (ELISA) with a commercial reagent (HITAZYME, Hitachi Chemical Co., Ltd, Tokyo, Japan).

RESULTS

Sensitivity and specificity of real-time PCR. In this PCR study, the C_T value for a positive result was defined as the point at which the horizontal threshold line was crossed. The analytical sensitivities of the PCR assay for six CAP pathogens, *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, and *S. pyogenes*, are shown in Table 2. The limit of detection was as follows; 2 DNA copies for *S. pneumoniae*, 10 copies for *H. influenzae*, 5 copies for *M. pneumoniae*, 3 copies for *C. pneumoniae*, 2 copies for *L. pneumophila*, and 18 copies for *S. pyogenes*. The correlation coefficient between C_T and bacterial cell counts was high for all microorganisms: $r = 0.9987$ for *S. pneumoniae*; $r = 0.9992$ for *H. influenzae*; $r = 0.9984$ for *S. pyogenes*; $r = 0.9970$ for *M. pneumoniae*; $r = 0.9987$ for *C. pneumoniae*; and $r = 0.9971$ for *L. pneumophila*. Those six

CAP pathogens could be detected on the order of 10¹ copies per reaction, with 10⁰ copy per reaction yielding no C_T (data not shown).

On the other hand, the specificity of six MB probe and primer sets were examined against 27 gram-positive and -negative microorganisms selected from laboratory stock cultures as amplification-negative controls as shown in Table 3. From those organisms, no nonspecific positive results were obtained.

Sensitivities and specificities of real-time PCR for clinical specimens. The sensitivities and specificities of the real-time PCR assay for *S. pneumoniae*, *H. influenzae*, *S. pyogenes*, and *M. pneumoniae* from 429 clinical specimens were determined, comparing these results with the results of conventional culture tests (Table 4). The relative sensitivities and the specificities of this real-time PCR assay were as follows: 96.2% and 93.2% for *S. pneumoniae*, 95.8% and 95.4% for *H. influenzae*, 100% and 100% for *S. pyogenes*, and 100% and 95.4% for *M. pneumoniae*, respectively.

The sensitivity and specificity of real-time PCR for *M. pneumoniae* relative to those of the serologic assay (Table 5) were also measured, and they were 90.2% and 97.9%, respectively.

In Japan, culture assay of *C. pneumoniae* has not been performed routinely in clinical laboratories but has instead been determined by the titer of antibody, in which a fourfold titer rise between the acute phase and the convalescence phase indicates a positive sign of *C. pneumoniae* infection. In all of six

TABLE 3. Specificity panel: amplification-negative-control organisms

Genus	Species
<i>Streptococcus</i>	<i>S. agalactiae</i> , <i>S. dysgalactiae</i> subsp. <i>equisimilis</i> , <i>S. mitis</i> , <i>S. milleri</i> , <i>S. salivarius</i> , <i>S. oralis</i> , <i>S. mutans</i> , <i>S. sanguis</i> , <i>S. bovis</i>
<i>Enterococcus</i>	<i>E. faecalis</i> , <i>E. faecium</i> , <i>E. avium</i>
<i>Staphylococcus</i>	<i>S. aureus</i> , <i>S. epidermidis</i> , <i>S. haemolyticus</i>
<i>Moraxella</i>	<i>M. catarrhalis</i>
<i>Neisseria</i>	<i>N. meningitidis</i>
<i>Haemophilus</i>	<i>H. parainfluenzae</i> , <i>H. haemolyticus</i>
<i>Escherichia</i>	<i>E. coli</i>
<i>Klebsiella</i>	<i>K. pneumoniae</i> , <i>K. oxytoca</i>
<i>Pseudomonas</i>	<i>P. aeruginosa</i>
<i>Citrobacter</i>	<i>C. freundii</i>
<i>Mycoplasma</i>	<i>M. orale</i> , <i>M. hominis</i> , <i>M. salivarium</i>

TABLE 4. Sensitivities and specificities of real-time PCR compared with those of conventional culture

Species and real-time PCR result	No. (%) of samples also showing culture result		Total no. of samples
	Positive	Negative	
<i>S. pneumoniae</i>			
Positive	202 (96.2)	15 (6.8)	217
Negative	8 (3.8)	204 (93.2)	212
Total	210	219	429
<i>S. pyogenes</i>			
Positive	4 (100.0)	0 (0.0)	4
Negative	0 (0.0)	425 (100.0)	425
Total	4	425	429
<i>H. influenzae</i>			
Positive	161 (95.8)	12 (4.6)	173
Negative	7 (4.2)	249 (95.4)	256
Total	168	261	429
<i>M. pneumoniae</i>			
Positive	36 (100.0)	18 (4.6)	54
Negative	0 (0.0)	375 (95.4)	375
Total	36	393	429

C. pneumoniae cases, both the titer and real-time PCR gave positive results.

In one case of this real-time PCR study, *L. pneumophila* was identified from an adult patient who was suspected of having *L. pneumophila* pneumonia based on the clinical symptoms, WBC, CRP and the chest X-ray, though culture for sputum was negative, and later, *L. pneumophila* serogroup 5 was actually detected in the water from the patient's bathroom.

Correlation between C_T value of real-time PCR and numbers of CFU of bacterial cells. C_T values derived from the real-time PCR assay were compared with numbers of CFU of *S. pneumoniae* (Fig. 1) and *H. influenzae* (Fig. 2). The CFU in 100 clinical samples randomly selected were categorized using sheep blood agar for *S. pneumoniae* and by the use of chocolate agar II for *H. influenzae*. Correlation coefficients between C_T values and numbers of CFU were high for *S. pneumoniae* ($r = 0.9968$) and *H. influenzae* ($r = 0.9954$).

DISCUSSION

In general clinical practice for infectious disease, chemotherapy for CAP must be started based on clinical symptoms, radiograph findings, and laboratory results at the time of hospitalization, considering the severity of the symptoms. One of the measures to decrease health care costs and to improve benefits for patients is to identify the causative microorganisms rapidly and precisely, thus enabling the appropriate antibiotic to be selected at the beginning of hospitalization. However, conventional culture techniques for microorganisms, still the "gold standard" in clinical laboratories, are time consuming. In

TABLE 5. Correlation between results of real-time PCR and those of serologic assay for *M. pneumoniae*

Real-time PCR result	No. (%) of samples also showing serologic assay result		Total no. of samples
	Positive	Negative	
Positive	46 (90.2)	8 (2.1)	54
Negative	5 (9.8)	370 (97.9)	375
Total	51	378	429

the case of patients who were treated by antibiotics prior to hospitalization, the culture method may give false-negative results of microorganisms, and thus, it is possible that causative agents cannot be determined from the remaining antibiotic activity in clinical specimens.

For identification of *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila*, the culture method takes a week or more, and therefore, real-time PCR, iCycler real-time PCR, and nucleic acid sequence-based amplification have been used, improving the sensitivity and the specificity.

By molecular genetic assays, it is possible to detect microorganisms with high sensitivity and specificity, since bacteria damaged by antibiotics and remaining DNA of dead bacteria killed by antibiotics are detected. Thus, simultaneous detection of the CAP pathogens *S. pneumoniae*, *H. influenzae*, and *S. pyogenes*, in addition to *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila*, is desirable for rapid diagnosis of CAP and for the selection of appropriate antibiotics, supporting traditional laboratory methods. Real-time PCR with MB probes allows us to monitor in vitro DNA amplification successively, eliminating nonspecific amplification and the need for gel electrophoresis, and with the remarkable progress of PCR machines and reagents, real-time PCR is now available in clinical laboratories.

In the present study, we developed real-time PCR with gene-specific MB and primers to afford simultaneous detection of six CAP pathogens from clinical specimens with high sensitivity (95.8% to 100%) and high specificity (93.2% to 100%). Among 19 *Legionella* species, which are documented as human pathogens, 91.5% of cases are caused by *L. pneumophila* (32, 41). The urine antigen detection assay has been used in many laboratories, but the assay detects only a limited number of the serogroups of *L. pneumophila*. By conventional PCR assay with the 16S rRNA (26), *Legionella* species are detected; therefore, combined detection of the *mip* and 16S rRNA genes may enable us to detect all of the *Legionella* species and to differentiate *L. pneumophila* among *Legionella* species.

In the preparation of MB oligonucleotides, the MB annealing temperature is designed to be 7 to 10°C higher than that of the primers to assure high sensitivity and specificity (data not shown).

To determine causative agents, sputum culture has been employed for adults; however, expectoration is impossible for infants and children. Alternatively, nasopharyngeal secretions are readily obtainable from children with respiratory tract infections (RTIs) (10), but the test results must be analyzed carefully, because healthy children also carry *S. pneumoniae* (28) and *H. influenzae* in nasopharyngeal secretions. In the PCR assay, a positive result should be carefully analyzed as to

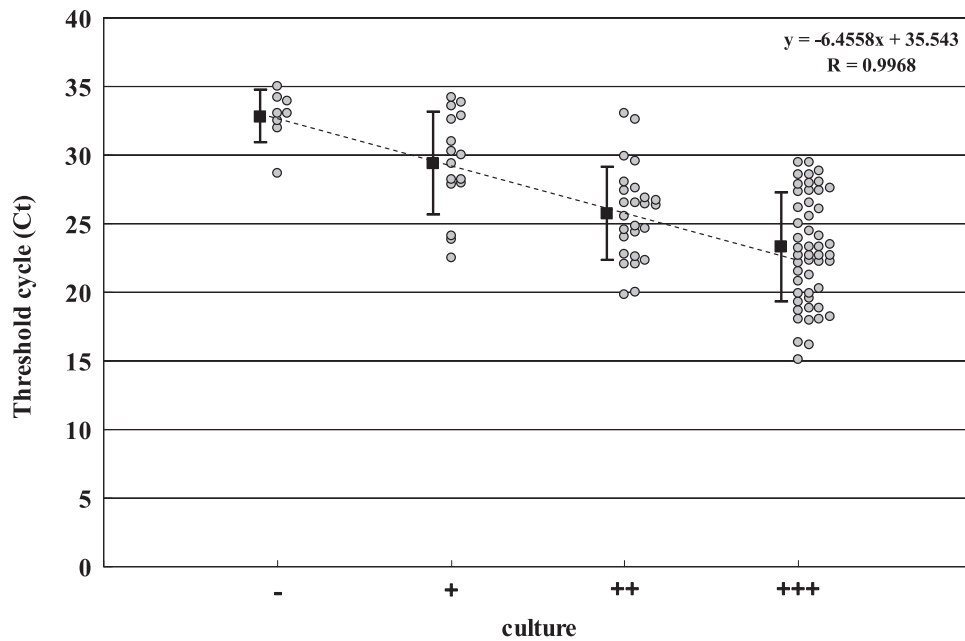


FIG. 1. Correlation of C_T values derived from the real-time PCR assay and culture count results for *S. pneumoniae* ($n = 100$). Negative (-), no colonies observed; +, $<10^3$ /sample (1 to 24 CFU per plate); ++, 10^3 to $<10^4$ /sample (25 to 240 CFU per plate); +++, $\geq 10^4$ /sample (≥ 241 CFU per plate). Each square represents the mean \pm standard deviation.

whether it means that an etiologic agent has been identified, considering chest radiograph findings, clinical symptoms, and clinical laboratory tests, such as WBC and CRP tests.

S. pyogenes is the most common causative agent of pharyngotonsillitis, accounting for 15% to 30% of cases in children and 5% to 10% in adults (3). The agent is rarely a causative agent of CAP, but it is known to cause a very severe CAP infection (6, 23). Accordingly, to supplement conventional cul-

ture, new diagnostic tools have been studied. A rapid immunoassay for *S. pyogenes* is recommended in the United States, but in all cases of negative results, the specimens are cultured (1). If a diagnosis of CAP with *S. pyogenes* can be obtained rapidly and precisely by real-time PCR, the most appropriate chemotherapeutic agent can be administered quickly.

Here, our data demonstrate that a real-time PCR assay of main CAP pathogens with pathogen-specific MBs shows high

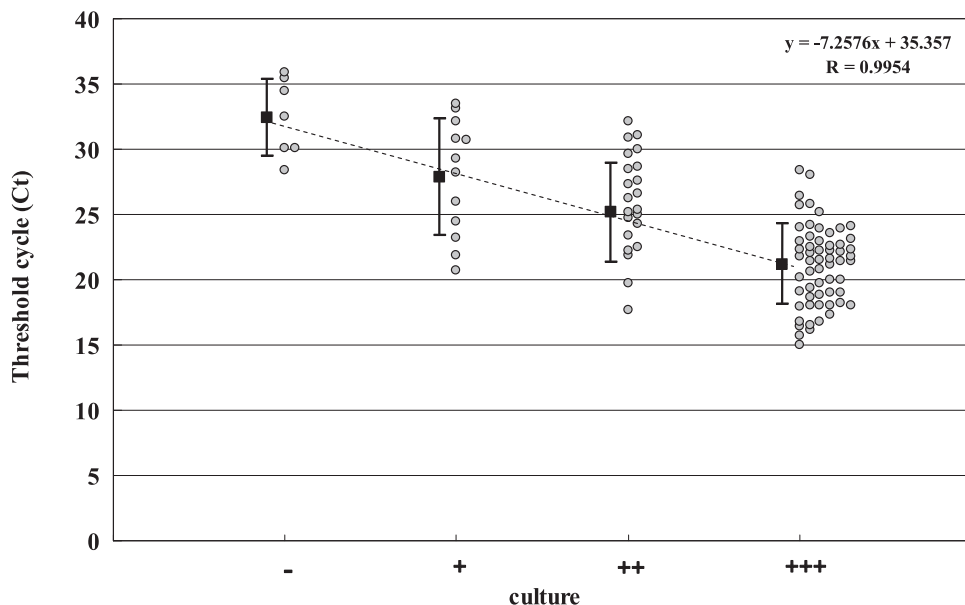


FIG. 2. Correlation of C_T values derived from the real-time PCR assay compared to culture count results for *H. influenzae* ($n = 100$). Negative (-), no colonies observed; +, $<10^3$ /sample (1 to 24 CFU per plate); ++, 10^3 to $<10^4$ /sample (25 to 240 CFU per plate); +++, $\geq 10^4$ /sample (≥ 241 CFU per plate). Each square represents the mean \pm standard deviation.

sensitivity and high specificity, with the results for six CAP pathogens agreeing well with those of conventional cultural and immunological methods. Furthermore, cases where there were discrepancies between real-time PCR results and those of cultures, namely, 8 strains of 100 *S. pneumoniae* strains (Fig. 1, culture negative) and 7 strains of 100 *H. influenzae* strains (Fig. 2, culture negative) were investigated by interviewing physicians, and it was revealed that in almost all of those cases, patients were treated with antibiotics prior to hospitalization.

On the other hand, 8 strains of 210 *S. pneumoniae* strains (Table 4) and 7 strains of 168 *H. influenzae* strains (Table 4) gave real-time-PCR-negative results with cultural-positive results. Those discrepancies may be due to PCR inhibitors which are derived from contamination of the blood from specimens.

Our data demonstrate that real-time PCR with pathogen-specific MB can detect microorganisms in a few hours; and thus, by this assay it is possible to assess the time course of empirical chemotherapy, thus supporting infection management. Ultimately, we expect that the real-time PCR technique described here will be expanded to a multiplex PCR to detect several RTI causative viruses (11) as a general diagnostic method for lower RTIs, used together with conventional culture techniques.

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