

삼출성 중이염 소아의 중이액에서 폐구균의 분자적 진단

변성완¹ · 김한울^{2,3} · 윤서희^{2,3} · 박인호³ · 김경호^{2,3}

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Molecular Diagnosis of *Streptococcus pneumoniae* in Middle Ear Fluids from Children with Otitis Media with Effusion

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Purpose: The long-term administration of antibiotics interferes with bacterial culture in the middle ear fluids (MEFs) of young children with otitis media with effusion (OME). The purpose of this study is to determine whether molecular diagnostics can be used for rapid and direct detection of the bacterial pathogen in culture-negative MEFs.

Methods: The specificity and sensitivity of both polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) to the *lytA* gene of *Streptococcus pneumoniae* were comparatively tested and then applied for pneumococcal detection in the clinical MEFs.

Results: The detection limit of the PCR assay was approximately 10⁴ colony forming units (CFU), whereas that of LAMP was less than 10 CFU for the detection of *S. pneumoniae*. Both PCR and LAMP did not amplify nucleic acid at over 10⁶ CFU of *H. influenzae* or *M. catarrhalis*, both of which were irrelevant bacterial species. Of 22 culture-negative MEFs from children with OME, LAMP positivity was found in twelve MEFs (54.5%, 12/22), only three of which were PCR-positive (25%, 3/12). Our results showed that the ability of LAMP to detect pneumococcal DNA is over four times higher than that of PCR ($P < 0.01$).

Conclusions: As a high-resolution tool able to detect nucleic acid levels equivalent to <10 CFU of *S. pneumoniae* in MEFs without any cross-reaction with other pathogens, *lytA*-specific LAMP may be applied for diagnosing pneumococcus infection in OME as well as evaluating the impact of a pneumococcal conjugate vaccine against OME.

Key Words: *Streptococcus pneumoniae*, Otitis media with effusion (OME), Loop-mediated isothermal amplification (LAMP), Polymerase chain reaction (PCR), Molecular diagnosis

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Introduction

Isolation of bacteria from middle ear fluid (MEF) by either culture or detection of bacterial antigens has been a reliable standard for the etiologic diagnosis of otitis media (OM). *Streptococcus pneumoniae* is the most common pathogen isolated from the MEFs of subjects with otitis media with effusion (OME),

followed closely by *Haemophilus influenzae* and *Moraxella catarrhalis*¹⁻⁴. In addition, other bacteria isolated include Group A streptococci, α -hemolytic streptococci, and *Staphylococcus aureus*. However, a large number of MEF samples are culture-negative or devoid of bacterial antigens despite the presence of polymorphonuclear leukocytes in MEF samples, which implicates a bacterial etiology in OM cases⁵.

A key reason for the presence of culture-negative MEF may be the increase in antibiotic usage for acute otitis media (AOM). For instance, almost all out-patient children under 3 years of age routinely receive antibiotic treatment for AOM before undergoing tympanostomy tube placement^{6,7}. The function of antibiotics, to either inhibit bacterial growth or to allow the host innate immune system to clear the bacterial pathogen, leads to a collection of culture-negative MEF samples from subjects with recurrent AOM or OME, resulting in the difficulty of diagnosing OM pathogens. In addition, a collection of large numbers of culture-negative MEF samples due to antibiotic treatment may also affect the evaluation of vaccine effectiveness against OM in subjects with vaccination.

Although *Streptococcus pneumoniae* is isolated in both OME and recurrent AOM, with a relatively lower rate of culture in OME than AOM, pneumococcal nucleic acid has been found in a large number of culture-negative MEF samples from OME⁸. This indicates that the prevalence of OME and recurrent AOM due to pneumococcal infection may be higher than the prevalence we can determine through culture-based pneumococcal isolation from MEFs. Therefore, the molecular diagnostic method of detecting pneumococcal nucleic acids may be optimal for diagnosing pneumococcal infection in OME or recurrent AOM.

Previous studies have introduced two different types of molecular tests that are able to detect pneumococcal nucleic acids in clinical samples: one type is a polymerase chain reaction (PCR) targeting pneumolysin (*ply*), autolysin (*lytA*) or pneumococcal surface protein A (*pspA*) used in several pneumococcal speciation studies⁸⁻¹¹, and the other is a loop-mediated isothermal amplification (LAMP) technique targeting *lytA* in the ce-

rebrospinal fluid (CSF)¹². Thus, in this present study, we focused on two molecular tests, PCR and LAMP, which commonly target the *lytA* gene, and we evaluated whether these two molecular tests could be applied to identify pneumococcal infection in OME and recurrent AOM.

Materials and methods

1. Collection of middle ear effusion (MEE) specimens

Twenty-two MEF samples were collected from children with OME (thirteen boys and nine girls; ages, 1-7 years (median age, 3 years) who underwent tympanostomy in 2012 (Feb-Oct) at the Department of Otorhinolaryngology at the Ewha Womans University Mokdong Hospital, Seoul, Korea. The middle ear effusions were aseptically collected from the middle ear cavity during tympanostomy, sent to the Ewha Center for Vaccine Evaluation and Study (ECVES), and stored frozen at -70°C before use. All MEF samples were culture-negative on blood agar plates when incubated in a CO₂ incubator at 37°C for over 18 hours. The viscous MEF was liquefied by the addition of Sputasol™ solution (Oxoid, England) prior to both PCR and LAMP^{13,14}. The study protocol was approved by the Institutional Review Board of the Ewha Womans University Mokdong Hospital (IRB No. ECT 11-13-43). Written informed consent was obtained from parents or legal guardians before tympanostomy.

2. Bacterial strains

Streptococcus pneumoniae TIGR4 (serotype 4) was used as a reference strain in testing the specificity of PCR and LAMP, and the three counter-reference strains used were *Haemophilus influenzae* type b Egan, *Moraxella catarrhalis* (ATCC 25238) and *Staphylococcus aureus* (ATCC 29737), which are frequently isolated in chronic OME. Additional clinical isolates were obtained from the Ewha Womans University Mokdong Hospital during 2001-2014. All bacteria were cultured on blood

agar plates (Table 1).

3. Molecular diagnostic assays

To assess whether the molecular diagnostic assays were able to detect pneumococcal infection in culture-negative OME, two different types of molecular tests (PCR and LAMP) were chosen from previous studies^{12, 15}. Both assays were based on the *lytA* gene, which is specific for all strains of *S. pneumoniae*. The oligonucleotide primer sets for both PCR and LAMP were purchased from Macrogen Inc. (Seoul, Korea), as shown in Table 2. All of the reactions were performed as previously described^{12,15}.

Briefly, the PCR mixture contained, in a 50- μ L reaction volume, 0.04 mM *lytA* primer, 200 mM dNTP, 5 μ L of

10x Ex Taq buffer, 5 U of Takara *ExTaq* polymerase (Takara Bio Inc., Shiga, Japan), and 2 μ L of MEF, which was 5-fold diluted in SputasolTM solution. The reaction conditions were 98°C for initial denaturation for 3 min, 30 cycles of 98°C for 15 sec, 53°C for 15 sec, 72°C for 30 sec, and final extension at 72°C for 10 min.

LAMP was performed as follows: the reaction mixture contained 1.6 mM each of FIP and BIP primer, 0.2 mM each of F3 and B3 primer, 0.4 mM of LB primer, 8 U of the *Bst* DNA polymerase large fragment (Enzymomics, Daejeon, Korea), 1.4 mM dNTP, 0.8 M betaine (Sigma, St. Louis, MO, USA), 2.5 μ L of 10X reaction buffer, and 2 μ L of MEFs, which were 5-fold diluted in SputasolTM solution as template DNA. Before the addition of *Bst* DNA polymerase, the reaction was initially denatured at 98°C for 10 min, incubated at 63°C for 35 min with the *Bst* DNA polymerase for DNA extension, and then heated at 80°C for 2 min to terminate the reaction. All amplification products from both the PCR and LAMP were analyzed by electrophoresis with a 1-2% agarose gel containing RedSafeTM (iNTRON, Seongnam-si, Korea) at 4 V/cm for 30 min. To verify the amplified DNA products, the representative band was confirmed by sequencing.

4. Statistical analysis

The detection sensitivity of conventional PCR in MEF specimens was compared to that of LAMP by a Fisher exact test using GraphPad Prism v.4 (GraphPad software Inc., La Jolla, CA, USA), and *P* values of <0.05 were

Table 1. Differential Detection of *Streptococcus pneumoniae* and Non-pneumococcal Reference and Clinical Isolates by Polymerase Chain Reaction (PCR) and Loop-Mediated Isothermal Amplification Assay (LAMP)

Strains and isolates	PCR	LAMP	Reference
<i>Streptococcus pneumoniae</i> serotype 4 TIGR	+	+	19)
<i>Streptococcus pneumoniae</i> nontypable MNZ11/NCC1*	+	+	20)
<i>Moraxella catarrhalis</i> ATCC 25238	-	-	21)
<i>M. catarrhalis</i> EMC_001-004, clinical isolates [†]	-	-	this study
<i>Staphylococcus aureus</i> ATCC 12598	-	-	22)
<i>S. aureus</i> EMC_001-004, clinical isolates [†]	-	-	this study
<i>Haemophilus influenzae</i> type b Eagan [‡]	-	-	23)
<i>H. influenzae</i> type b EMC_001-004, clinical isolates [‡]	-	-	this study

*NCC1: null capsule clade 1.

[†]Vaccine strain.

[‡]Clinical isolates during 2000-2014.

Table 2. List of Primers Used in this Study

Primer	Description*	Molecular assay	DNA sequence (5'→3')	Reference
P5322	<i>lytA</i> ⁸⁴⁰⁻⁸⁶⁰	PCR	CAA CCG TAC AGA ATG AAG CGG	15)
P3322	<i>lytA</i> ⁵⁴²⁻⁵⁶³		TTA TTC GTG CAA TAC TCG TGC G	
Sp5-FIP	<i>lytA</i> ⁸²⁶⁻⁸⁴⁷	LAMP assay	<u>CCG CCA GTG ATA ATC CGC TTC A[†]C CTC AAC TGG GAA TCC GC</u>	12)
Sp5-BIP	<i>lytA</i> ⁷⁸²⁻⁸⁰³		<u>TCT CGC ACA TTG TTG GGA ACG G[†]CC AGG CAC CAT TAT CAA CAG G</u>	
Sp5-F3	<i>lytA</i> ⁸⁹²⁻⁹¹¹		GCG TGC AAC CAT ATA GGC AA	
Sp5-B3	<i>lytA</i> ⁷¹⁸⁻⁷³²		AGC ATT CCA ACC GCC	
Sp5-LB	<i>lytA</i> ⁷⁶³⁻⁷⁸⁰		TGC ATC ATG CAG GTA GGA	

*Numbers indicate nucleotide location in GenBank No. AE008540.

[†]The sequence of *lytA* gene is underlined.

Abbreviations: PCR, polymerase chain reaction; LAMP assay, Loop mediated isothermal amplification assay.

considered statistically significant.

Results

To establish a molecular test for the rapid and direct diagnosis of *S. pneumoniae*, a major cause of culture-negative OME, we utilized PCR and LAMP, both targeting the pneumococcal *lytA* gene, as the two molecular tests had already been verified in previous publications as molecular diagnostic methods able to detect the *lytA* gene, representing the existence of *S. pneumoniae* in various specimens including sputum, blood and CSF⁸⁻¹²⁾.

1. Specificity and sensitivity of molecular diagnostic assays in MEF

Although the species specificity of the primers against the *lytA* gene sequence in both PCR and LAMP had been tested against diverse species, including *S. pneumoniae* and *H. influenzae*¹²⁾, we re-confirmed the specificity against two additional reference strains, *M. catarrhalis* and *S. aureus*, because they are well-known pathogens frequently found in OME. When 10⁶ CFU of bacteria were added to each reaction mixture, PCR did not amplify DNA in the non-pneumococcal strain, even after 35 reaction cycles, while pneumococcal strains were well amplified. Similarly, LAMP amplified the pneu-

mococcal DNA in 60 min of reaction (Table 2). The sequence analysis of the amplified DNA from both assays confirmed that the DNA products were correctly amplified from the primer-targeted *lytA* gene from *S. pneumoniae* (data not shown).

In determining the detection limit of both assays, we amplified DNA from 10-fold serially diluted pneumococcal bacteria in sterile MEF, which was negative by both PCR and LAMP. Our results showed that the detection limit of the LAMP assay was less than 10 CFU of *S. pneumoniae* in MEF, whereas that of PCR was approximately 10⁴ CFU (Fig. 1). These results were highly consistent with a previous study in CSF¹²⁾. Conclusively, both the PCR and LAMP assay are highly specific for *S. pneumoniae* in MEF, but LAMP is a thousand times more sensitive than PCR for pneumococcal detection in MEF samples.

2. Diagnosis of culture-negative OME by both PCR and LAMP

To assess whether the culture-negative OME was caused by pneumococcal infection, both PCR and LAMP were used with 22 MEF samples from culture-negative OME. From a total of 22 MEF samples, twelve samples were positive by LAMP (54.5%, 12/22), whereas only three were positive by PCR (13.6%, 3/22), all of which were positive by LAMP as well. The remaining

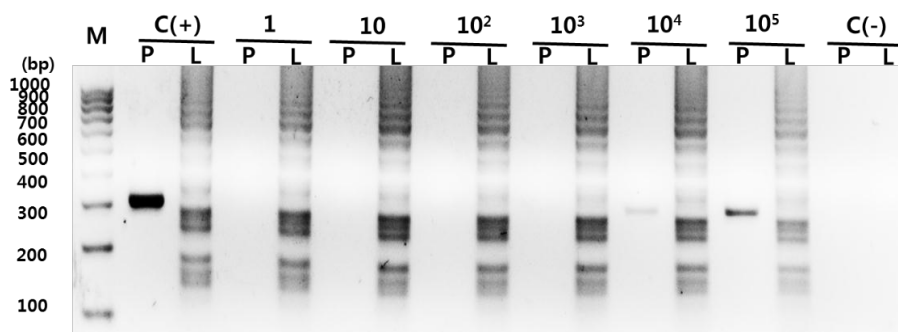


Fig. 1. Sensitivities of polymerase chain reaction (PCR) and loop-mediated isothermal amplification assay (LAMP) against *Streptococcus pneumoniae* (SPEC6B) spiked in sterile middle ear fluid. P and L indicate PCR and LAMP, respectively, and C (+) and C (-) indicate a positive control for pneumococcal DNA and a negative control, respectively. Bacteria was serially diluted in the middle ear fluid and used for two molecular diagnostic methods. The detection limits of PCR and LAMP are 10⁴ CFU and less than 10 CFU, respectively.

Table 3. Detection of *Streptococcus pneumoniae* by both Polymerase Chain Reaction (PCR) and Loop-Mediated Isothermal Amplification Assay (LAMP) in 22 Middle Ear Fluids from Otitis Media with Effusion

Result	LAMP		Total
	Positive, n (%)	Negative, n (%)	
PCR positive, n (%)	3 (13.6)	0(0)	3 (13.6)
PCR negative, n (%)	9 (40.9)	10 (45.5)	19 (86.4)
Total	12 (54.5)	10 (45.5)	22 (100)

Abbreviation: n, number.

12 MEF samples were negative by both PCR and LAMP (Table 3). Our results reflect that there are different detection limits between PCR and LAMP.

Discussion

The isolation of bacteria from MEF samples has been relied on as a basic standard for detecting the etiology of OME. Following the introduction of diverse vaccines directed against the pathogens causing OME, regardless of the presence of live bacteria, bacterial detection through the identification bacterial debris, such as fragmented nucleic acids in MEF samples, from subjects with OME has become a challenge. Thus, in order to overcome the limitations of pneumococcal identification by culture or antigen detection, we tested two different molecular tests (PCR and LAMP) specifically targeting the pneumococcal *lytA* gene, using MEF samples artificially spiked with *S. pneumoniae* and clinical MEF samples from OME. As a result, we found that LAMP was more efficient for detecting *S. pneumoniae* than PCR in terms of both specificity and sensitivity in the MEF environment.

As a molecular test, LAMP appears to be more useful than culture in assessing the impact of PCV, especially in OME cases associated with a long-term use of antibiotics, because antibiotics are frequently prescribed according to the clinical guideline for AOM, and 10–20 % of AOM cases can result in either OME or recurrent AOM. In general, the use of antibiotics can be a key reason for culture-negative MEF samples from both AOM and OME cases. In this study, regardless of the

lack of growth of pneumococcal bacteria due to use of various bactericidal and/or bacteriostatic antibiotics, we showed that a small amount of pneumococcal nucleic acid existed in MEFs. The amount of nucleic acid was not great enough to be detected by PCR, but it was still great enough to be detected by LAMP with a high detection limit (<10 CFU), as shown in Fig. 1 above.

Unlike PCR, which had cross-reactions with other oral streptococci such as *S. pseudopneumoniae*, *S. mitis* and *S. oralis*, LAMP did not have any cross-reactions with these oral streptococci as previously described¹²⁾. In addition, we found that LAMP did not react with the nucleic acid from other main OME-pathogens, such as *H. influenzae* and *M. catarrhalis*, even at 10^6 CFU in MEF samples (Table 2). Our results indicate that LAMP would be a highly reliable molecular test for diagnosing pneumococcal OME.

Pneumococcal conjugate vaccines (PCV) are primarily directed against invasive pneumococcal diseases in young children, but they can also reduce the incidence of pneumococcal OM. According to previous studies, however, the use of PCV reduced the overall AOM incidence by 6–9%^{16,17)}, whereas it is not clear whether PCVs reduce OME or recurrent AOM. Generally, 10–20% of all cases of OM can result in either OME or recurrent AOM¹⁸⁾, and *S. pneumoniae* is isolated by culture in both OME and recurrent AOM at a relatively lower rate than in AOM. Nonetheless, pneumococcal nucleic acid has been found in a large number of culture negative MEF samples from OME⁸⁾, indicating that the prevalence of *S. pneumoniae* in either OME or recurrent AOM may be higher than the prevalence able to be determined by culture-based bacterial isolation. LAMP is therefore a highly useful tool in assessing the impact of PCV against OME or recurrent AOM, as it has both high specificity and sensitivity against a small amount of pneumococcal DNA in MEF samples. In spite of the usefulness of LAMP in diagnosing pneumococcal infection in OME, however, there is a lack of function in applying LAMP to assess the impact of PCV against OME, as all PCVs have been developed to protect against pneumococcal diseases caused by vaccine serotypes. Thus, it is necessary for LAMP to focus on

the pneumococcal serotypes causing OME to evaluate the impact of PCV. Therefore, we speculate that LAMP would be more useful if it could differentiate between pneumococcal DNA samples associated with serotypes.

In conclusion, we performed two molecular tests to determine if they can be applied to identify pneumococcal infection in MEF samples from OME. Our results demonstrated that LAMP is more efficient at detecting pneumococcal DNA in culture-negative MEF samples than PCR and that the actual pneumococcal prevalence in OME was clearly higher than the pneumococcal prevalence that was shown by culture. However, it should be noted that our results are limited in the terms of the small number of clinical MEF samples from OME that were tested. Thus, further studies with more clinical specimens will be required for the establishment of LAMP for multiple-purpose diagnoses.

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요약

목적: 장기간의 항생제 치료는 중이염 어린이 환자의 중이액으로부터 원인균이 배양되는 것을 방해한다. 본 연구는 배양 음성 중이액으로부터 분자적 진단에 의한 신속한 균 검출 가능성 여부를 확인하고자 하였다.

방법: 폐구균 *lytA* 유전자를 표적으로 하는 PCR과 LAMP로 민감도와 특이도를 비교 결정하고, 임상 중이액에서의 폐구균 검출에 적용하였다.

결과: PCR 기법에 의한 폐구균 검출 최소한계는 약 10^4 집락형성단위(CFU)이고, LAMP의 검출 최소한계는 10 CFU에서 결정되었다. 한편 두 가지 검사법 모두 *Haemophilus influenzae*와 *Moraxella catarrhalis*에 대해 10^6 CFU 이상에서도 DNA를 증폭하지 않았다. 22개의 배양음성 중이액 중에서 12개 검체가 LAMP-양성(54.5%, 12/22)으로 확인되었고, 이들 12개 LAMP-양성 검체 중, 3개의 검체만이 PCR-양성으로 확인되었다(25%, 3/12). 본 연구의 결과는 LAMP 기법의 폐구균 검출 해상력이 PCR 기법에 비교하여 4배 이상 높음을 보여준다($P < 0.01$).

결론: *lytA*-특이 LAMP 기법은, 중이액 내의 타 병원균과는 교차반응 없이 10 CFU 폐구균의 DNA를 검출할 수 있는 고해상 기술로서, 중이액 폐구균 검출 및 폐구균 백신의 보급에 따른 백신 효과 평가에 적용이 기대된다.