

Detecting Lymphotoxin-alpha Gene Mutation, a Genetic Risk Factor in Community-acquired Pneumonia, Using Allele-specific PCR

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ABSTRACT: Genetic mutation in the Lymphotoxin-alpha (LTA) gene, LTA 250G>A (Substitution, position 250, G→A) is known to cause severe inflammatory responses to infectious diseases, and one of the major inflammatory conditions this mutation is known to cause is enhanced risk of septic shock in community-acquired pneumonia. Previously proposed methods for detecting this mutation are time consuming and require special instruments. To readily detect this mutation while preserving high accuracy, we used multiplex PCR. The results indicate that the PCR condition proposed in this study allows us to detect both LTA wildtype (wt) and LTA 250G>A with high accuracy. Both genotypes can be distinguished by different sizes of DNA amplification in gel electrophoresis. We were able to confirm this using four different human cell lines: A172 (LTAwt), SK-MEL2 (LTAwt), A375sm (LTAwt), and MCC13 (LTA 250G>A). This novel method can be used for rapid and accurate detection of LTA 250G>A, which is a genetic risk factor for community-acquired pneumonia. This is a simple and inexpensive method for mutation detection, which is accessible to minimally equipped laboratories.

KEYWORDS: Molecular Biology, Genetic Mutation, LTA mutation, community-acquired pneumonia, allele-specific PCR.

■ Introduction

Community-acquired pneumonia (CAP) encompasses cases of infectious pneumonia in patients who live in a community.¹ The disease is commonly caused by pathogens such as *Streptococcus pneumoniae*, influenza A, *Mycoplasma pneumoniae* and *Chlamydomphila pneumoniae*.² It is the third most common reason for hospital admission for adults, and in the United States, it is the leading cause of death from infectious diseases. CAP is also one of the leading causes of severe sepsis, which has a considerably high fatality rate.³

Proinflammatory genes such as lymphotoxin-alpha (LTA) code for pro-inflammatory cytokines that play a central role in inflammatory diseases of infectious origin, such as CAP. Overexpression of proinflammatory cytokines can have a deleterious effect on the host, leading to several disorders including sepsis, ischemia, and hemorrhage.⁴ LTA 250G>A has been identified as an important genetic mutation that worsens the severity of different inflammatory conditions. Among the many inflammatory conditions that it is known to exacerbate, LTA+250 AA genotype predisposes community-acquired pneumonia patients at greater risk for septic shock.⁵ A previous study also indicates that LTA+250 AA genotype site is associated with higher serum tumor necrosis factor-alpha levels and a higher mortality rate in children with bacteremia.⁶

Allele-specific polymerase chain reaction (ASPCR) is a technique of polymerase chain reaction (PCR) that allows the direct detection of any point mutation in human DNA by amplified products in agarose gel electrophoresis. ASPCR is designed with a set of oligonucleotide primers that generates a 3' mismatch with the DNA template, which does not allow the primer extension by DNA polymerase.⁷

Genetic mutation in the LTA (250G>A) is known to be one of the greatest risk factors for septic shock in community-acquired pneumonia.⁸ However, previous methods for detection require extended workflow time and sophisticated equipment, making it more difficult for the patients to readily access pre-diagnosis. Here, a novel method of LTA mutation detection using allele-specific PCR is proposed. This method only requires four DNA primers, a PCR machine, and minimal agarose gel electrophoresis equipment, which enables molecular diagnostic assays to be analyzed rapidly in 3 hours without any need for expensive lab equipment.

■ Methods

Cell culture of human cell lines:

Four human cell lines were obtained from Korea Cell Line Bank (Seoul, Korea): A172, SK-MEL2, A375SM, and MCC13. RPMI-1640 medium with 10% FBS and 1% penicillin and streptomycin supplements were used. The cells were maintained at a 37 °C in a CO2 incubator.

Genomic DNA purification from human cell lines:

1.2 x 10⁶ of human cells were harvested for genomic DNA extraction. DNA was isolated using QIAamp DNA Mini Kit (Qiagen). After the cells were resuspended with 200 µL phosphate-buffered saline (PBS), 200 µL lysis buffer was added. After incubating the lysate at 56 °C for 10 min, 200 µL of ethanol was added. Then, samples were loaded on the Mini spin column. After the centrifugation was applied for 1 min, the flow-through solution was removed. Then the column was washed with 500 µL AW1 buffer and 500 µL AW2 buffer. The purified DNA was eluted with a 200 µL AE buffer.

Allele-specific PCR:

The primer sequences are presented in Figure 1. 20 µL PCR reaction was performed as follows: 1 x reaction buffer, 2 mM MgCl₂, Taq DNA polymerase, 250 µM of each dNTP (Bioneer), 1 pmol of each primer, and 100 ng genomic DNA template. Then, the following PCR condition was used for the target gene amplification: 95 °C for 5 min, 35 cycles of 95 °C for 20 sec, 56 °C for 30 sec, and 72 °C for 30 sec with a final extension at 72 °C for 5 min.

Agarose gel electrophoresis:

After 1.5% agarose gel was prepared with Red-safe (Intron) staining solution, the samples were loaded onto the gel with 6X DNA loading buffer (Bioneer). After the gel was run at 100 V for 30 min, the gel was visualized by a Blue light illuminator (B-box).

Results and Discussion

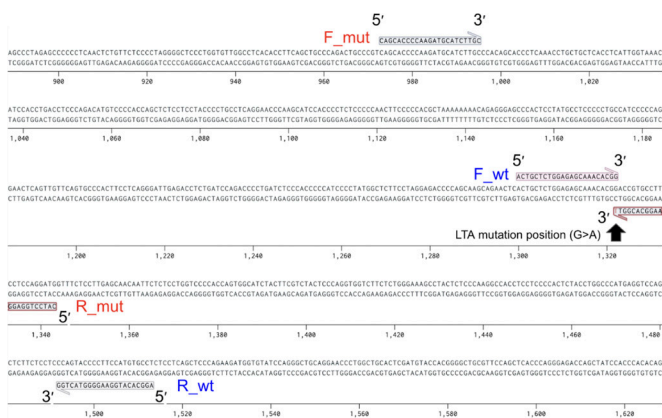


Figure 1: Primer design of LTA 250 G>A allele-specific PCR. Forward primer wild type (F_wt) and Reverse primer wild type (R_wt) generate 214 bp for detecting wild-type allele G. Forward primer mutant (F_mut) and Reverse primer mutant (R_mut) generate 371 bp for detecting mutant allele A.

The method presented here allowed efficient discrimination of LTA mutation by allele-specific PCR in a single reaction with standard PCR conditions. A common reverse primer and two forward allele-specific primers with different tails amplify two allele-specific PCR products of different lengths: 214 bp for detecting wild-type allele G and 371 bp for detecting mutant allele A (Figure 1).

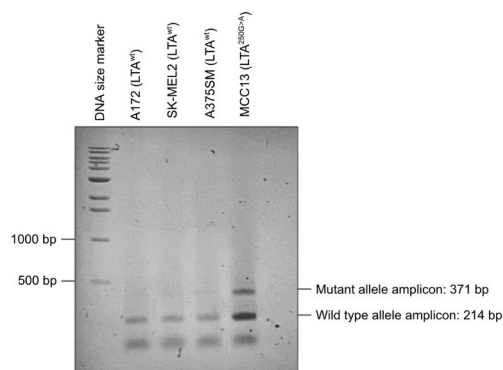


Figure 2: Allele-specific PCR results using four different human cell lines: A172 (LTAwt), SK-MEL2 (LTAwt), A375SM (LTAwt), and MCC13 (LTA 250G>A). The mutant allele amplicon was only detected in an MCC13 DNA sample.

To confirm the specificity of the assay, one LTA 250G>A mutation containing cell line (MCC13) and three cell lines with LTA wild type (A172, SK-MEL2, A375SM) were used in this allele-specific assay. A wild type allele band (214 bp) was detected in all four cell lines. A 371 bp mutant allele band was detected only in MCC13 indicating that MCC13 had heterozygous mutation (LTAwt/250G>A) (Figure 2). This result was consistent with the mutation data provided by the Catalogue Of Somatic Mutations In Cancer (COSMIC) which is a mutation database that includes about 6 million coding mutations across 1.4 million tumor samples.

Conclusion

ASPCR has been used in DNA-based diagnostic techniques for the diagnosis of genetic and infectious diseases. In this research, the accuracy and specificity of our novel ASPCR was confirmed by the detection of LTA 250G>A by analyzing the PCR products in agarose gel electrophoresis. Our result was consistent with the mutation database provided by COSMIC. This novel assay can be easily performed by using basic laboratory equipment. This method can potentially be applied to detect many other single nucleotide polymorphisms or point mutations found in genetic diseases. To fully confirm the applicability of this result, it would be necessary to test more than four cell lines and corroborate the specificity of LTA 250G>A mutation detection. Also, there are numerous other mutations that are associated with community-acquired pneumonia. Further studies may be carried out to detect a more comprehensive set of CAP-associated mutations in one single PCR reaction.

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