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Construction and Identification of a Positive Control Recombinant Plasmid for Respiratory Syncytial Virus Nucleic Acid Detection

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Abstract

Objective: Human respiratory syncytial virus (HRSV) is one of the major viruses of acute respiratory tract disease among infants and young children. Establishing a positive control for HRSV PCR detection is very important for quality control of HRSV PCR detection. Methods: We first designed a specific primer to amplify the HRSV N gene fragment, and inserted it into the pMDTM19-T vector to obtain a recombinant plasmid. The recombinant plasmid was then verified by PCR amplification, Enzyme digestion, sequencing and real-time PCR. Results and Conclusion: Amplification product length and Digestion length of positive control were consistent with the length of the target sequence and a significant amplification curve appeared, suggesting that the positive control can perform good laboratory quality control on PCR detection of HRSV.

Keywords

Human respiratory syncytial virus, N gene, Real-time PCR.

1. Introduction

Human respiratory syncytial virus (HRSV) is the predominant pathogen identified in hospitalized infants and young children with Acute respiratory tract infection (ARTI)[1]. It is estimated that HRSV causes approximately 30 million ALRI events, result 3 million hospitalizations and more than 60000 deaths in children under 5 years old[2; 3]. HRSV is a negative-sense, single-stranded RNA virus, belongs to the *Pneumoviridae* family. The HRSV genome contains 10 genes, which encode 11 proteins: NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2, and L[4]. According to the antigenic and genetic variability, HRSV strains are classified into subtypes A and B (HRSVA and HRSVB)[5; 6]. In the A and B subtypes, the nuclear protein (N) gene is the most conserved gene within the same subtype[7]. For now, the diagnosis of HRSV mainly includes the following methods: (1) isolation and culture of viruses; (2) serology and antigen detection; and (3) molecular detection, including PCR, RT-PCR and qPCR methods[8]. Virus isolation and culture has limited its application due to its long identification time and serology and antigen detection has a lower sensitivity. Molecular detection technology, especially qPCR technology, has become the most important diagnostic technology due to its advantages of fastness, sensitivity and convenience[9]. In the present study, we constructed a recombinant plasmid inserted with an amplified fragment of the N gene as a positive control in the HRSV detection process, which is pivotal for good laboratory quality control in PCR detection.

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2. Materials and Methods

2.1 Materials

pMD^{TM}19-T Vector was purchased from Takara. Primers and probes were synthesized in Biotech.

2.2 Construction of recombinant plasmid

The N gene sequences of the HRSV subtypes in the GenBank database was analyzed, and the upstream and downstream primers were designed for the conserved regions of the N gene (Table 1). The N gene of HRSV subtypes were amplified using PCR, and the amplification reaction was carried out by following thermal profile: 94 ˚C 3 min, 35 cycles of 94 ˚C 30 s, 55 ˚C 60 s, and 72 ˚C 1 min. PCR products were purified on 2% agarose gels and then ligated to the pMD^{TM}19-T Vector. After that, the pMD^{TM}19-T Vector was transformed into DH5α E. coli competent cells and the bacterial solution was uniformly spread on a plate containing Amp, X-gal and IPTG, and a single white colony was picked.

Table 1. Primer sequences of N gene of HRSV subtypes A and B

Primer	Primer Sequence (5'-3')
HRSVA-N-F	AGATCAACTTCTGTCATCCAGCAA
HRSVA-N-R	TTCTGCACATCATAATTAGGAGTATCAAT
HRSVB-N-F	GATGGCTCTTAGCAAAGTCAAGTTAA
HRSVB-N-R	TGTCAATATTATCTCCTGTACTACGTTGAA

2.3 Restriction enzyme identification

Added 4ul Recombinant plasmid, 1ul *EcoR* I, 1ul *Hind* III, 1ul 10×Green Fast Digest Buffer and 3ul ddH_2O to a reaction system, thoroughly mixed and then reacted at 37℃ for 1h. Electrophoresis was performed on a 2% agarose gel, and the expected band was cut and sent to sequencing.

2.4 Quantitative Real-time PCR

Subgroup-specific primer and probe sequences were show in Table 2. The recombinant plasmid was used as a cDNA template. The reaction system (20 ul) was as follows: 2ul cDNA, 1ul forward primer, 1ul reverse primer, 1ul probe, 10ul 2×TransStart Probe qPCR SuperMix and 5ul Nuclease-free Water and the amplification reaction was carried out by following thermal profile: 95 ˚C 10 min, 45 cycles of 95 ˚C 15 s and 55 ˚C 1min.

Table 2. subgroup-specific primer and probe sequences of N gene

Primer	Primer Sequences (5'-3')
HRSVA-F	AGATCAACTTCTGTCATCCAGCAA
HRSVA-R	TTCTGCACATCATAATTAGGAGTATCAAT
HRSVA-P	FAM-CACCATCCAACGGAGCACAGGAGAT-BHQ1
HRSVB-F	GATGGCTCTTAGCAAAGTCAAGTTAA
HRSVB-R	TGTCAATATTATCTCCTGTACTACGTTGAA
HRSVB-P	FAM-TGATACATTAAATAAGGATCAGCTGCTGTCATCCA-BHQ1

3. Results

3.1 Restriction enzyme digestion of recombinant plasmid

We first performed colony PCR on the extracted single white colony using the upstream and downstream primers of pMD^{TM}19-T vector (Primer M13-47, Primer RV-M). As shown in Fig. 1A,

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the gene bands amplified by PCR were consistent with the molecular weight of the target band. (A subtype: 239 bp, B subtype: 259 bp). The plasmid was extracted from the bacterial strain with the desired amplification band, and double-digested with *EcoR* I and *Hind* III. The results showed that the molecular weight of the restriction band was consistent with the target band as shown in Fig. 1B (Subtype A: 145 bp, B subtype 165 bp). The corresponding plasmid was sent for sequencing. The sequencing results were aligned by NCBI and showed that the HRSV A and B subtype positive control recombinant plasmids were successfully constructed.

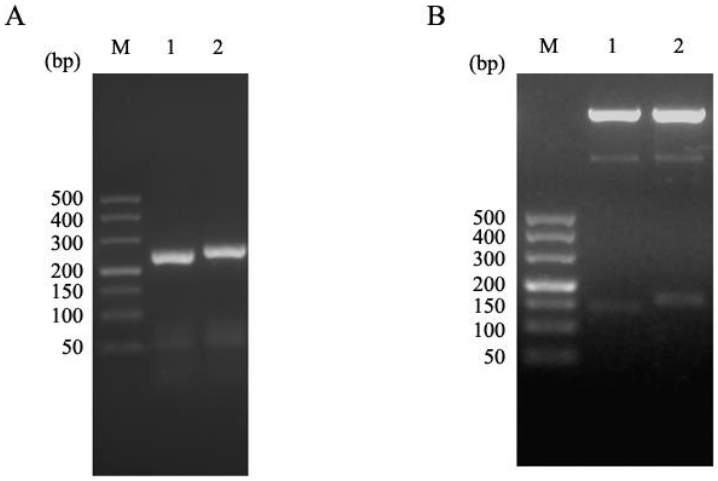

Fig. 1 (A) Colony PCR of recombinant Plasmid. (B) Restriction enzyme identification of recombinant Plasmid. M: DL500 DNA Marker; 1: HRSVA; 2: HRSVB.

3.2 Amplification curve of Quantitative Real-time PCR

In the PCR detection process, in order to ensure the validity of the detection reagent, a positive control was usually set. To verify whether the recombinant plasmid we constructed can be used as a positive control for the detection of HRSV nucleic acid, we performed a real-time PCR assay. As shown in Fig. 2, when a specific primer and probe targeting the HRSV N gene were used to react with the recombinant plasmid, a significant S-type amplification curve appeared, indicating that the recombinant plasmid we constructed can be used as a positive control for HRSV nucleic acid assay.

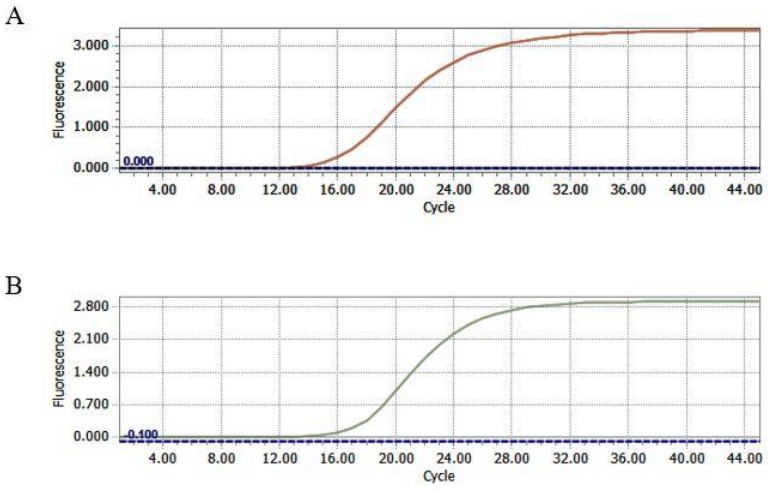

Fig. 2 (A) Amplification curve of HRSVA. (B) Amplification curve of HRSVB.

4. Discussion

Acute respiratory tract infection (ARTI), one of the most common infectious disease worldwide, is a major cause of morbidity and mortality in young children[10]. HRSV is one of the most common respiratory pathogen worldwide[11]. In recent years, molecular detection technology has been widely

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used in laboratory diagnosis and screening of certain viruses. However, how to ensure the reliability and accuracy of the experimental results is the focus of attention. In our study, we successfully constructed a recombinant plasmid inserted with the amplified fragment of HRSV N gene as a positive control in the detection of HRSV nucleic acid and further verified its availability by fluorescence quantitative PCR experiments.

Acknowledgments

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