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# Development of a SYBR Green real-time RT-PCR assay for the detection of avian encephalomyelitis virus

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Avian encephalomyelitis virus (AEV) causes epidemic diseases in poultry worldwide. A SYBR Green real-time reverse transcription-polymerase chain reaction (rRT-PCR) assay was developed for the rapid detection and quantitation of AEV in this study. A pair of specific primers was designed in the highly conserved VP1 gene of this virus. When comparing this assay with conventional RT-PCR, the rRT-PCR assay was 100 times more sensitive and could detect levels as low as 10 standard DNA copies of the AEV SX strain. The specificity of this technique was evaluated in five other avian pathogens. The AEV RNA was detected as early as three days post-infection in chicken embryos. All 18 clinical chicken brains collected from an AEV outbreak in Northwestern China were detected to be positive (100%) using the rRT-PCR assay. However, only 5 of the 18 samples were positive (28%) using the conventional RT-PCR. The results were confirmed by virus isolation in chicken embryos. This high sensitivity, specificity, and simplicity of the SYBR Green rRT-PCR approach can be a more effective method than the conventional one for AEV diagnosis and surveillance.



## 1. Introduction

Avian encephalomyelitis (AE) is an important disease that occurs in chicken, pheasant, turkey, and quail (Markson and Blaxland, 1958; Itakura and Goto, 1975). Avian encephalomyelitis virus (AEV), which is also known as epidemic tremor and a member of the family Picornaviridae, is characterized by a positive single-stranded RNA genome (Marvil et al., 1999; Shafren and Tannock, 1992). It is the only species of the genus Tremovirus (ICTV, 2012. www.ictvonline.org). Young chicks show neurological symptoms that are characterized by ataxia, paresis or paralysis, and rapid tremors of the head and neck, and have high morbidity and variable mortality (McNulty et al., 1990; Olitsky, 1939). Accordingly, AEV has caused considerable economic losses. No neurologic sign can be observed in adult laying birds, but the virus causes a slight reduction in egg production (Markson and Blaxland, 1958; Itakura and Goto, 1975). Currently, no treatment for AEV is available. Control and prevention of this disease are achieved by vaccination of the flocks. Therefore, a rapid and highly sensitive technique is required for AEV diagnosis and surveillance before the virus spreads widely.

The definitive methods for identifying AEV include virus isolation (Tannock and Shafren, 1985; Wills and Moulthrop, 1956), fluorescent-antibody technique (Miyamae, 1974, 1977), enzyme-linked immunosorbent (Shafren and Tannock, 1988, 1991), and agar gel precipitin (AGP) assays (Girshick and Crary, 1982; Lukert and Davis, 1971). However, these methods have certain limitations: time consuming, labor intensive, and low specificity. Recently, a conventional RT-PCR method based on VP2 gene has been reported as a rapid, sensitive, and specific technique (Xie et al., 2005). However, the result of the conventional RT-PCR was assessed by gel electrophoresis, which is time consuming, prone to contamination, and unsuitable for large-scale investigations (Liu et al., 2002; Mackay et al., 2002; Reid et al., 2007). Moreover, the determination of the amount of virus in different tissues and cells is helpful for investigating the nosogenesis, virus replication, host–virus interactions, tropism, and screening of anti-viral drugs, but the current RT-PCR does not have this capability (Friedrichs et al., 2004; Günther et al., 2004).

In recent years, the real-time RT-PCR (rRT-PCR) has been widely used for both the detection and quantification of other pathogenic microorganisms simultaneously (Gurukumar et al., 2009; Mendy et al., 2006). The use of a SYBER Green rRT-PCR for identifying AEV has not been reported so far. This study attempts to create a SYBER

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Green rRT-PCR assay for the molecular diagnosis of AEV that is more sensitive and specific than virus isolation and serological assays to provide more tools for researchers who work with AEV.

## 2. Materials and methods

### 2.1. Viruses

The virulent AEV strain SX was isolated from brain tissue of chicken flocks infected naturally in Shaanxi Province of China (Zhang et al., 2000). Commercial vaccine strain 1143 was from Intervet International B.V. Boxmeer, Holland. Infectious bronchitis virus (IBDV), Newcastle disease virus (NDV), Marek's disease virus (MDV), Avian influenza virus (H9N2), and infectious bursal disease virus (IBDV) were isolated by our laboratory.

### 2.2. Primer design

A pair of rRT-PCR primers based on the highly conserved VP1 gene of AEV strains 1143 (GenBank accession no. NC003990) was designed using Primer Premier software (Premier Biosoft International, Palo Alto, USA) as follows: AE 5′-GAATTAGCTCCTGGTAAACCTCG-3′ (covering nucleotides 2115–2127) and reverse AE 5′-TATTATCGCAACACCCTCAGG-3′ (complementary to nucleotides 2399–2382). The oligos were synthesized by Genscript (Genscript Biotech Corporation, Nanjing, China) and a 288 bp product was amplified.

The primers for the established conventional RT-PCR in the detection of AEV from chicken embryos and clinical samples were previously described (Xie et al., 2005). The PCR yielded a 619 bp product.

### 2.3. Preparation of standard plasmid DNA templates

The genome RNA of AEV strain SX was extracted using RNAiso Plus (Takara Biotech Corporation, Dalian, China) according to the manufacturer's instructions and reverse transcribed into cDNA using the EasyScript One-Step RT-PCR SuperMix (Transgen Biotech Corporation, Beijing, China). The obtained cDNA was subsequently used for RT-PCR with the primers of rRT-PCR. The amplicons were then inserted into the pMD-19T cloning vector (Takara Biotech Corporation, Dalian, China) and transformed into *Escherichia coli* DH5α competent cells (Takara Biotech Corporation, Dalian, China). The recombinant plasmid DNA was extracted using the TIANprep plasmid extraction kit (Tiangen Corporation, Beijing, China) and sequenced by Genscript (Genscript Biotech Corporation, Nanjing, China). The plasmid DNA concentrations and purity were measured by ultraviolet absorbance at 260 nm and 280 nm with a Smartspec 3000 spectrophotometer (Bio-Rad, Hercules, USA). The copy numbers of the recombinant plasmid were calculated as described previously (Ke et al., 2006).

### 2.4. Optimization of SYBR Green real-time RT-PCR

All reactions were performed using a Bio-Rad icycler IQ5 Real-time PCR Detection System (Bio-Rad, Hercules, USA) and an UltraSYBR Mixture (Cwbiotech, Beijing, China). rRT-PCR was performed with a volume of 20 μL containing 10 μL of 2 × UltraSYBR Mixture and 0.8 μL standard plasmid, which was diluted in a 10-fold dilution series ranging from $1.0 \times 10^1$ copies/μL to $1.0 \times 10^7$ copies/μL. The final primer concentration ranged from 0.1 μM/L to 0.7 μM/L. Each program was composed of an initial step at 95 °C for 10 min and 40 cycles: 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 50 s. Fluorescence was measured by a program that had been set in advance. The melting curves were generated by monitoring the fluorescence of the SYBR Green signal from 55 °C to 95 °C. Conditions were selected to ensure that both an exponential increase in fluorescence and an AEV-specific melting peak were observed.

### 2.5. Establishment of the standard curve

The rRT-PCR standard curve was generated under optimized conditions. The standard plasmid DNA were diluted in a 10-fold dilution series ranging from $1.0 \times 10^1$ copies/μL to $1.0 \times 10^7$ copies/μL and tested in triplicate within the SYBR Green rRT-PCR assay. A Bio-Rad iCycler IQ5 detection software (Bio-Rad, Hercules, USA) was used to generate the standard curve and to calculate the correlation coefficient ($R^2$) and efficiency of the standard curve.

### 2.6. Sensitivity, specificity, and reproducibility analysis

Ten-fold serial dilutions from $1.0 \times 10^1$ copies/μL to $1.0 \times 10^7$ copies/μL were assayed by rRT-PCR to determine their sensitivity. The SYBR Green rRT-PCR products were visualized by gel electrophoresis on a 2% agarose gel (Biowest, Madrid, Spain) to compare the sensitivity with the established rRT-PCR.

AEV strains SX and RNA or DNA extracted from five other avian viruses, namely, IBV, NDV, AIV (H9N2), IBDV, and MDV, were subjected to rRT-PCR to confirm the specificity of the technique. RNA-free water was used as the negative control (NC).

To check the reproducibility of this new assay, standard plasmid DNA of three different 10-fold serial concentrations were assayed by rRT-PCR. The intra-assay was conducted with three replicates of three dilution samples at the same time. The inter-assay was performed by three independent assays every other week. Both coefficient variations (CV) were calculated by dividing the standard deviation of each tested sample by its mean and multiplying the result by 100 (Pignatelli et al., 2010).

### 2.7. Detection of AEV from chicken embryos

Twenty 7-day-old specific-pathogen-free (SPF) chicken embryos (Lv. fang Biotech. Yangling, China) were inoculated into the yolk sac with 0.2 ml of $10^{-3}$ dilution of AEV strain 1143. Ten SPF chicken embryos served as the negative control with 0.1 M phosphate-buffered saline (pH = 7.4). Brain, liver, and allantoic fluids were collected at three and five days post-inoculation (PI). These tissues were disrupted three times by freezing and thawing and centrifuged at 1000 × *g* for 30 min at 4 °C. The RNA of these samples was extracted and processed by established rRT-PCR and conventional RT-PCR.

### 2.8. Detection of clinical samples

Chicken brains ($n = 18$) were collected from an AEV outbreak in Shaanxi Province, China. Both SYBR Green rRT-PCR and RT-PCR were performed.

## 3. Results

### 3.1. Preparation of standard plasmid DNA templates

The 288 bp VP1 gene was amplified by RT-PCR with AEV specific primers and cloned into plasmid pMD-19T. The fragment was sequenced, confirming that it had 100% nucleotide identity with the expected VP1 sequence of the AEV strain SX. The concentration of the recombinant plasmid was measured as 126 ng/μL, and the copy numbers were calculated as $3.99 \times 10^{11}$ copies/μL.