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Development of a one-step real-time RT-PCR assay using a minor-groove-binding probe for the detection of duck Tembusu virus

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A B S T R A C T

The design and development of a 3′-conjugated minor-groove-binding (MGB) probe for a real-time RT-PCR assay allowing for the rapid, sensitive, and specific detection of duck Tembusu virus (DTMUV) RNA are described. This assay targeted the 3′ terminal non-coding region (NCR) of the TMUV genome and detected 1×10^1 copies of RNA per reaction without cross-reaction with other duck pathogens. The linear range of detection was 2×10^1–2×10^8 copies/μl. The assay was rapid, requiring just over 2.0 h, including the nucleic acid extraction step. Therefore, this assay is an excellent tool for research routine diagnostic applications, and study of the epidemiology of TMUV infections among duck flocks.

1. Introduction

In 2010, a novel flavivirus emerged in eastern China and caused an extensive outbreak among egg-laying and breeder ducks. As a result, there was a significant decline in egg production within 1–2 weeks after infection. There was a high morbidity (up to 90%) and mortality rate (5–30 percent). This viral disease has resulted in a serious economic loss for the Chinese duck industry. The causative agent has been isolated and sequenced. Based on the species criterion of the flavivirus genus (Kuno et al., 1998), the virus was classified as a new Tembusu virus (TMUV), the Ntaya virus (NTAV) group, which is a mosquito-borne flavivirus cluster (Cao et al., 2011). This was the first reported case of a zoonotic flavivirus causing severe duck disease.

TMUV is a spherical, enveloped virus of approximately 45 nm in diameter (Yun et al., 2012b). The genome consists of single-stranded, positive-sense RNA of 10,990 bp with a unique open reading frame (ORF) encoding one large polyprotein (Yun et al., 2012a). The polyprotein is cleaved co- and post-translationally by viral and cellular proteases to form 3 structural proteins and 7 nonstructural proteins. The ORF is flanked by a type-1 capped 5′ terminal non-coding region (NCR) and a 3′ terminal NCR.

Flaviviruses can cause serious diseases in both humans and animals. However, flaviviruses that cause only poultry disease are rare. They include a chick-origin TMUV among broiler chicks originally named Sitiawan virus (Kono et al., 2000) and the Israel turkey meningoencephalitis virus among geese and turkeys (Barnard et al., 1950). The duck TMUV (DTMUV) has proven to be highly pathogenic for Pekin ducks, Cherry Valley Pekin ducks, and Shaoxing ducks. Clinical investigations demonstrated the DTMUV can also affect several other species of ducks and geese (Cao et al., 2011; Yun et al., 2012a). However, it remains unclear whether the pathogenicity of DTMUV towards birds and human differs between strains.

Detection of TMUV infection among duck flocks and differentiating it from other diseases resulting in poor egg production are major challenges that necessitate appropriate diagnostic methods. Currently, TMUV is diagnosed based on epidemiological information, clinical symptoms, pathological changes, and viral isolation.

Novel probes, termed minor-groove-binding (MGB) probes, have been developed (Afonina et al., 2002; Belousov et al., 2004). MGB probes contain an MGB moiety, a molecule that stabilizes the hybridization of the probe with single-stranded DNA targets. By doing so, it increases the specificity and sensitivity of the hybridization by raising the melting temperature and reducing the length of the probe (Kutyavin et al., 2000). Hybridization of MGB probe to a specified target increases the distance between the fluorophore

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Table 1
Primers and probes designed in the study.

Primer	Sequence (5′–3′)	Positions in genome[a]	Amplicon size (bp)
F1	GCATTTGTTTGAAGAGATAG	10440–10459	551
R1	AGACTCTGTGTTCTACCACC	10971–10990	
F2	AAGTCAGGCCAGGGAATCC	10467–10485	96
R2	CATGCACCCAGATTTGTTAACC	10542–10562	
P2	FAM-CCGTCATCCAACATC-MGB	10495–10509	

[a] Position of primers and probes within the genome sequence of DTMUV (relative to accession number JF270480 and JF459991).

and its quencher, thus resulting in a detectable increase in fluorescence. This provides greater accuracy in quantitation; therefore, MGB TaqMan probes are now used for wide range of real-time PCR applications (Caroline et al., 2002; Ala et al., 2004; McMenamy et al., 2011). The present study describes the development and optimization of an MGB-probe assay for rapid and sensitive detection of DTMUV.

2. Materials and methods

2.1. Virus strains and sample preparation

The DTMUV strains used in this study (YY5, ZJ-6) were isolated using 9-day-old embryonated specific-pathogen free (SPF) eggs. Infectious allantoic sac was harvested 3–5 days post-inoculation. The virus was then cultured in a BHK-21 cell line. A monolayer of BHK-21 cells was inoculated with 100 μl of egg-propagated virus and incubated at 37 °C for 1 h to allow virus adsorption. The infectious culture supernatant of BHK-21 cells was harvested 48–72 h post-inoculation.

To assess the specificity of the MGB assay for detecting DTMUV, duck enteritis virus (DEV), duck parvovirus (DPV), duck hepatitis A virus type 1 (DHAV-1), avian influenza virus (AIV, H9N2), duck paramyxovirus (DPMV), and duck reovirus (DRV) were propagated, and the nucleic acid was extracted (Liu et al., 2007; Yang et al., 2008). Total RNA from BHK-21 cell lines was used as a negative control.

2.2. RNA extraction

Total RNA was isolated from the 140-μl cell culture supernatant or from 140 μl of allantoic fluid, and BHK-21 cell using the QIAamp Viral RNA kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

2.3. Primer and probe design

DTMUV YY5 and ZJ-6 were previously sequenced by the Avian Disease Lab of Zhejiang Academy of Agricultural Sciences (GenBank accession nos. JF270480 and JF459991). Conventional RT-PCR was carried out using DTMUV (YY5) as a template with primers targeting a region in the 3′ NCR (F1 and R1) to generate a product of 557 bp. The product was sequenced and was aligned with the other DTMUV sequences in GenBank. Based on the sequencing results, specific primers (F2 and R2) and a TaqMan-MGB fluorescent probe were designed for use in real-time RT-PCR (rRT-PCR), and generated a 96 bp amplicon. The probe was labeled with the fluorescent reporter FAM at the 5′ terminus; the non-fluorescent quencher was positioned at the 3′ terminus. Table 1 lists the sequences of the primers and probe.

BLAST analyses were performed on the primer and probe sequences to identify any potential cross-homologies with other nucleotide sequences in Genbank (http://www.ncbi.nlm.nih.gov/). The primers and MGB probe were obtained from GeneCore (Shanghai, China).

2.4. Preparation of an RNA standard by in vitro transcription

Conventional RT-PCR was performed to generate the standard viral RNA using primers F1 and R1, as described above. The amplicon was purified from an agarose gel with the Takara Agarose Gel DNA Purification kit according to manufacturer's protocol (Takara, Dalian, China). The purified 551-bp cDNA fragment was cloned into the pGEM®-T vector according to manufacturer's instructions (Promega, Madison, WI, USA). The identity of the insert was confirmed by sequencing, and the plasmid was subjected to in vitro transcription using the MEGAscript® T7 in vitro transcription kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). The transcribed RNA was treated with TURBO DNase. The RNA was then purified using a MEGAclear™ Purification Kit according to the manufacturer's instructions (Ambion, Austin, TX, USA) to remove excess nucleotides and pyrophosphates. The concentration of the purified RNA was measured in duplicate by spectrophotometry at 260 nm (Bio-Rad, Hercules, CA, USA). The concentration was adjusted to a uniform number of RNA molecules (Fronhoffs et al., 2002). Copy numbers of the RNA standards were calculated. Serial dilutions from 10^{10} to 10^{0} copies/μl were prepared in DEPC-water and were stored in RNase-free tubes (Eppendorf, Cambridge, UK).

2.5. Real-time RT-PCR with the MGB probe

The reaction was conducted in a total volume of 20 μl containing 10 μl 2× One-Step RT-PCR Buffer III (TaKaRa, Dalian, China), 0.4 μl EX Taq HS DNA Polymerase (5 U/μl, TaKaRa, Dalian, China), 0.4 μl RT Enzyme MixII (TaKaRa, Japan), 10 μM primers (F2 and R2), 10 μM TaqMan MGB probe, 2 μl RNA template, and DEPC-treated water to a final volume of 20 μl. The concentration of primers and the probe were optimized. Real-time PCR was performed in a 96-well plate using the ABI 7300 Real-Time PCR System (ABI, Forster, CA, USA) with the following thermal cycling conditions: 5 min at 42 °C, 10 s at 95 °C, and 40 cycles of 5 s at 95 °C and 31 s at 60 °C. Each assay included at least 2 negative controls (DEPC water) in place of an RNA template. The fluorescence data were analyzed after the completion of 40 cycles.

2.6. Sensitivity of the one-step rRT-PCR assay

To determine the detection limit of the rRT-PCR assay, serial 10-fold dilutions of standard RNA (1×10^{8}–1×10^{1} copies/μl) were prepared with DEPC-treated water. The standard RNA was amplified under the optimum conditions with 2 μl per reaction. The results for each concentration were measured by fluorometric analysis.

2.7. Reproducibility of the one-step rRT-PCR assay

The reproducibility of the assay was determined using a standard method (Mohamed et al., 2006). The RNA standard was diluted to 1×10^{7}, 1×10^{5}, 1×10^{3}, and 1×10^{1} copies/μl. Each dilution was tested under optimum conditions in triplicate using 2 μl of RNA per reaction. This was repeated 3 times. To determine the intra-assay and inter-assay variation, the mean, standard deviation (S.D.), and coefficient of variation (CV) were calculated separately for each standard RNA dilution using threshold cycle (Ct) values. To determine the intra-assay reproducibility, the amplifications were repeated on three different days. The intra- and inter-assay