Rapid detection and quantification of different European IBV strains by quantitative real-time RT-PCR

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Infectious bronchitis virus (IBV) is a highly infectious and contagious pathogen of chickens worldwide. While infectious bronchitis (IB) is considered primarily a disease of the respiratory system, different IBV strains may show variable tissue tropisms and also affect the oviduct and the kidneys, with serious consequences. It is important to rapidly differentiate IB from other respiratory diseases. In this study, we would like to assess the accuracy of a quantitative RT-PCR (qRT-PCR) targeting the 5´-UTR conserved region of IBV genome to detect and quantify the most widespread field IBV European strains. Allantoic fluid of specific-pathogen-free (SPF) chicken embryos infected with the M41 reference strain and different European IBV serotypes, including Italy 02, QX and 793B isolates were tested. In addition, field-collected tissue samples of the trachea, kidney and caecal tonsils from chickens infected with different European IBV serotypes were also assessed by qRT-PCR. The assay amplified all IBV isolates with the limit of detection of 10 template copies per reaction. Field samples were also positive. The real time PCR targeting 5´ untranslated region of IBV can be successfully used to detect IBVs directly from tissue samples infected with different European field isolates.

Keywords: infectious bronchitis virus, qRT-PCR, poultry.

El virus de la bronquitis infecciosa aviar (IBV) es un patógeno altamente infeccioso y contagioso que afecta a pollos mundialmente. Aunque la bronquitis infecciosa se considera principalmente una enfermedad del tracto respiratorio, algunas cepas de IBV muestran variable tropismo al oviducto y riñones, produciendo serias consecuencias. Es importante diferenciar rápidamente la bronquitis infecciosa de otras enfermedades respiratorias. En el presente estudio, el objetivo fue valorar la capacidad de una RT-PCR cuantitativa (qRT-PCR), que presenta como diana la región conservada 5'-UTR del genoma de IBV, para detectar y cuantificar las cepas de campo de IBV más prevalentes en Europa. El líquido alantoideo de embriones de pollo libres de patógenos específicos (SPF) infectados con la cepa de referencia M41 y diferentes aislados de IBV europeos, incluyendo Italy 02, QX y 793B, fueron testados. Además, muestras de tráquea, riñón y tonsila cecal obtenidas de campo de pollos infectados con diferentes serotipos de IBV europeos también fueron incluidas en el estudio. La qRT-PCR amplificó todos los aislados de IBV con un límite de detección de 100 copias de genoma por reacción. Las muestras de campo también resultaron positivas. La qRT-PCR que presenta como diana la región 5'-UTR del genoma de IBV puede ser utilizada satisfactoriamente para detectar IBV directamente en muestras de campo obtenidas de animales infectados con diferentes aislados europeos de IBV.

Palabras clave: virus de bronquitis infecciosa, qRT-PCR, aves de producción.

Introduction

Avian infectious bronchitis (IB) is a disease of serious economic importance for the poultry industry worldwide. The disease affects the respiratory and reproductive tract as well as the renal system of chickens and hens, causing respiratory disease, reduction in weight-gain and usually life-long decrease of egg laying performance. The causative agent, infectious bronchitis virus (IBV), a member of *Coronaviridae* family, has a positive-sense single stranded RNA genome of approximately 27.6 kilobases (kb) in length (Jackwood and Wit, 2013). A large number of serotypes and genotypes of IBV are present worldwide. These different subtypes are thought to be generated by nucleotide insertions, deletions, point mutations or even recombination mainly in the S1 subunit gene (Moore *et al.*, 1998). Because some serotypes do not cross-protect against antigenically unrelated serotypes (Kwon *et al*., 1993), IBV outbreaks occur regularly despite widespread use of vaccination (Karaca *et al*., 1993).

Traditionally, IBV serotypes were identified by the virus-neutralization (VN) test or by the hemagglutination-inhibition (HI) test. However, these techniques are labor-intensive and time consuming (Kwon *et al*., 1993). Nowadays, molecular biology techniques such as reverse transcription-polymerase chain reaction (RT-PCR) for amplification of the S1 subunit followed by nucleotide sequencing have become more commonly used because of their short turn-around time and high degree of specificity. In 2006, Callison and coworkers developed a real-time quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) diagnostic test to detect and quantify the most common IBV in the United States. The assay was designed to amplify a 143-bp product in the 5′-UTR of the IBV genome. In this study, we would like to assess the accuracy of this qRT-PCR targeting the 5´-UTR conserved region of IBV genome to detect and quantify the most widespread field IBV European strains.

Materials and methods

Viruses. A reference Massachusetts IBV strain as well as pathogenic viral stocks of the 4/91, Italy 02 and QX serotypes available in our laboratory were used.

Clinical samples. Clinical specimens obtained from 2012 to 2014 that were previously subjected to IBV detection and genotyping tests were included in this study. Tracheal, kidney and cecal tonsil tissue samples which were ensured to be positive Mass, 4/91, Italy 02 and QX IBV subtypes were tested.

Primers and probe. We used primers and probe for a universal IBV qRT-PCR assay designed previously (Callison *et al*., 2006). A forward primer IBV5_GU391 (5´-GCT TTT GAGCCT AGC GTT-3´) located at nucleotide positions 391–408 of the IBV M41 strain genome sequence (GenBank accession no. AY851295); a reverse primer IBV5_GL533 (5´-GCC ATG TTG TCA CTG TCT ATT G-3´) located at nucleotide positions 533–512 of the IBV M41 strain genome sequence, and a Taqman® dual-labeled probe IBV5_G probe (5´-FAMCACCAC CAG AAC CTG TCA CCT C-BHQ1-3´) located at nucleotide positions 494–473 of the IBV M41 strain genome sequence were used to amplify and detect a 143-bp fragment of the 5´-UTR gene.

IBV quantitation standard. To amplify the target gene, RT-PCR was performed using 2 µl of M41 strain RNA, 2.5 µl of 10X PCR buffer (Applied Biosystems, CA, US), dNTP (Applied Biosystems, CA, US), 10 µmol each of the primers and 5 U AmpliTaq Gold (Applied Biosystems, CA, US) in a

total volume of 25 µl. Sample RNA was first reverse transcribed to cDNA at 48°C for 30 min. PCR cycling conditions began with an initial hold at 95°C for 10 min, followed by 35 cycles consisting of 94°C for 30 s, 50°C for 30 s and 68°C for 15 s and a final step of 68ºC for 10 min.

RT-PCR amplification product was examined by agarose-gel electrophoresis. After ethidium bromide staining, the band was visible only at the expected molecular weight. Subsequently, the 5´-UTR PCR amplicon (nt. 391–533 of the IBV M41 strain genome sequence) was cloned in a pGEM-T Easy plasmid vector (Promega, WI, US) and then transfected into competent *E.coli* cells. Plasmid DNA was purified from E.coli using the PCR purification kit (Qiagen, CA, US). The cloned UTR region was sequenced and confirmed to be identical to the IBV M41 strain genome sequence present in Genbank (GenBank accession no. AY851295). Concentration of the plasmid was determined by photometry at 260 nm and calculated as genome equivalents (copies)/ml as the molecular weight of the plasmid was known. Serial dilutions of the plasmid ranging from 1×10^{1} to 1×10^{8} copy/ml were used for generating the standard curve**.**

RNA extraction and qRT-PCR assay. Viral RNA was extracted from 150 µl of allantoic fluids or homogenized tissue samples using Nucleospin RNA kit (Macherey- Nagel, Düren, Germany) according to manufacturer´s instructions. As previously described by Roh *et al.* (2014), the AgPath-IDTM One-step RT-PCR kit (Ambion Inc., TX, US) was used to perform qRT-PCR assays according to manufacturer's instructions. The 25-ml qRT-PCR reaction mixture included 12.5µl 2X RT-PCR buffer, 1 µl of each primer with a concentration of 10µM, 0.4 µl of probe, 1 ml of 25X RT PCR enzyme mix and 5 ml of viral RNA. The qRT-PCR reactions were performed on the Applied Biosystems H 7500 Fast Realtime PCR system (Life Technologies Ltd., Carlsbad, CA) under the following conditions: one cycle of 50 C for 30 min and 95 C for 15 min followed by 40 cycles of 94 C for 1 sec and 60 C for 60 sec. The assay included negative controls for the qRT-PCR reaction and for RNA extraction. Viral RNA copies in allantoic fluids and clinical tissue samples were determined by extrapolation to a standard curve generated upon amplification of serial dilutions of the IBV 5´ UTR gene of M41 reference strain.

Results and discussion

The qPCR assay has engendered wider acceptance compared to other techniques due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination (Mackay *et al.*, 2002). The results obtained by the use of universal primers and probe sets in qRT-PCR assays for IBV genome detection are comparable to those obtained by challenge-virus detection in embryonated eggs. Furthermore, in some IBV types the qRT-PCR assay has been reported to be more sensitive (Roh *et al*, 2014.). Despite the use of primers targeting the S1 gene of IBV genome, the extensive variability and mutation rates in this gene make it easier to produce false negative results in RT-PCR techniques. In comparison, 5´UTR of the IBV genome is known to be the highly conserved (Callison *et al*., 2006). In our study, a real time Taqman®-based RT-PCR assay targeting 5´UTR region of IBV genome was used for detection and quantification of IBV genomic RNA directly from tissue samples (trachea, kidney, cecal tonsil) collected from broilers infected with different European IBV serotypes.

The assay amplified a 143-bp product in the 5´UTR region of IBV genome. A standard curve obtained upon amplification of serial dilutions of the IBV 5´ UTR gene of M41 reference strain was then generated. Despite the difference in initial RNA concentrations, the qRT-PCR primer-probe set specific for 5´UTR was capable to detect Massachusetts reference IBV isolate and also different European IBV field isolates from allantoic fluids. More important, it was able to quantify the IBV genome directly from the tissue samples tested. The unknown quantities of virus in the allantoic fluids and clinical specimens were plotted against the standard curve (Figure 1). The allantoic fluid from Massachusetts reference IBV isolate and three European IBV isolates belonging to QX, Italy 02 and 4/91 genotypes presented Ct values between 11,52 and 13,92. All IBV tissue samples subjected to the qRT-PCR assay also presented a specific fluorescence signal and Ct values between 16,80 and 31,84,

depending of the type of sample tissue. Kidney showed the lower Ct value (16,80), whereas cecal tonsils presented the higher ones (30,15 to 31,84).

Additionally, the assay had a limit of detection and quantification of 100 template copies per reaction. These results are in agreement with the obtained by Callison *et al.* (2006), whom reported the capacity of this assay to detect the 15 American strains of IBV tested as well as two Turkey coronavirus strains.

In conclusion, the qRT-PCR targeting 5´ untranslated region of IBV can be successfully used to detect IBVs directly from tissue samples infected with different European field isolates.

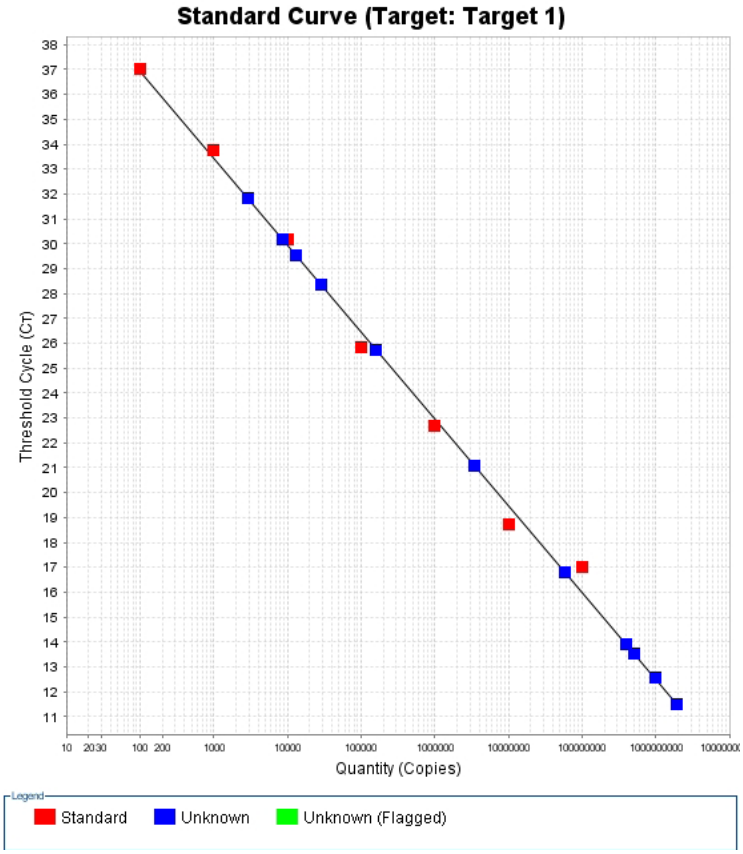

Figure 1. Standard curve generated using serially diluted M41 IBV 5 UTR . Ct, cycle threshold.

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