Polish Journal of Veterinary Sciences Vol. 20, No. 3 (2017), 599–601

DOI 10.1515/pjvs-2017-0073

Short communication

Differentiation of infectious bronchitis virus vaccine strains Ma5 and 4/91 by TaqMan real-time PCR

T. Stenzel, D. Dziewulska, M. Śmiałek, B. Tykałowski, J. Kowalczyk, A. Koncicki

Department of Poultry Diseases, Faculty of Veterinary Medicine,
University of Warmia and Mazury in Olsztyn, Oczapowskiego 13, 10-719 Olsztyn, Poland

Abstract

The aim of this study was to develop rapid molecular assays for differentiating vaccine strains Ma5 and 4/91 of the infectious bronchitis virus (IBV). Specific primers and probes for S1 and N genes were designed based on the nucleotide sequences of both vaccine strains. Cross-reactivity was not observed. Assay sensitivity was 2.373 x 10^3 copies of the Ma5 strain, and 3.852 x 10^3 copies of the 4/91 strain. Samples belonging to a known genotype demonstrated that the designed assays supported rapid and sensitive detection of Ma5 and 4/91 vaccine strains of IBV.

Key words: infectious bronchitis virus, Ma5, 4/91, TaqMan real-time PCR, vaccination

Introduction

Infectious bronchitis (IB) is a highly contagious viral disease that occurs globally in chickens (Cavanagh and Gelb 2008). It is caused by an infectious bronchitis virus (IBV) of the family *Coronaviridae*. The IBV is a single-stranded RNA virus (Boursnell et al. 1987) with high genetic diversity, which increases the variability of the antigenic properties of different IBV strains (Cavanagh and Gelb 2008, Sjaak et al. 2011). Genetic diversity in IBV strains results from mutations in the gene encoding the S1 subunit of the spike glycoprotein which is responsible for viral immunogenicity and contains virus-neutralizing epitopes (Koch et al. 1990, Cavanagh et al. 1992). These mutations partially explain IB outbreaks in vaccinated flocks of chickens (Wickramasinghe et al. 2014). The selection of appropriate vaccine IBV strains and effective vaccination methods play a crucial role in IB prevention. Vaccines with selected genotypes impart effective protection against homologous viral strains and partial protection against strains with other genotypes (Sjaak et al. 2010). However, vaccination with two genetically distinct vaccine strains such as Mass (of Massachusetts-like strain) and 4/91 (of 4/91-like strain) can provide broad cross-protection against heterologous IBV strains (Cook et al. 1999). The genotype of IBV isolates can be verified by analyzing the sequence of the S1 gene, but a molecular assay for differentiating IBV strains is difficult to design due to high variability in this genomic region. The nucleocapsid (N) gene is more conserved, but its sequence can also be used in genotyping (Meir et al. 2010). The aim of this study

Correspondence to: T. Stenzel, e-mail: tomasz.stenzel@uwm.edu.pl

Table 1. The characterization of IBV strains used during investigation and results of TaqMan qPCR amplification and S1 gene sequencing. The RNA of strains marked with an asterisk was isolated directly from vaccines, and the RNA of the residual strains was obtained from the National Veterinary Research Institute in Pulawy (Poland).

Strain	Source	Original classification	TaqMan qPCR amplification		S1 gene sequencing
			Ma5	4/91	
Nobilis Ma5*	vaccine	Mass-like	+	–	Ma5
PL.G072/12	tracheal swabs	Mass-like	+	–	Ma5
PL.G206/14 K1	embryos	Mass-like	+	–	–
PL.IB.12.06.1980	embryos	Mass-like	+	–	–
Nobilis 4/91*	vaccine	4/91-like	–	+	4/91
PL.G255K/97	embryos	4/91-like	–	–	Variant strain partially similar to the 4/91-like vaccine strain
PL.G049/12	tracheal swabs	4/91-like	–	–	–
PL.G096/15	intestines	4/91-like	–	+	4/91
PL8G089/15	intestines	4/91-like	–	+	4/91

was to develop a rapid and sensitive method for differentiating IBV vaccine strains Ma5 and 4/91.

Materials and Methods

Genetic material was isolated from Nobilis IB Ma5 and Nobilis IB 4/91 (MSD Animal Health) commercial vaccines with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. A 1700 bp fragment of the S1 gene and a 1300 bp fragment of the N gene were amplified according to the procedure described by Meir et al. (2010) with the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies) for the reverse transcription step and with the Hot Star Taq Plus Master Mix kit (Qiagen) for PCR. PCR products were sequenced by Genomed (Poland), and specific primers and probes for TaqMan real-time PCR (quantitative PCR, qPCR) were designed based on the obtained sequences. The following primers and probes were used: for the 4/91 strain amplifying a 111 bp fragment of the N gene: 4/91F 5' – CAAGTCAGCAGCATGGATATTG – 3', 4/91R 5' – GCTGGTCCTGTTC CAGTATAG – 3' and 4/91Probe: 5' – 6-FAM AGACGCCAACACCGGTATAAACCA BHQ-1 – 3'; for the Ma5 strain amplifying a 101 bp fragment of the S1 gene: Ma5F 5' – CTCTTCACCTGGG TGTATTGTT – 3', Ma5R 5' – AGACAAAGCCATACCTGATGAC – 3' and Ma5Probe: 5' – 6-FAM TGGTGGTCGTGTTG TTAATGCTT CTTCT BHQ-1 – 3'. The composition of the reaction mixture was as follows: 10 µL of TaqMan® Fast Universal PCR Master Mix (2X), no AmpErase® UNG (Life Technologies), 1.8 µL of each primer (conc. 10 µM), 2 µL of the probe (conc. 2.5 µM), 1.4 µL of deionized water and 3 µL of cDNA. TaqMan real-time PCR was conducted in the LightCycler 96 (Roche) thermocycler at 95°C for 30 s, followed by 45 cycles at 95°C for 15 s, and 60°C for 60s. The reaction mixture for Ma5 and 4/91 assays was identical, and both assays were performed as uniplex q-PCR in the same run.

Assay sensitivity was determined by preparing the standard curve. In the first step, S1 and N gene fragments were amplified according to the procedure described by Meir et al. (2010). The amplicons contained sequences complementary for Ma5 and 4/91 strain primers and probes. Buffer and nucleotides residues were removed with a commercial kit (Clean-Up, A&A Biotechnology), and amplicon concentration was measured using a spectrophotometer (NanoDrop 2000, Thermo Scientific). The gene copy number was calculated based on amplicon concentration and size with a copy number calculator (University of Rhode Island, Genomics and Sequencing Center). Standard ten-fold serial dilutions of amplicons were used as template DNA. Aliquots of each dilution (initial dilution: 10^9, final dilution: 10^2) were subjected to TaqMan real-time PCR to determine the LOD (Limit of Detection) of DNA copies. After reaction optimization Polish IBV field strains belonging to Ma5-like (n=3) and 4/91-like (n=4) strains were tested by both assays. Field strains were obtained from the National Veterinary Research Institute in Pulawy (Poland) as eluted RNA. The samples are listed in Table 1. The S1 gene of the analyzed vaccine and field strains was additionally amplified by RT-PCR described by Meir et al. (2010) to verify the results of TaqMan real-time PCR, and the products were sequenced (Genomed). The resulting sequences were aligned in the Lasergene v 8.1.5 application (DNASTAR) and compared using the Clustal W method and Mega 5.2 software (Tamura et al. 2011).

Results and Discussion

Primers and molecular probes for differentiating the analyzed vaccine strains were designed according to the method of Meir et al. (2010) which supports the development of a sensitive molecular assay for IBV detection based on the amplified fragment of the N gene. We were unable to design primers and molecular probes based on a single gene or its fragment without cross amplification between Ma5 and 4/91 vaccine strains. For this reason, we designed primers and probes amplifying a fragment of the S1 or N gene in only one of the tested isolates. For the Ma5 vaccine strain, this was a 101 bp fragment of the S1 gene, and for the 4/91 vaccine strain, this was a 111 bp fragment of the N gene. The amplification of vaccine genetic material revealed that both assays were specific for the analyzed IBV vaccine strain. Assay sensitivity was determined at 2.373 x 10^3 copies of the Ma5 virus and 3.852 x 10^3 copies of the 4/91 virus. Three of the tested Polish field isolates (PL.IB.12.06.1980, PL.G206/14K1 and PL.G072/12) responded positively in the Ma5 assay and two (PL8G089/15 and PLG096/15) responded positively in the 4/91 assay (Table 1). Two field isolates of 4/91-like strains were not amplified in either assay. The amplification of the 1700 bp product of the S1 gene in three samples (one positive in the Ma5 assay and two not amplified in either assay) also failed, probably due to the high variability of the S1 gene or sample degradation. Sequencing and phylogenetic analyses revealed that all samples that were successfully amplified in 4/91 or Ma5 assays belonged to those strains. Only one sample that was not amplified in either assay was successfully sequenced, and the phylogenetic analysis revealed that this strain belonged to a variant genotype that was partially related to the 4/91 vaccine strain. The designed assays supported rapid differentiation of IB viruses belonging to Ma5 and 4/91 vaccine strains. In both assays, the problems with field strain amplification could be attributed to the high variability of the IB virus (Ladman et al. 2006) and high assay specificity for vaccine strains, probably because molecular probes were designed based on the genetic material isolated from commercial vaccines. Our findings suggest that the designed molecular assays could be useful for laboratory investigations of the examined vaccine strains and for verifying the effectiveness of IBV vaccinations based on Ma5 and 4/91 strains.

Acknowledgments

The authors would like to thank Dr. Katarzyna Domańska-Blicharz of the National Veterinary Research Institute in Pulawy (Poland) for providing genetic material of Polish field IBV strains for this study.

Publication supported by KNOW (Leading National Research Centre) Scientific Consortium "Healthy Animal – Safe Food", decision of the Ministry of Science and Higher Education No. 05-1/KNOW2/2015.

References

Boursnell ME, Brown TD, Foulds IJ, Green PF, Tomley FM, Binns MM (**1987**) Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J Gen Virol 68: 57-77.

Cavanagh D, Gelb J (**2008**) Infectious bronchitis. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE (eds). Diseases of Poultry 12th ed., Blackwell Publishing Professional, Ames, Iowa, USA, pp 117-135.

Cavanagh D, Davis PJ, Cook JK, Li D, Kant A, Koch G (**1992**) Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathol 21: 33-43.

Cook JK, Orbell SJ, Woods MA, Huggins MB (**1999**) Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. Avian Pathol 28: 477-485.

Koch G, Hartog L, Kant A, van Roozelaar DJ (**1990**) Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. J Gen Virol 71: 1929-1935.

Ladman BS, Loupos AB, Gelb J Jr. (**2006**) Infectious bronchitis virus S1 gene sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. Avian Pathol 35: 127-133.

Meir R, Maharat O, Farnushi Y, Simanov L (**2010**) Development of a real-time TaqMan RT-PCR assay for the detection of infectious bronchitis virus in chickens, and comparison of RT-PCR and virus isolation. J Virol Methods 163: 190-194.

Sjaak de Wit JJ, Cook JK, van der Heijden HM (**2011**) Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathol 40: 223-235.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (**2011**) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731-2739.

Wickramasinghe IN, van Beurden SJ, Weerts EA, Verheije MH (**2014**) The avian coronavirus spike protein. Virus Res 194: 37-48.