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Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens

Scott A. Callison [a], Deborah A. Hilt [a], Tye O. Boynton [b], Brenda F. Sample [c], Robert Robison [d], David E. Swayne [e], Mark W. Jackwood [a,*]

[a] *Department of Population Health, Poultry Diagnostic and Research Center, 953 College Station Road, University of Georgia, Athens, GA 30602, USA*
[b] *Department of Microbiology, University of Georgia, Athens, GA 30602, USA*
[c] *Agriculture Research Laboratory, University of Delaware, Georgetown, DE 19947, USA*
[d] *Maryland Diagnostic Laboratory, Salisbury, MD 21801, USA*
[e] *USDA, Agricultural Research Service, Southeast Poultry Research Laboratory, 934 College Station Road, Athens, GA 30605, USA*

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Abstract

It is important to rapidly differentiate infectious bronchitis virus (IBV) from disease agents like highly pathogenic avian influenza virus and exotic Newcastle disease virus, which can be extremely similar in the early stages of their pathogenesis. In this study, we report the development and testing of a real-time RT-PCR assay using a Taqman®-labeled probe for early and rapid detection of IBV. The assay amplifies a 143-bp product in the 5′-UTR of the IBV genome and has a limit of detection and quantification of 100 template copies per reaction. All 15 strains of IBV tested as well as two Turkey coronavirus strains were amplified, whereas none of the other pathogens examined, tested positive. Evaluation of the assay was completed with 1329 tracheal swab samples. A total of 680 samples collected from IBV antibody negative birds were negative for IBV by the real-time RT-PCR assay. We tested 229 tracheal swabs submitted to two different diagnostic laboratories and found 79.04% of the tracheal swabs positive for IBV by real-time RT-PCR, whereas only 27.51% of the samples were positive by virus isolation, which is the reference standard test. We also collected a total of 120 tracheal swabs at six different time points from birds experimentally infected with different dosages of IBV and found that, independent of the dose given, the viral load in the trachea plateau at 5 days post-inoculation. In addition, an inverse relationship between the dose of virus given and the viral load at 14 days post-inoculation was observed. Finally, we tested 300 total tracheal swab samples, from a flock of commercial broilers spray vaccinated for IBV in the field. The percentage of birds infected with the IBV vaccine at 3, 7, and 14 days post-vaccination was 58%, 65%, and 83%, respectively, indicating that only slightly more than half the birds were initially infected then the vaccine was subsequently transmitted to other birds in the flock. This observation is significant because coronaviruses, which have a high mutation rate, can revert to pathogenicity when bird-to-bird transmission occurs. The real-time RT-PCR test described herein can be used to rapidly distinguish IBV from other respiratory pathogens, which is important for control of this highly infectious virus. The test was extremely sensitive and specific, and can be used to quantitate viral genomic RNA in clinical samples.

Keywords: Real-time RT-PCR; IBV; Diagnostic test; Coronavirus

1. Introduction

Infectious bronchitis (IB) is a highly infectious disease of the upper-respiratory tract in chickens (Cavanagh and Naqi, 2003). It can also affect the kidneys and reproductive tract. It is of economic importance to the poultry industry due to the high morbidity and production losses associated with the disease. The etiologic agent of IB is infectious bronchitis virus (IBV), an enveloped, positive-sense, single-stranded RNA virus that belongs to the Coronaviridae family. The virus contains four structural proteins; nucleocapsid surrounding the viral RNA, an integral membrane glycoprotein, a small envelope protein, and a spike glycoprotein located on the surface of the viral envelope, which contains epitopes that induce virus-neutralizing and serotype-specific antibodies.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IB, infectious bronchitis; IBV, infectious bronchitis virus; PDRC, Poultry Diagnostic and Research Center; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcriptase-polymerase chain reaction

* Corresponding author. Tel.: +1 706 542 5475; fax: +1 706 542 5630.
E-mail address: mjackwoo@uga.edu (M.W. Jackwood).

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Although control of IBV is primarily through the use of live attenuated vaccines, the disease is difficult to control because different serotypes of the virus do not cross-protect. Therefore, it is imperative to quickly and accurately detect the presence of the virus within an infected poultry flock so that subsequent flocks can be properly vaccinated. It is also important to rapidly differentiate IBV infections from other upper-respiratory diseases like avian influenza, Newcastle disease, infectious laryngotracheitis, and avian mycoplasmosis so that appropriate measures against those diseases can be taken in a timely manner.

Current diagnostic assays for IBV include virus isolation in embryonating eggs, tracheal organ culture, or cell culture immunoassays, and molecular assays that detect the viral RNA (Gelb and Jackwood, 1998). Virus isolation is considered the reference standard; however, it is expensive and time consuming because several passages may be required to detect the virus. Immunoassays use IBV-specific monoclonal antibodies to detect the virus in direct or indirect fluorescent antibody and enzyme-linked immunosorbent assay (ELISA) formats. Although faster and simpler than virus isolation, immunoassays tend to lack specificity and sensitivity and none detect all strains or types of IBV (Karaca and Naqi, 1993; Karaca et al., 1992; Naqi et al., 1993). Molecular assays for the detection of IBV are commonly used because they provide highly specific and sensitive results in a timely manner. Molecular assays use the reverse transcriptase-polymerase chain reaction (RT-PCR) to detect viral RNA directly from a clinical sample or from virus isolated in a laboratory host system. When RT-PCR is used to amplify the spike glycoprotein of IBV, it can be coupled with restriction fragment length polymorphism (RFLP) or nucleic acid sequencing to identify the type of the virus (Cavanagh et al., 1990; Jackwood et al., 1997; Keeler et al., 1998; Kingham et al., 2000; Kwon et al., 1993).

The constant threat of globally important diseases like avian influenza and Newcastle disease, which must be differentiated from IB, in diagnostic investigations, makes it extremely important to rapidly identify the causative agent of any upper-respiratory disease or changes in egg shell quality and egg production in chickens. In this study, we developed a TaqMan®-based real-time RT-PCR assay for rapid detection of IBV viral RNA directly from clinical samples. We examined the sensitivity and specificity of the test and evaluated it using known negative, known positive, and clinical samples from commercial chickens.

2. Materials and methods

2.1. Virus isolation and known strains

Virus isolation was performed in specific pathogen-free (SPF) 9–11-day-old embryonating chicken eggs as previously described (Gelb and Jackwood, 1998). Other viral and mycoplasma strains used in this study are listed in Table 1.

Table 1
Virus and mycoplasma strains used in this study

Pathogen[a]	Strain	Source[b]	IBV real-time assay
IBV	Massachusetts 41	PDRC	+
IBV	Arkansas DPI	PDRC	+
IBV	Arkansas 99	PDRC	+
IBV	Connecticut 46	PDRC	+
IBV	Delaware 072	PDRC	+
IBV	Holland 120	PDRC	+
IBV	Nebraska 95	PDRC	+
IBV	GA/470/98	PDRC	+
IBV	Florida	PDRC	+
IBV	GAV 4595	PDRC	+
IBV	Gray	PDRC	+
IBV	Holte	PDRC	+
IBV	Iowa 97	PDRC	+
IBV	JMK	PDRC	+
IBV	SE17	PDRC	+
AIV	HK/1073/99 H9N2	SEPRL	–
AIV	TK/PA/7975/97 H7N2	SEPRL	–
AIV	TK/VA/15851/02 H7N2	SEPRL	–
NDV	B1	SEPRL	–
NDV	LaSota	SEPRL	–
APV	–	SEPRL	–
TCoV	Rb	PDRC	+
TCoV	Tx	PDRC	+
ILTV	Vaccine	PDRC	–
Avian adenovirus	Serotype 1	PDRC	–
MG	Vaccine	PDRC	–

[a] IBV: infectious bronchitis virus, AIV: avian influenza virus, NDV: Newcastle disease virus, APV: avian paramyxovirus, TCoV: Turkey coronavirus, ILTV: infectious laryngotracheitis virus, and MG: *Mycoplasma galisepticum*.
[b] PDRC: Poultry Diagnostic and Research Center (Athens, GA), SEPRL: Southeast Poultry Research Laboratory (Athens, GA).

2.2. RNA extraction

Extraction of RNA from clinical samples was performed with the High Pure RNA Isolation Kit (Roche, Indianapolis, IN) or the RNeasy Kit (Qiagen, Valencia, CA). Extractions from allantoic fluid and tracheal swabs taken from experimentally infected birds as well as RNA extracted for sensitivity and specificity analysis and for test evaluation was conducted with the MagMax 96 Total RNA isolation kit (Ambion, Austin, TX) following the manufacturer's suggestions.

2.3. IBV real-time RT-PCR assay

A sequence alignment (Fig. 1) of the 5′-untranslated region (UTR) of six strains of IBV (GenBank accession

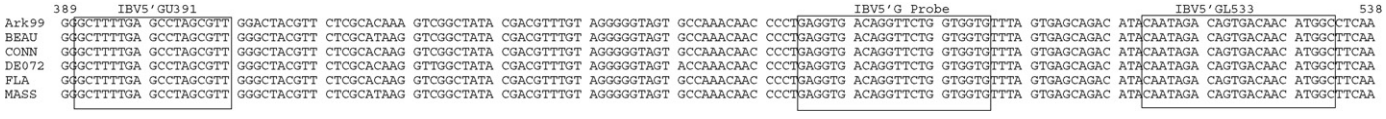

Fig. 1. Alignment of the 5′-UTR for six different strains of IBV with the forward primer (IBV5′GU391), reverse primer (IBV5′GL533), and the probe (IBV5′G Probe) boxed. Nucleotides 1 and 150 correspond to nucleotides 389 and 538, respectively, in GenBank accession no. AY851295.

nos. AY392051, M95169, AY392049, AY392054, AY392050, AY851295) was used to identify conserved sequences within this region to design primers and a probe for the real-time RT-PCR assay. A forward primer IBV5′GU391 (5′-GCT TTT GAG CCT 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′-FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1-3′) located at nucleotide positions 494–473 of the IBV M41 strain genome sequence were designed to amplify and detect a 143-bp fragment of the 5′-UTR gene. The primers were synthesized by Integrated DNA Technologies (Coralville, IA), and the probe was synthesized by BioSearch Technologies (Novato, CA). Primers and probe were utilized in a 25 μL reaction containing 12.5 μL of Quantitect Probe RT-PCR 2× mix (Qiagen, Valencia, CA), 0.25 μL of RT enzyme (Qiagen), primers to a final concentration of 0.5 μmol, probe to a final concentration of 0.1 μmol, 2.75 μL of water, and 5 μL of RNA template. The reaction was conducted in a SmartCycler (Cepheid, Sunnyvale, CA) at 50 °C for 30 min; 95 °C for 15 min with optics off; 40 cycles of 94 °C for 1 s followed by 60° C for 60 s with optics on. For each reaction, the cycle threshold (C_T) number was determined corresponding to the PCR cycle number at which the fluorescence of the reaction exceeded 30 U of fluorescence, which is the default value for the SmartCycler.

2.4. Limit of detection and quantification

Runoff RNA transcripts corresponding to the first 735 nucleotides of the Mass 41 IBV genome were generated to use as standards in the assay. Transcripts were generated from a plasmid containing the first ~5500 bp of the Mass 41 genome of IBV downstream of a T7 promoter, which was created using the 5′-primer A-Beau U30 (5′-TACACTAGCCTTGCGC-TAGA-3′) and 3′-primer A-Beau L5447 (5′-GCACGCCA-AAGTCCCATAG-3′). Briefly, RNA was extracted from the Mass 41 strain of IBV using the High Pure RNA Isolation Kit (Roche Diagnostics Corporation). The purified RNA was resuspended in diethylpyrocarbonate (DEPC) treated water and used in the RT-PCR reaction as previously described (Jackwood et al., 1997; Lee and Jackwood, 2001). The amplified product was cloned into the pCR-XL-TOPO vector (Invitrogen Inc., Carlsbad, CA) per the manufacturer's directions and sequenced to verify its accuracy. The plasmid was linearized at base pair 735 relative to the start of the genome with Xmn I (New England Biolabs, Beverly, MA). The linearized DNA was gel purified and used as template with a RiboMax T7 *In Vitro* Transcription System (Promega, Madison, WI) per the manufacturer's recommendations. The length of the runoff RNA transcripts was verified by agarose gel analysis, and the concentration was determined using a BioPhotometer (Eppendorf, Hamburg, Germany). Ten-fold serial dilutions of the runoff RNA transcripts containing 2×10^6 to 2×10^1 copies of template per microliter were made and 5 μL of each dilution was used as template in the assay. The limit of detection/quantification and reproducibility of the assay were determined by six independent runs. The limit of detection was defined as the lowest RNA concentration yielding a C_T value where the fluorescence of the reaction exceeded 30 U of fluorescence. The limit of quantification was defined as the lowest RNA concentration on the standard curve that maintained linearity. The average number of IBV genome copies for each group of experimental samples was estimated using the equation derived from the standard curve.

2.5. Sensitivity and specificity

To test the specificity of the assay, RNA or DNA from 11 different pathogens known to infect the avian upper-respiratory tract (Table 1) was used in the test. We further tested the specificity of the assay using negative samples obtained from 340 commercial layer chickens housed from 1 day of age in positive pressure Horsfal isolation units at Poultry Diagnostic and Research Center (PDRC, Athens, GA). The birds were monitored for serum antibody titers to IBV using the ProFlock® IBV ELISA kit (Synbiotics). A total of 123 serum samples were collected at 3 weeks (62 samples) and 6 weeks (61 samples) of age. Sterile polyester tipped applicators (MDCI Ltd., West Sussex, U.K.) were used to take tracheal swabs from each bird at 3 and 6 weeks of age (680 total samples). Each swab was placed into a 1.5 ml microcentrifuge tube containing 1 ml of sterile 1× PBS (pH 7.4). The tubes were mixed using a bench-top vortex, and stored at −70 °C until needed for RNA extraction.

The sensitivity of the assay was determined using clinical tracheal swab samples submitted to Delaware (Agriculture Research Laboratory, University of Delaware, Georgetown DE 19947, USA) and Maryland (Maryland Diagnostic Laboratory, Salisbury, MD 21801, USA) laboratories. Clinical samples consisting of 229 tracheal swabs from commercial chickens experiencing upper-respiratory disease, submitted as routine diagnostic cases to the Delaware (Georgetown, DE) and Maryland (Salisbury, MD) State Diagnostic Laboratories were tested by the real-time RT-PCR assay, as well as, by virus isolation at those laboratories (Gelb and Jackwood, 1998). The sensitivity was calculated with the formula: SE = TP/(TP + FN), where; SE = sensitivity, TP = True positives, which were samples positive by virus isolation (Gelb and Jackwood, 1998) and by the real-time RT-PCR assay, and FN = false negatives, which were samples positive by virus isolation and negative by the real-time RT-PCR assay.

2.6. Test evaluation

The real-time IBV RT-PCR test was evaluated with known positive samples obtained from IBV inoculated specific pathogen-free (SPF) chickens divided into four groups of 32 chickens each and housed in four positive pressure Horsfal isolation units located in four separate filtered air positive pressure rooms at PDRC (Athens, GA). At 1 day of age, blood and tracheal swab samples were taken from two birds in each group. The next day, each bird was inoculated intranasally with 50 μL of the Arkansas DPI strain of IBV with the following titers;

group 1 no virus, group 2 was given 1.6×10^1 embryo infectious dose$_{50}$ (EID$_{50}$)/mL, group 3 was given 1.6×10^3 EID$_{50}$/mL, and group 4 was given 1.6×10^5 EID$_{50}$/mL. The birds were examined twice daily for clinical signs and tracheal swabs were taken from five birds in each group on 1, 5, 10, 14, 21, and 28 days post-inoculation (d.p.i.). Tracheal swabs were placed in 200 μL of 1× PBS (pH 7.4) and stored at −70 °C until used for RNA extraction.

In addition, a total of 300 tracheal swab samples (100 birds swabbed at 3, 7, and 14 days post-vaccination) from a commercial broiler flock that was spray vaccinated with a combination Mass/Ark commercial vaccine at 14 days of age were also tested. After collection, each tracheal swab was placed into 1.5 ml microcentrifuge tubes containing 1 ml of sterile 1× PBS (pH 7.5). The tubes were mixed using a bench-top vortex and stored at −70 °C until used for RNA extraction.

3. Results

3.1. Primers and probe design

The primers and Taqman® probe targeted a highly conserved region of the 5′-UTR (Fig. 1) and amplified a 143-bp product (data not shown). The probe was designed to anneal to the same strand as the IBV5′GL533 primer. This strategy allowed for the fewest guanine residues within the probe sequence and placed the 5′-end of the probe only 17 bp away from the 3′-end of the IBV5′GL533 primer.

3.2. Limit of detection and quantification

The detection and quantification limits were determined using C_T values obtained for each reaction containing from 10^7 to 10^2 copies of the standard RNA. The values were plotted against the log of the number of template copies and a linear equation ($y = -0.282x + 11.861$) with a R^2 value = 0.997 was generated (Fig. 2). The assay maintained linearity for at least six orders of magnitude. Using the slope from the linear equation, the overall efficiency of the assay was estimated to be 91.43%.

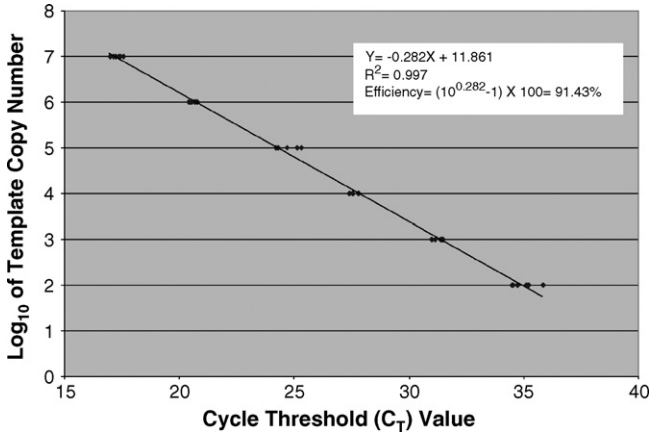

Fig. 2. The assay standard curve was generated by plotting the C_T values vs. $\log_{10}$ of 10-fold serial dilutions (10^7–10^2) of standard RNA corresponding to the 5′-UTR of the IBV genome. An overall reaction efficiency of 91.43% was estimated using the standard curve slope as indicated by the formula.

The assay was negative below 100 template copies. Therefore, the limit of detection and quantification were both determined to be 100 template copies. The standard deviation of the mean C_T values obtained for each reaction containing from 10^7 to 10^2 copies of the standard RNA calculated from six independent runs, ranged from 0.123 to 0.517 cycles.

3.3. Sensitivity and specificity

The assay amplified RNA from all 15 IBV strains and the two TCoV strains listed in Table 1, whereas no amplification signal was observed from any of the other pathogens tested. A search of the GenBank database using the BLASTN analysis program (http://www.ncbi.nlm.nih.gov/BLAST/) indicated that 16 additional strains of IBV would likely also be detected by the assay (SAIBBK, DQ288927; H52, AY392048; Partridge/GD/S14/2003, AY646283; Peafowl/GD/KQ6/2003, AY641576; CAL99, AY514485; K1699, AY561728; CU994, AY561727; CU570, AY561726; CU805, AY561725; CU-T2, AY561724; CU510, AY561723; CU705, AY561722; KO751, AY561721; MA5, AY561720; LX4, AY338732; BJ, AY319651).

Three hundred and forty commercial layers not exposed to IBV, were negative for IBV antibodies (61 serum samples) by commercial ELISA (Synbiotics) at 6 weeks of age when the experiment was terminated. Maternal antibodies were detected in 1 of 62 serum samples taken from those birds at 3 weeks of age. A total of 680 tracheal swabs (340 swabs at 3 weeks of age and 340 swabs at 6 weeks of age) taken from those birds were negative for IBV in the real-time RT-PCR assay.

A total of 181 out of 229 (79.04%) clinical samples submitted to the Delaware and Maryland Diagnostic laboratories were positive by the IBV real-time RT-PCR assay, whereas only 63 out of 229 (27.51%) samples were positive by VI. The two tests were the same for 109 out of 229 (47.60%) samples. Only one sample was IBV real-time RT-PCR negative and VI positive, while 119 samples were IBV real-time RT-PCR positive and VI negative. Using virus isolation as the reference standard, the calculated sensitivity for the assay was 0.98.

3.4. Test evaluation

Known positive tracheal swabs taken from three groups of chickens given different doses of the Arkansas DPI strain of IBV and one group of negative control birds are presented in Fig. 3. The mean copy number for each group was calculated using a standard curve (Fig. 2). At 1 d.p.i., viral load in the trachea correlated with the dose given to each group with a 28-fold difference of viral RNA measured between groups 2 and 3, and a 64-fold difference between groups 3 and 4. At 5 d.p.i., the virus load in the trachea plateaued at approximately 1×10^7 RNA copies regardless of dose. At 14 d.p.i., birds that received the lowest dose of virus (group 2), had the highest copy number of viral genomes (approximately 10,000 copies), whereas birds in group 4, which received the highest dose of virus, had the lowest number of viral RNA copies (approximately 600 copies). Three of the samples from the negative control birds in group 1,

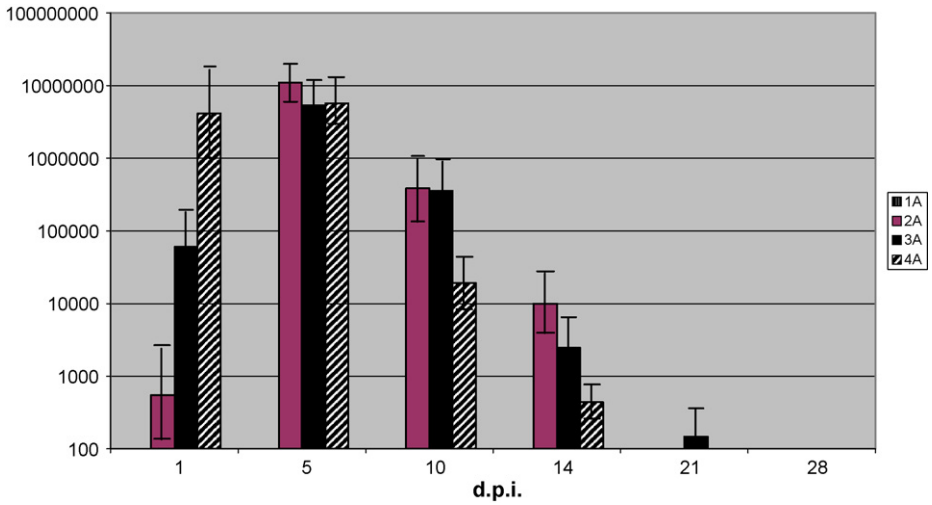

Fig. 3. The viral load in each sample was quantified using the standard curve (Fig. 2) and the average viral genome copy number per group was calculated. Group 1 (1A) received no virus, group 2 (2A) received a 50 μL dose of virus with a titer of 1.6×10^1 EID_{50}/mL, group 3 (3A) received a 50 μL dose of virus with a titer of 1.6×10^3 EID_{50}/mL, and group 4 (4A) received a 50 μL dose of virus with a titer of 1.6×10^5 EID_{50}/mL. Error bars indicate ± 1 S.D., and d.p.i. = days post-inoculation.

had recorded C_T values of 35.4, 28.25, and 36.84, at 1, 5, and 21 d.p.i., respectively. Two of those samples (35.4 and 36.84) were below our calculated limit of detection and thus considered negative. The other sample was retested and also found to be negative (data not shown).

Tracheal swabs taken from an IBV spray vaccinated commercial broiler chicken flock at 3 days post-vaccination, had 58 out of 100 samples test positive, whereas at 7 days post-vaccination, 65 out of 100 samples were positive and at 14 days post-vaccination, 83 out of 100 samples were positive.

4. Discussion

In this report we present the development and evaluation of a real-time Taqman®-based RT-PCR assay for the detection and quantification of IBV genomic RNA directly from tracheal swabs. The target region for the assay was the highly conserved 5′-UTR of the IBV genome, which resulted in amplification of all IBV strains tested. It also amplified the 5′-UTR of two strains of TCoV. This result was not unexpected because TCoV is closely related to IBV and likely arose from a recombination event within the spike glycoprotein gene (Guy, 2000; Jackwood et al., 2004). All other TCoV genes including the 5′-UTR appear to be similar to IBV (Guy, 2000). With the exception of the closely related TCoV, the assay was specific to IBV and did not detect any of the other pathogens tested.

Like all coronaviruses, IBV generates a 3′-co-terminal nested set of viral mRNAs when it replicates in the cell. Generally, only the full-length viral genome is packaged in the virus particle. By targeting the highly conserved extreme 5′-end of the genome, but not the leader sequence (nucleotides 1 to 64), which is found on all viral subgenomic mRNAs, the assay only utilizes full-length viral RNA as template, which should make it more sensitive than other RT-PCR methods that target the spike gene, and more accurate as a quantification tool for viral load in biological samples.

The limit of detection/quantification and reproducibility of the assay was evaluated by generating a standard curve with RNA run-off transcripts. The standard curve was generated from six independent runs on samples containing from 10^7 to 10^2 copies of RNA and the limit of detection and quantification were both determined to be 100 template copies. Using the standard curve equation, the efficiency of the assay was calculated to be 91.43%. In addition, the assay appears to be highly reproducible based on standard deviations of the C_T values, which ranged from 0.123 to 0.517 cycles.

We found that the real-time RT-PCR assay was extremely sensitive in a diagnostic laboratory setting. Of the clinical samples submitted to the Delaware and Maryland diagnostic laboratories, 79% were positive for IBV by the real-time assay whereas only 27% of those samples were positive by virus isolation. The window of time that IBV can be detected following infection with the real-time RT-PCR test described herein is 21 days post-infection, which likely represents a significant improvement over virus isolation and explains the higher rate of detection in the tracheal swab samples. For virus isolation, it is generally recommended that tissues such as cecal tonsil and kidney or cloacal swabs be taken in addition to tracheal swabs because the virus is known to persist there (Gelb and Jackwood, 1998). Not only does that practice significantly increases the work load it also can yield misleading results because vaccine viruses can also persist in those tissues and be shed in the feces (Cavanagh and Naqi, 2003).

It is important to evaluate any new diagnostic test with a number of known negative and positive samples. Known negative samples, obtained from commercial layer chicks maintained in isolation units and monitored for antibodies to IBV by ELISA, all tested negative for IBV in the real-time RT-PCR assay. Since we wanted to insure the birds remained free of IBV, we choose commercial layer chicks with maternal antibodies to the virus as an added insurance against infection. For known positive samples, we used SPF chicks with no maternal antibodies and

infected them with three different dosages of the Arkansas DPI strain of IBV. An additional negative control group was also maintained during that experiment. Three samples from the negative control group had C_T values of 35.4, 28.25, and 36.84. After retesting, all of the samples were below our calculated limit of detection and considered negative. Cross-contamination of samples especially during the RNA extraction process is a real concern with any RT-PCR reaction, but it is recognized that precautions should be taken to limit contamination at every step of the procedure.

The viral load in the trachea of birds given different dosages of IBV showed that regardless of the initial dose of virus, a similar maximal amount of viral RNA was detected at 5 d.p.i. More studies will be necessary to determine the course of virus replication *in vivo*, but it appears that the tracheal epithelium can support a finite amount of virus and that virus replication continues until that limit is reached. For the dosages used in our study, the maximum level of viral load was reached within 5 days post-infection. It is interesting to note and quite appropriate that the requirements for efficacy of IBV vaccines outlined in section 113.327 Title 9 of the Code of Federal Regulations (http://www.access.gpo.gov/nara/cfr/waisidx_99/9cfr113_99.html) requires that detection of challenge virus be conducted 5 d.p.i. We also observed that the level of viral RNA in the trachea of birds, which received a high dose of virus, declined more quickly than levels in birds receiving a low dose of the virus. Loss of ciliated columnar epithelium and presumably the associated virus replicating in those cells, is a common lesion with IBV infection. Birds receiving a high dose of IBV can develop clinical signs and lesions within 24 h, whereas natural spreading virus generally requires 36 h or more before clinical signs and lesions occur (Cavanagh and Naqi, 2003). Presumably, high dosages of the virus would accelerate the loss of the epithelial cells in the upper-respiratory tract leading to a more rapid decline in viral load.

When spray vaccinated commercial broilers were examined for IBV by the real-time RT-PCR assay we found that only 58 of 100 birds were positive at day 3 post-vaccination. By day 7, 65 of 100 birds were positive and by 14 days post-vaccination 83 of 100 birds were positive for IBV. The dynamics of vaccine spread in commercial chicken flocks following spray vaccination in the field is not known. Our data indicates that approximately half of the birds in the flock were initially exposed to the vaccine (day 3 post-vaccination data), which then spread to other birds in the flock so that by 14 days post-vaccination, more than 80% were exposed. More data will be required to verify this result, but it appears that exposure to IBV vaccine by in-house spray vaccination could be improved. Initially it could be considered advantageous for IBV vaccines to spread in a marginally exposed flock providing a mechanism for all the birds in the flock to become immunized, however; vaccine spread in a flock also presents opportunities for back passage of these rapidly mutating coronaviruses potentially leading to a reversion to pathogenicity.

It is important to rapidly differentiate infectious agents that cause respiratory diseases similar to low pathogenicity avian influenza and lentogenic and mesogenic Newcastle disease so that veterinarians can quickly respond to outbreaks. In addition, IBV remains an extremely important disease in commercial chickens and quickly diagnosing it is critical for control of this highly infectious virus. The real-time RT-PCR assay for IBV described herein can be conducted directly on tracheal swabs without the need for virus isolation. And although it does not differentiate between different types of IBV it is extremely sensitive and specific, and can be used to quantitate viral RNA in clinical samples.

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