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Validation of specific quantitative real-time RT-PCR assay panel for Infectious Bronchitis using synthetic DNA standards and clinical specimens

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ABSTRACT

Infectious bronchitis (IB) is a highly contagious upper respiratory tract disease of chickens caused by infectious bronchitis virus (IBV), which has various serotypes that do not cross-protect. Vaccine control strategies for this virus are only effective when designed around the currently circulating serotypes. It is essential to not only rapidly detect IBV but also to identify the type of virus causing disease. Six TaqMan™-based quantitative real-time RT-PCR assays (Universal, Ark, Mass, DE/GA98, GA07, GA08) were developed and examined the sensitivity and specificity for each assay. Assays were developed targeting the hypervariable region in the S1 gene subunit. The analytical sensitivity of TaqMan™-based quantitative real-time RT-PCR assays (qRT-PCR) assays was evaluated using synthetic DNA standards that were identical with the target sequence and specificity was further validated using clinical and biological specimens. All developed assays performed equivalently when using synthetic DNA templates as standard material, as it achieved linearity over a 5 $\log_{10}$ dynamic range with a reproducible limit of detection of ≤10 target copies per reaction, with high calculated amplification efficiencies ranging between 90%–115%. Further validation of specificity using clinical and biological specimens was also successful.

1. Introduction

Infectious bronchitis (IB) is a highly contagious upper respiratory tract disease of chickens that is caused by avian coronavirus infectious bronchitis virus and constitutes significant economic loss in the industry (Cavanagh and Naqi, 2003). Although a respiratory disease, IB can also affect the female reproductive tract, leading to poor production and egg quality. Some strains cause severe nephritis that results in significant mortality in young birds (Cook et al., 2012). Infectious bronchitis virus (IBV) is a lipid-enveloped positive-sense single-stranded RNA virus of the family *Coronaviridae* genus *Gammacoronavirus* (Masters, 2006; Jackwood, 2012). The major determinant of IBV serotype specificity is the spike protein, which is the most significant protein for virus identification as it contains epitopes for serotype-specific antibodies (Cavanagh and Naqi, 2003; Jackwood, 2012). Many serotypes exist across the globe, and cross-protection between serotypes is poor as the degree of amino acid identity between the S1 proteins of different IBV strains decreases (Cavanagh et al., 1997; Cavanagh, 2007). Thus, constant worldwide surveillance and identification of IBV types is fundamentally important.

Vaccines play a critical role in the control of IBV in poultry (Devlin et al., 2016), and vaccination against multiple IBV serotypes in commercial poultry operations is routinely practiced. Currently, more than 50 antigenic and genetic types of this virus have been officially reported and, among them, the Arkansas (Ark), Massachusetts (Mass), Delaware (DE) and Georgia 98 (GA98) types are frequently isolated in the field, and are also the commonly used vaccine types in the United States (Jackwood et al., 2005; Jackwood, 2012). In recent years, two new IBV variants namely Georgia 07 (GA07) and Georgia 08 (GA08) have emerged (Kulkarni and Resurreccion, 2010; Jackwood, 2012; Kulkarni, 2016). According to a recent comprehensive phylogeny-based classification system for IBV based on the complete nucleotide sequence of the S1 gene, IBV was categorized into 6 main genotypes (GI to GVI), along with 32 sub-genotypic lineages and some potential groups that were presented as unique variants (Valastro et al., 2016). The Mass, Ark, GA07 and GA08 IBV types examined in this study are in the same GI group and 1, 9, 25 and 27 sub-genotypic lineages respectively. The DE and GA98 viruses are in the GIV group sub-genotypic lineage 1 (Valastro et al., 2016). The Mass IBV vaccine was produced and used as the first and only available vaccine for many years, however new IBV antigenic types have steadily emerged and new IBV vaccines have been produced in an attempt to control them. Because different IBV types do

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not cross protect, it is imperative to detect and differentiate the IBV types within an infected poultry flock accurately and rapidly so effective vaccination can be implemented. However, diagnosis of IBV infections using traditional methods like viral culture and serology are insensitive, laborious and time-consuming to be applicable in clinical detection.

To aid accurate and rapid diagnosis of IBV in the field we developed quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assays that would quickly identify specific IBV types and could be conducted on clinical samples. Real-time RT-PCR has become one of the most common methods of gene quantitation due to its broad dynamic range, high sensitivity, and high sequence-specificity (Wong and Medrano, 2005) in addition to functional simplicity and short run times. Real-time RT-PCR has been useful for detecting viral agents of infectious diseases (Mackay et al., 2002). In this study, TaqMan™-based quantitative real time RT-PCR methods for rapidly detecting and typing IBV were evaluated using synthetic DNA templates that represent IBV serotypes found in the field. The purpose of using synthetic DNA templates was to provide authentic standards to quantify the presence of the target S1 gene for serotyping assays, and the 5′-untranslated region (UTR) for IBV screening tests. Evaluation of amplification efficiency using synthetic DNA is sensitive, accurate and has various advantages as the sequence of the synthetic DNA template can be freely designed without contamination, and qualitative misinterpretations of the experimental results are rare (Abe et al., 1999; Moriya et al., 2006). An internal positive control (IPC) assay was also developed to monitor potential reaction inhibitors. This is a non-target template present in the same well as the sample, which is co-amplified simultaneously with the target sequence of interest (Oikonomou et al., 2008), thus preventing false-negative reporting due to PCR inhibition (Hoorfar et al., 2004; Nordstrom et al., 2007). Lastly, clinical specimens obtained from experimentally and naturally infected birds, as well as virus stocks, were processed for validating the specificity of these assays. Statistical analysis of these assays was based on the MIQE guidelines (Bustin et al., 2009).

2. Material and methods

2.1. Design of primers and probes

The IBV types analyzed in this study were Ark, Mass, DE, and GA98, which are the most frequently isolated IBV types and commonly used vaccine types in the United States along with 2 relatively new types GA07 and GA08 (Kulkarni and Resurreccion, 2010; Jackwood, 2012; Roh et al., 2014; Kulkarni, 2016). Primers and probes for each test are listed in Table 1. The universal assay targeted the 5′-UTR region while the type-specific assays targeted the hypervariable region in the S1 gene subunit of IBV. All hydrolysis, minor groove binding (MGB) probes used in this study were labeled at the 5′ end with the reporter dye 6-carboxyfluorescein and MGB quencher at the 3′ end (Applied Biosystems, Foster City, CA, USA). The universal and most of the type- specific primers and probes were previously reported (Callison et al., 2006; Roh et al., 2013, 2014). Primers and probe sets for GA07 and GA08 were newly designed in this study. A GA08 variant forward primer (GA08-V-F) was included to ensure that all existing GA08 IBV types circulating in the field would be detected including GA08 variant types and Mass and DE/GA98 IBV type-specific probes were slightly modified to increase specificity and to synchronize thermocycling conditions with other type-specific assays. The DE and GA98 IBV types shared the same primer and probes, due to genetic similarities within the hypervariable S1 gene subunit (Roh et al., 2014). Specificity of the primers and probes was verified by an in-depth *in silico* examination with the use of the BLAST search tool at NCBI (www.ncbi.nlm.nih.gov), and by processing viral nucleic acid extracted from known negative clinical samples that did not contain the target sequence. Another set of primers and probe targeting endogenous avian RNA that exists in avian originated samples for use as an internal positive control (IPC) were also designed for the universal assay. The probe for the IPC was tagged with VIC dye at the 5′ end and MGB quencher at the 3′ end (Applied Biosystems, Foster City, CA, USA).

2.2. Preparation of synthetic DNA standards

Double-stranded synthetic DNA standards were designed and synthesized based on the sequence of the hypervariable region in the S1 gene subunit of multiple serotypes (Mass, Ark, GA07, GA08, DE, GA98), including the universal 5′UTR region of IBV (Integrated DNA Technologies, Coralville, IA, USA) based on an in-depth *in silico* examination with the use of the BLAST search tool at NCBI (www.ncbi.nlm.nih.gov). As the primer pairs for each assay were designed to generate an amplicon of around 90–260 base pairs, synthetic DNA standards were designed accordingly (Table 1). The synthetic DNA standards were serially diluted 10-fold from 10^5 to 10^1 copies per 5 µl. The 25 µl qRT-PCR reactions were prepared as follows: 5 µl of synthetic DNA standard, 10 µl of RealPCR™ RNA master mix (IDEXX Laboratories, Westbrook, ME, USA), forward and reverse primers (both with final concentrations of 0.4 µM), probe (final concentration of 0.1 µM) and RNase/DNase free water (Integrated DNA Technologies, Coralville, IA, USA). This reaction combination was the same for all IBV type-specific tests. The GA08 type IBV assay included an additional forward primer (0.4 µM in reaction) to account for a genetic variant (Table 1). The IBV universal assay included primers and probe targeting endogenous avian RNA for use as an internal positive control (IPC) in avian originated samples. In samples where endogenous avian RNA was not expected to be present, a separate synthetic IPC template representing the endogenous avian RNA was spiked into the reaction mixture. The 25 µl reaction mixture for the universal assay was the same as type-specific assays except an additional pair of forward primer (0.4 µM in reaction), reverse primer (0.4 µM in reaction) and probe (0.1 µM in reaction) targeting the IPC template along with 2 µl of the IPC (10^3 target copy numbers) itself was added. All materials regarding the IPC were provided by IDEXX laboratories. The gene and sequence information of the IPC is proprietary. Each assay included positive and negative controls.

2.3. Preparation and processing of clinical and biological samples

Panels of 30 clinical and biological avian samples that were positive for each corresponding IBV serotype were prepared and processed for validation of the type-specific assays as well as 20 known negative samples that included non-target serotypes and a non-IBV avian respiratory virus. For the universal assay, we processed 60 IBV positive samples containing Mass, Ark, GA98, DE, GA13, CONN, GA07, and GA08 type IBVs. Clinical samples refer to swabs that were collected from live infected birds whereas biological samples consisted of virus stocks and organic tissue samples (Table 2). All samples used in this study were obtained from our laboratory archives. Briefly, viral nucleic acid was extracted from samples with the use of MagMAX-96 Total RNA Isolation Kit (Ambion, Inc., Austin, TX) and the MagMAX™ Express 96 automated nucleic acid purification machine (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' protocols. The extracted nucleic acid was resuspended in 50 µl of elution buffer, and 5 µl was subsequently used for the qRT-PCR assays. Reactions were prepared as described above using extracted nucleic acid and the IPC template mimicking the target sequence of the specific endogenous avian RNA was excluded from universal assays at this step. Each assay included positive and negative controls.

2.4. Thermocycling conditions for qRT-PCR

Amplification and detection were conducted in an Applied Biosystems® 7500 fast Real-Time PCR system (Applied Biosystems,

Table 1
Primers and probe used in this study.

Primers/Probes	Target	Sequences(5'–3')	Amplicon size	Reference
IBV 59 GU391		GCTTTTGAGCCTAGCGTT		
IBV 59 GL533	Universal	GCCATGTTGTCACTGTCTATT	143bp	(Callison et al., 2006)
IBV 59 G Probe		FAM-CACCACCAGAACCTGTCACCTC-MGBNFQ[a]		
Ark-F'		GGTGAAGTCACTGTTTCTA		
Ark-R'	Arkansas	AGCACTCTGGTAGTAATAC	94bp	(Roh et al., 2013)
Ark-Probe		FAM-TRTATGACAACGAATC-MGBNFQ		
Mass-F'		CGTKTACTACTAYCAAAGTGC		
Mass-R'	Massachusetts	CCATGAATARTACCAACARTACAC	138bp	Modified from (Roh et al., 2014)
Mass-Probe		FAM-AGGTGAAGAGCCTGCATTATTAGATTC- MGBNFQ		
DE/GA98-F'		AGGCGTTTGTACTGYATA		
DE/GA98-R'	Delaware /Georgia 98	GCCATGCCTTAAAATTTG	197bp	Modified from (Roh et al., 2014)
DE/GA98-Probe		FAM- ACTATGCAAYTATGACCRGTTCCACCAC-MGBNFQ		
GA07-F'		ACAAGGGGGTGCGTATGC		
GA07-R'	Georgia 07	TGCGTAACAAACACAGTAAAGTCT	213bp	This study
GA07-Probe		FAM-TGCATCAGTATGTACT-MGBNFQ		
GA08-F'		GCAGGCTCCTCATCTTCTTG		
GA08-V-F'[b]		GCAGGTACTGCCCAAAGTTG	262bp	This study
GA08-R'	Georgia 08	CAGGCCCACTACCGTTTTG		
GA08-Probe		FAM-TAAGTCAGGTGCCAAGGA-MGBNFQ		

[a] FAM, 6-carboxyfluorescein; BHQ, black-hole quencher; MGB, minor groove binder.
[b] Forward primer for GA08 variant.

Table 2
Known positive clinical and biological tissue samples used for this study.

Target Virus	Clinical samples	Biological tissue samples		
	Tracheal swabs	Virus stocks	Cecal Tonsil	Trachea
Universal*	12	30	5	13
Mass	30	0	0	0
Ark	12	0	5	13
GA98/DEL	30	0	0	0
GA07	0	30	0	0
GA08	0	0	0	30

* Universal IBV positive samples consisted of Mass, Ark, GA98, DE, GA13, CONN, GA07, GA08.

Foster City, CA, USA) with the following conditions: 50 °C for 15 min and 95 °C for 1 min. followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s with optics on. Thermal cycling conditions for all assays were identical.

2.5. Statistical analysis

The amplification efficiency was calculated using the 7500 Fast Software v2.0.6 (Applied Biosystems, Foster City, CA, USA). As PCR efficiency is generally established through the standard curve method (Larionov et al., 2005; Callison et al., 2007; Faye et al., 2013), the standard curve of each test was generated by plotting C_T values against relative input copy numbers. Briefly, generation of the standard curve involves a series of samples serially diluted in 10-fold, each analyzed in triplicate. The formula used for calculating PCR efficiency is E(Efficiency) $= [10(-1/\text{slope})] - 1$. The real-time PCR software provides a standard curve and slope by measuring the quantification cycle which is represented as CT values. The amplification efficiency of each test was determined based on the slope of the log-linear portion of the standard curve. The coefficient of determination (R^2) was calculated for each assay.

3. Results

3.1. Primer and probe design

All primer and probe designs used in this study were validated *in-silico* from alignments of currently available IBV sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank). For the GA08 type assay, an extra forward primer targeting the variant type GA08 was added. The DE/GA98 type assays shared the same set of primers and probe due to the similarities in the target sequence (Roh et al., 2014).

Table 3
Efficiency of IBV qRT-PCR assays.

Target	Mean C_T values[a] for corresponding synthetic DNA standard copy number					Slope [b]	Efficiency (%) [c]	R^2 [d]
	10^5	10^4	10^3	10^2	10^1			
Universal [e]	23.69 ± 0.01	26.96 ± 0.01	30.33 ± 0.06	33.55 ± 0.39	37.12 ± 1.20	−3.35	98.8	0.99
Ark	22.85 ± 0.14	26.06 ± 0.22	29.24 ± 0.33	32.03 ± 0.59	35.04 ± 0.63	−3.04	113.3	0.99
Mass	22.69 ± 0.03	25.99 ± 0.14	29.32 ± 0.17	32.50 ± 0.53	37.10 ± 1.82	−3.53	91.9	0.99
DE/GA98	23.40 ± 0.11	27.74 ± 0.14	30.82 ± 0.04	34.36 ± 0.36	37.49 ± 1.05	−3.48	93.8	0.99
GA07	25.63 ± 0.38	28.68 ± 0.24	31.89 ± 0.08	35.13 ± 0.44	37.71 ± 0.18	−3.06	112.2	0.99
GA08	23.40 ± 0.08	26.66 ± 0.05	30.04 ± 0.07	33.24 ± 0.21	36.95 ± 0.34	−3.37	98.1	0.99
GA08 Variant[f]	22.54 ± 0.06	25.72 ± 0.17	29.25 ± 0.15	32.83 ± 0.51	35.38 ± 0.19	−3.28	101.8	0.99

[a] Mean C_T values of triplicate runs ± Standard deviation.
[b] Slope calculated from Y = Y intercept − slope $\log_{10}$.
[c] PCR Efficiency = $[10^{(-1/\text{slope})}] - 1$.
[d] Coefficient of determination.
[e] Co-amplified with Internal positive control.
[f] Forward primer targeting GA08 variants was included in original GA08 assay.

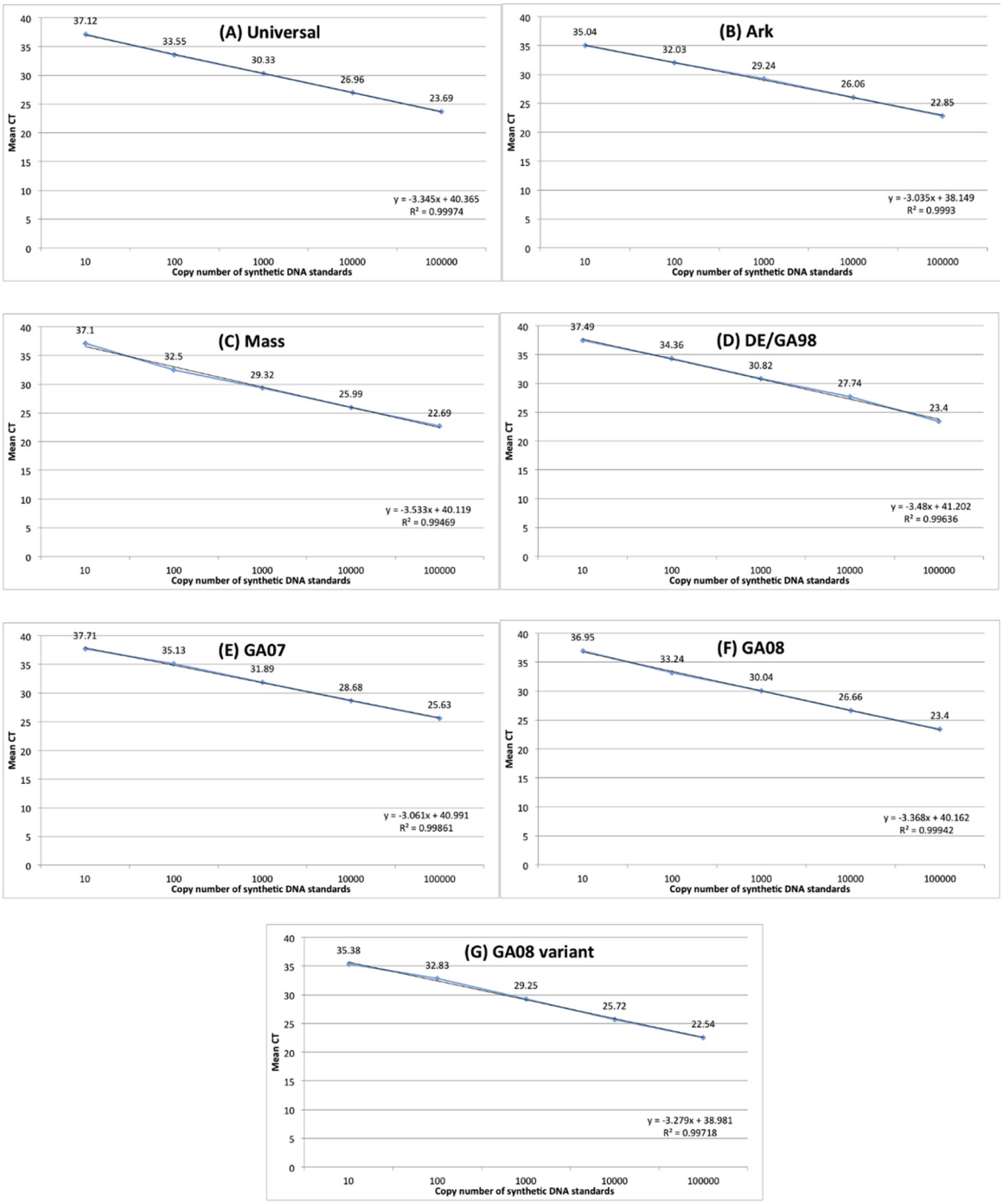

Fig. 1. Analytical sensitivity of the qRT-PCR assays. Standard curves for (A) Universal, (B) Ark, (C) Mass, (D) DE/GA98, (E) GA07, (F) GA08, (G) GA08 variant assays presenting the mean C_T plotted against the relative input copy numbers (log_{10}) of synthetic DNA standards. Synthetic DNA standards were serially diluted by 10- fold at a 5 log_{10} range, starting from 10^5 copies down to $\leq$10 copies per reaction.

3.2. Sensitivity of qRT-PCR assays using synthetic DNA standards

To determine the dynamic range, limit of detection (LOD) and quantitative capabilities of the developed IBV universal and type-specific assays, standard curves were generated using synthetic DNA standards with sequences matching the target sequence on 5′- UTR region or the hypervariable region in the S1 gene subunit. The specificity of the primers and probe designed for each assay was examined in detail *in silico* and found to be specific. The dynamic range of the universal IBV qRT-PCR assays spanned 5 log_{10} units from 10 to 10^5 copies per reaction with a slope of -3.35 and an R^2 value of 0.99, at a LOD of $\leq$10 copy numbers and with an average calculated efficiency of 98.8 % (Table 3). The assay detected 10 copy numbers in all triplicate runs in the universal assay. The artificially added IPC templates were successfully amplified and did not appear to interfere with the amplification of the target template regarding the universal assay (data not shown). The mean C_T values, slopes of the standard curve and R^2 value of the other type-specific assays are shown in Table 3. All type-specific assays retained linearity for 5 orders of magnitude at a LOD of $\leq$10 copy numbers per reaction and amplification efficiency was calculated by using the slope from the linear equation. The average efficiencies of all type-specific assays were within the acceptable range (Zhang and Fang,

Table 4
Sensitivity of the IBV universal and type-specific assays using clinical and biological samples.

Assay type	Known positive samples		Sensitivity (%)[b]
	No. positive	No. negative	
Universal[a]	60	0	100 (60/60)
Ark	30	0	100 (30/30)
Mass	30	0	100 (30/30)
DE/GA98	29	1	97 (29/30)
GA07	30	0	100 (30/30)
GA08	28	2	93 (28/30)

[a] Known positive sample group for the universal assay consisted of Mass, Ark, DE, GA98, GA07, GA08, CONN[b], GA13 type IBV.
[b] Percentage of positive samples within a given subset.

Table 5
Specificity of the IBV universal and type-specific assays using clinical and biological samples.

Virus	Type	Specific IBV qRT-PCR assay					
		Universal	Ark	Mass	DE/GA98	GA07	GA08
IBV	Ark	+	+	–	–	–	–
	Mass	+	–	+	–	–	–
	DE	+	–	–	+	–	–
	GA98	+	–	–	+	–	–
	GA07	+	–	–	–	+	–
	GA08	+	–	–	–	–	+
	GA13	+	–	–	–	–	–
	CONN[b]	+	–	–	–	–	–
NDV[a]	Lasota	–	–	–	–	–	–

[a] New castle disease virus.
[b] Connecticut-type IBV.

2006; Zheng et al., 2013; Svec et al., 2015; Zhang et al., 2016). The R^2 of all type-specific assays (Fig. 1) were ≥ 0.99, which indicates that results are highly reproducible.

3.3. Retrospective validation of universal and type-specific assays using clinical and biological specimen

To further verify the diagnostic application of the qRT-PCR assays, panels of 30 positive samples corresponding to each target serotype were tested for each type-specific assay, as well as 20 known negative samples that included non-target serotypes and non-target avian respiratory viruses. Samples consisted of tracheal and choanal swabs, and organ samples collected from experimentally and naturally infected birds as well as virus stocks grown in embryonated eggs. Sensitivity was determined as the percentage of positive samples detected within a subset of known positive samples (Table 4). Specificity of the universal assay was verified by testing 60 samples (Table 2) that were positive for Mass, Ark, GA98, DE, GA13, CONN, GA07, GA08 type IBVs and 20 negative samples containing non-IBV avian respiratory viruses. As DEL/GA98 type-specific assays shared the same primers, probes and target sequences, they were verified with positive samples for GA98 and DE type IBV. Samples were considered negative when the C_T values were ≥40. The universal assay successfully detected IBV in all IBV positive samples regardless of their serotypes, and none of the non-IBV avian respiratory viruses were detected in the IBV negative samples (Table 4,5). The endogenous avian RNA IPC in the samples were successfully co-amplified with the target sequence in the universal assay and did not show interference with assay performance (data not shown). The retrospective validation results of type-specific assays using clinical specimens are also presented in Table 4. Ark, Mass and GA07 type-specific assays successfully detected the target IBV type in 100 % of known positive samples, with the DE/GA98 assay detecting 97 % and GA08 assay detecting 93% (Table 4). No cross detection was observed with the non-target serotypes, including between different viruses in the same sub-genomic lineage (eg. Mass and Conn in the GI-1 lineage), indicating a high specificity of the developed type-specific assays (Table 5).

4. Discussion

Infectious bronchitis is responsible for significant economic losses to the poultry industry throughout the world and rapid, accurate identification of the currently circulating serotypes is essential for implementing an effective vaccine control strategy (Cavanagh, 2003). For that purpose, we propose a panel of qRT-PCR assays for IBV that would be suitable for rapid and type specific diagnostic purposes. Instead of cDNA templates from IBV, synthetic DNA templates mimicking IBV target sequences were utilized for standardization and verification of the developed assays. Synthetic DNA templates are known to have advantages over cDNA templates that are prepared from biological samples in terms of qRT-PCR standardization as they present a measurable amplification efficiency compared to authentic cDNA templates (Moriya et al., 2006). Using such material provided a more stringent validation criteria for evaluation of LOD and analytical specificity. Even though the thermocycling conditions included an reverse transcription (RT) step as IBV was an RNA virus, the purpose of using synthetic DNA was to evaluate how well the primer and probe designs performed in binding and amplifying cDNA. Additionally, the RT step was validated by using viral RNA extracted from clinical and biological samples.

All developed IBV universal and type-specific assays performed equivalently when testing synthetic DNA templates, with linear detection over a 5-log range and amplification efficiencies ranging from 91.9%–113.3% (Table 2, Fig. 1). All assays showed LOD of ≤10 copy numbers per reaction with an R^2 ≥0.99 indicating high reproducibility. One hundred percent of the known positive samples were determined as positive by the universal and Mass, Ark, GA07 type-specific assays whereas the DE/GA98 assay detected 97% (29/30), and GA08 detected 94% (28/30) as positive out of the known positive sample panel. An internal positive control (IPC) was also designed to be co-amplified in each reaction mixture to aid in the accurate reporting of results by preventing false negative reports. The endogenous avian RNA IPC was only used in the universal assay as the performance of the type-specific assays were interfered with by the presence of the avian RNA IPC primers and probe (data not shown). The IPC was designed to generate a mean C_T value of 27–29 with 10^3 copy numbers under non-inhibitory conditions. The mean C_T values of the IPC were spread over a broader range with a higher standard deviation (SEM) when testing with biological and clinical samples, which seemed reasonable as the quantities of the target endogenous avian RNA would have varied in each sample (data not shown).

The current assay panel that was developed and validated in this study hold several advantages in terms of field diagnostics. They can identify multiple IBV serotypes in the same clinical sample, making it possible for the set of assays to be utilized as a vaccine monitoring tool in flocks that were vaccinated with multiple types of IBV. One of the other achievements of this study was that all qRT-PCR assays shared the same thermocycling parameters, thus allowing to test for various types of IBV on the same test plate in a single session. Although concurrent detection of different IBV field types in the same sample is possible by running separate tests using different primer/probe combinations on the same sample, multiplexing the assays was unsuccessful as the decrease of sensitivity and efficiency was in the unacceptable range (data not shown). It should be noted that the selection of field samples from our laboratory archives was simply based on their serotypes for the purpose of validation and not on an epidemiological perspective, which was outside the scope of this work. In addition, the sensitivity and efficiency of the universal and type-specific Mass and DE/GA98 assays were comparable to the assays that were previously developed (Roh

et al., 2014), but run times were much shorter due to the changes in the reaction conditions.

In summary, a panel of highly sensitive and specific qRT-PCR assays for universal detection and typing of IBV were developed and validated with synthetic DNA standards, clinical and biological specimens. The tests can be used to rapidly identify specific IBV types in clinical samples, detect more than one IBV type in the same sample and determine the relative amount of each IBV type in a sample. The provision of these assays will facilitate IBV diagnostics in the field in terms of accuracy, functional simplicity and rapidity as well as aid in evaluating type specific IBV vaccine takes following administration of different live attenuated vaccines.

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