DEVELOPMENT OF DIAGNOSTIC PANELS FOR RAPID IDENTIFICATION OF AVIAN RESPIRATORY DISEASES VIRUSES AND EXPRESSION OF INFECTIOUS BRONCHITIS SPIKE PROTEIN-PSEUDOTYPED VIRUS PARTICLES FOR DIAGNOSTIC SEROLOGY

by

JONGSEO MO

(Under the Direction of Mark W. Jackwood)

ABSTRACT

Respiratory viral diseases are common in commercial poultry, and major diseases such as infectious bronchitis (IBV), Newcastle disease (NDV), infectious laryngotracheitis (ILTV), and avian metapneumovirus (AMPV) can severely affect the performance of poultry, causing high costs to the industry resulting in significant economic loss and even trade restraints and embargos on poultry products. Constant surveillance and identification of the etiologic agent within an infected poultry flock are crucial, so appropriate countermeasures can be promptly implemented. To aid accurate and rapid diagnosis of respiratory viruses in the field, a panel consisting of ten sets of TaqMan™-based quantitative RT-PCR assays that shared the same thermocycling conditions with fast run times was developed and the potential of introducing serotyping ELISA for IBV was examined. Internal positive controls (IPC) were incorporated in five of TaqMan™-based quantitative RT-PCR assays, and the analytical sensitivity and specificity of all tests were evaluated rigorously using synthetic DNA standards that were identical with the target sequence and by using clinical and biological tissue specimens. All developed IBV screening and serotyping qRT-PCR assays achieved linearity over a 5 $\log_{10}$

dynamic range with a reproducible limit of detection of ≤10 target copies per reaction, and amplification efficiencies ranging between 90%-115%. NDV, ILTV, AMPV-A, AMPV-B qRT-PCR assays also achieved the same dynamic range and limit of detection, with amplification efficiencies ranging between 86.8%-98.2%. Further validation of specificity using clinical and biological specimens was also successful and met all the initial requirements. In a separate, but complementary, study, the potential of VSV particles expressing IBV spike proteins as serotyping ELISA antigens was examined. Genes of the IBV spike protein were incorporated into the outer shell of the viral envelope of VSV and the functionality of these VSV particles bearing different IBV spikes (Mass, Ark) as serotyping ELISA antigens was studied. This proof of concept study proved some potential of the IBV spike pseudotyped VSV particles as serotyping antigens. The primary focus of this whole study was the development and provision of a board panel of diagnostic assays and practical concepts that could aid in identifying respiratory viral etiologic agents that are distributed worldwide.

INDEX WORDS: Infectious bronchitis, Infectious laryngotracheitis, Newcastle disease, Avian metapneumovirus, Real-time polymerase chain reaction, Spike protein, Pseudotyping, Vesicular stomatitis virus, Enzyme linked immunosorbent assay

DEVELOPMENT OF DIAGNOSTIC PANELS FOR RAPID IDENTIFICATION OF AVIAN RESPIRATORY DISEASES VIRUSES AND EXPRESSION OF INFECTIOUS BRONCHITIS SPIKE PROTEIN-PSEUDOTYPED VIRUS PARTICLES FOR DIAGNOSTIC SEROLOGY

by

JONGSEO MO

B.S., Kyung Hee University, South Korea, 2009

D.V.M., Jeju National University, South Korea, 2013

M.S., Kangwon National University, South Korea, 2015

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2018

© 2018

Jongseo Mo

All Rights Reserved

DEVELOPMENT OF DIAGNOSTIC PANELS FOR RAPID IDENTIFICATION OF AVIAN RESPIRATORY DISEASES VIRUSES AND EXPRESSION OF INFECTIOUS BRONCHITIS SPIKE PROTEIN-PSEUDOTYPED VIRUS PARTICLES FOR DIAGNOSTIC SEROLOGY

by

JONGSEO MO

Major Professor:	Mark W. Jackwood
Committee:	Brian J. Jordan
	Daniel R. Perez
	Maricarmen Garcia
	Melinda Brindley

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2018

DEDICATION

To my wonderful family, In-Pil Mo, my father and fellow colleague who inspired me for my whole life to follow his footsteps on the same road to poultry diseases, My mother Ki-Ok Park for her unwavering support and love for pursuing my dreams, and my brother Jong-Suk Mo, another colleague and fellow traveler on the same road to science.

ACKNOWLEDGEMENTS

First of all, I would like to acknowledge Dr. Mark Jackwood, my advisor and mentor who has always inspired me and will continue to do so in this field of research. Joining your lab was one of the most joyful moments in my life, and I want to thank you again for granting me the most excellent opportunity to be your student,

Next, I would like to acknowledge my advisory committee, Dr. Brian Jordan, Dr. Daniel Perez, Dr. Maricarmen Garcia, Dr. Melinda Brindley, who have dedicated their precious time guiding me to go throughout this journey,

My current and former lab colleagues and sisters, Christina, Emily, Grace, Julia and Laura. It was a great joy to work with you, and hope our paths will cross again,

Our seasoned research specialists Debbie, Sunny and Sylva (Garcia lab) who was a joy to be with, and had never hesitated to help me when I needed it the most,

My current and former peers at the PDRC, Gaby, Daniel, Ade, Jeff, Lucas, Silvia, Maria, Claire, Karen, Carmen, Salman, Mohammadreza, Gustavo, Rafael, Ana Maria, William,

To all my other friends, graduate students, undergrads who have crossed paths with me,

To Mike and Valerie from IDEXX, it was a complete pleasure to work with you and thanks for giving me the opportunity to get involved in this great project,

My former advisor Dr. Sung and mentor Dr. Kwon who assisted me in coming to UGA,

Lastly, I send my deepest gratitude to my family.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Respiratory viral diseases are common in poultry and constitute an important cause of economic loss throughout the industry. Among such viral diseases, infectious bronchitis, Newcastle disease, infectious laryngotracheitis and avian metapneumovirus are the viruses that frequently affect the respiratory tract of chickens. Infectious bronchitis is a highly contagious upper respiratory tract disease of chickens that is caused by avian coronavirus infectious bronchitis virus (IBV) that can negatively affect production and egg quality (12). Infectious laryngotracheitis (ILTV) is an upper-respiratory disease of poultry from the family *Herpesviridae* that causes sporadic cases of disease around the world associated with acute respiratory signs (5, 10). Newcastle disease virus (NDV), which was formally known as *avian paramyxovirus 1* (APMV-1), can lead to trade embargoes and quarantine depending on the type and virulence of the strain (35). Lastly, avian metapneumovirus (AMPV) a member of *Paramyxoviridae,* is the etiologic agent for severe rhinotracheitis in turkeys, causing increased mortality in conjunction with secondary bacterial infections (19, 32).

It is crucial to rapidly detect and differentiate these viruses within an infected poultry flock so that appropriate measures against those diseases can be implemented promptly. In other words, demonstrating the presence of the virus and identifying the etiologic agent along with differentiating between possible candidates, which can be similar in the early stages of the presentation of the disease is essential (9, 11, 33). As vaccines play a critical role in the

containment of such viral diseases in commercial poultry (3, 14, 16, 27), it is advantageous to identify the etiologic agent accurately and rapidly. However, diagnosis of avian respiratory virus infections using traditional methods such as viral culture and/or serology are insensitive, laborious and time-consuming for routine clinical detection (6, 9, 13, 18, 35). Thus, the two primary goals of this research are to develop diagnostic tools for rapid identification of these viruses and to express spike protein of different common IBV serotypes as pseudotyped virus particles that can be applied for diagnostic serology.

Real-time PCR has nowadays become one of the most common methods of gene quantitation due to its large dynamic range, high sensitivity, and high sequence-specificity (36). Real-time PCR has been very useful for detecting viral agents of infectious diseases (25). The first and second aim of this research was to develop of TaqMan®-based RT-PCR methods for rapidly detecting and serotyping IBV as well as ILTV, NDV, AMPV. The purpose of using synthetic DNA templates was to provide authentic standards to generate a standard curve to quantify the presence of the target gene of each virus. An internal positive control was incorporated in some assays to verify the integrity of the test, and its effect on sensitivity and specificity was evaluated. Lastly, clinical and biological tissue specimens from naturally and experimentally infected birds were processed for validating the authenticity of these assays.

The third aim was to generate VSV particles that are pseudotyped with IBV spike proteins derived from different serotypes. Spike proteins which are located on the outside of all coronaviruses, are responsible for inducing serotype and virus-neutralizing antibodies (21). Vesicular stomatitis virus (VSV) has been widely used to produce pseudotyped virus particles, which have been used as a research tool to study many aspects of negative-strand RNA virus entry and replication, as well as for immunological assays and other purposes (2, 4, 15, 26). One

of the substantial advantages of using VSV is that they are not particularly selective in regards to the type of membrane protein that can be incorporated into the viral envelope (34). Thus, it is possible to generate a pseudotype virus that has the envelope protein of the spike protein of IBV assembled into the VSV membrane. Another advantage of pseudotyping IBV is that it only requires knowledge of the nucleotide sequence of the spike gene. IBV spike-pseudotyped virions can be used for multiple purposes including the development of serotype-specific diagnostics. Previously developed enzyme-linked immunosorbent assay (ELISA) kits targeting IBV specific antibodies are mostly based on coated whole virus particles, with few tests utilizing conserved structural and nonstructural proteins as coated antigen (7, 17, 24). These kits can quantify antibody levels against IBV, and facilitate large-scale monitoring of flocks, but focus only on universal detection of IBV and are not serotype specific. Since IBV spike-pseudotyped VSV particles only contain the spike protein, which is the major determinant of IBV serotype specificity, serotyping of serum samples may be possible and more accurate due to the absence of other cross-reactive IBV proteins.

The overall goal of this research was to develop diagnostic tools that can be applied for clinical poultry respiratory virus detection, serotyping and other purposes. Specifically, this research aims (I) to develop real-time PCR assays for IBV screening and serotyping, and evaluate its sensitivity & specificity by using synthetic DNA templates and clinical/biological tissue specimens, (II) to develop real-time PCR assays for NDV, ILTV and AMPV and evaluate its sensitivity & specificity by using synthetic DNA templates, internal positive controls, and clinical/biological tissue specimens, Lastly, (III) generate pseudotyped VSV particles that carry IBV spike proteins using an in vitro culture system and characterize produced particles for the incorporation of IBV spike proteins as ELISA antigens.

SPECIFIC AIMS

It is crucial to rapidly detect and differentiate major respiratory viruses such as IBV, NDV, ILTV and AMPV within an infected poultry flock so that appropriate measures against those diseases can be implemented promptly. Demonstrating the presence of the virus and identifying the etiologic agent along with differentiating between possible candidates, which can be similar in the early stages of their pathogenesis is essential for disease control. The overall goal of this research was to develop molecular methods and diagnostic tools to aid in this pursuit. The specific aims of this study are: aims (I) to develop real-time PCR assays for IBV screening and serotyping, and evaluate its sensitivity & specificity by using synthetic DNA templates and clinical/biological tissue specimens, (II) to develop real-time PCR assays for NDV, ILTV and AMPV and evaluate its sensitivity & specificity by using synthetic DNA templates, internal positive controls, and clinical/biological tissue specimens, Lastly, (III) generate pseudotyped VSV particles that carry IBV spike proteins using an in vitro culture system and characterize produced particles for the incorporation of IBV spike proteins as potential ELISA antigens.

Specific Aim 1. Developing real-time PCR assays for IBV screening and serotyping, and evaluate its sensitivity & specificity by using synthetic DNA templates and clinical/biological tissue specimens. There are various advantages of using real-time RT-PCR over other methods to quantify gene expression due to its functional simplicity, together with its combination of high sensitivity, specificity and short run times. Real-time PCR has been very useful for detecting viral agents of infectious diseases (25). For the first step, synthetic DNA sequences each mimicking the hyper-variable region in the S1 gene subunit of multiple serotypes (Mass, Ark,

GA07, GA08, Del/GA98), including the universal 5'UTR region of IBV, was used as templates for the assays to evaluate the amplification efficiency of synthetic DNA when paired with corresponding type-specific primer/probe sets. Type-specific primers and probes designed by our laboratory were utilized (31). An internal positive control (IPC) was also added to verify the integrity of the universal screening test, as well as to observe its effect on the test performance itself. An IPC is a non-target DNA template present in the same well as the sample, which is co-amplified simultaneously with the target sequence of interest (30). Thus, a control signal should always be produced even in the absence of the target sequence. This can prevent false-negative reporting due to PCR inhibition (20, 29). 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 (1, 28). Lastly, the sensitivity and specificity of the developed IBV serotype specific real-time RT-PCR test was determined by using known negative, known positive, and clinical samples from chickens. Evaluation of these assays was based on the MIQE guidelines, which stand for Minimum Information for Publication of Quantitative Real-Time PCR Experiments (8).

Specific Aim 2. Developing real-time PCR assays for NDV, ILTV and AMPV and evaluate its sensitivity & specificity by using synthetic DNA templates, internal positive controls, and clinical/biological tissue specimens. For rapid and accurate clinical detection of major poultry respiratory virus such as NDV, ILTV and AMPV, quantitative real-time PCR assays incorporated with internal positive controls were developed and rigorously evaluated. ILTV (9) and NDV (23) specific primers and probes sequences that were widely used for diagnostics were

selected based on other literature, with AMPV-A and B primer, probes newly designed for this study. Similar with specific aim 1, synthetic DNA templates were designed and synthesized based on the target sequence in the gC gene of ILTV, matrix (M) and polymerase (L) gene of NDV and the nucleoprotein (N) gene of AMPV-A and B respectively after an in-depth *in silico* examination and used for evaluation of analytical sensitivity. Internal positive control (IPC) was also added to all assays. The sensitivity and specificity of the developed assays were determined in retrospect by using known negative, known positive clinical samples from experimentally infected chickens. Evaluation of these assays was based on the MIQE guidelines (8).

Specific aim 3. Generate pseudotyped VSV particles that carry IBV spike proteins using an in vitro culture system and characterize produced particles for the incorporation of IBV spike proteins as potential ELISA antigen. The pseudotyping technique that was used for specific aim 3 was based on a VSV pseudotyping system using VSV-ΔG, an envelope gene-deficient VSV (34). Budding of VSV virions out of the cell surface does not require the VSV G protein (22) and can be replaced by a heterologous glycoprotein, which in this case would be the IBV spike protein. As VSV is not particularly selective to the type of membrane protein that can be incorporated into the viral envelope (34), a VSV pseudotype carrying the IBV spike can be generated. An *in vitro* cell culture system was used to generate pseudotyped VSV particles carrying IBV spike proteins. Plasmids carrying IBV spike protein genes from different serotypes were transfected into mammalian cells in an *in vitro* culture. Subsequently, the transfected cells were infected with envelope gene-deficient VSV (VSV-ΔG). Virus particles that bud out of the host cells will passively carry the IBV spike protein, which is expressed on the membrane of host cells. To determine the specificity of the VSV particles each bearing Mass and Ark spike as

serotyping ELISA antigens against homologous and heterologous serum, the dilution linearity of each ELISA assay correlating with the serial dilution of IBV spike pseudotyped VSV antigens was demonstrated. Demonstration of dilution linearity of the serially diluted VSV spike pseudotypes containing the specific serotyping antibody of interest was critical to validating the specificity and accuracy of the given ELISA results.

REFERENCES

1. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, Tanaka S, Yoshiba M, Kohara M. 1999. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. Journal of Clinical Microbiology 37:2899-2903.

2. Aiken C. 1997. Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. Journal of virology 71:5871-5877.

3. Alexander D. 2000. Newcastle disease and other avian paramyxoviruses. Revue Scientifique et Technique-Office International des Epizooties 19:443-455.

4. Anderson DB, Laquerre S, Goins WF, Cohen JB, Glorioso JC. 2000. Pseudotyping of glycoprotein D-deficient herpes simplex virus type 1 with vesicular stomatitis virus glycoprotein G enables mutant virus attachment and entry. Journal of virology 74:2481-2487.

5. Bagust T, Jones R, Guy J. 2000. Avian infectious laryngotracheitis. Revue Scientifique et Technique-Office International des Epizooties 19:483-488.

6. Brittain-Long R, Nord S, Olofsson S, Westin J, Anderson L-M, Lindh M. 2008. Multiplex real-time PCR for detection of respiratory tract infections. Journal of Clinical Virology 41:53-56.

7. Bronzoni RV, Fatima M, Montassier S, Pereira GT, Gama NM, Sakai V, Montassier HJ. 2005. Detection of Infectious Bronchitis Virus and Specific Anti-Viral Antibodies Using a Concanavalin A–Sandwich–ELISA. Viral immunology 18:569-578.

8. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry 55:611-622.

9. Callison S, Riblet S, Oldoni I, Sun S, Zavala G, Williams S, Resurreccion R, Spackman E, Garcia M. 2007. Development and validation of a real-time Taqman® PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. Journal of virological methods 139:31-38.

10. Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, Jackwood MW. 2006. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. Journal of virological methods 138:60-65.

11. Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, Jackwood MW. 2006. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. Journal of virological methods 138:60-65.

12. Cavanagh D, Naqi S. 2003. Infectious bronchitis. Diseases of poultry 11:101-119.

13. Cook JK, Cavanagh D. 2002. Detection and differentiation of avian pneumoviruses (metapneumoviruses). Avian Pathology 31:117-132.

14. Devlin JM, Vaz PK, Coppo MJ, Browning GF. 2016. Impacts of poultry vaccination on viruses of wild bird. Current Opinion in Virology 19:23-29.

15. Fukushi S, Mizutani T, Saijo M, Matsuyama S, Miyajima N, Taguchi F, Itamura S, Kurane I, Morikawa S. 2005. Vesicular stomatitis virus pseudotyped with severe acute respiratory syndrome coronavirus spike protein. Journal of general virology 86:2269-2274.

16. García M. 2017. Current and future vaccines and vaccination strategies against infectious laryngotracheitis (ILT) respiratory disease of poultry. Veterinary microbiology 206:157-162.

17. Gibertoni AM, Maria de Fátima SM, Sena JA, Givisiez PE, Furuyama CR, Montassier HJ. 2005. Development and application of a Saccharomyces cerevisiae-expressed nucleocapsid protein-based enzyme-linked immunosorbent assay for detection of antibodies against infectious bronchitis virus. Journal of clinical microbiology 43:1982-1984.

18. Gunson R, Collins T, Carman W. 2005. Real-time RT-PCR detection of 12 respiratory viral infections in four triplex reactions. Journal of clinical virology 33:341-344.

19. ho Lee E, Song M-S, Shin J-Y, Lee Y-M, Kim C-J, Lee YS, Kim H, Choi YK. 2007. Genetic characterization of avian metapneumovirus subtype C isolated from pheasants in a live bird market. Virus research 128:18-25.

20. Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. Journal of clinical microbiology 42:1863-1868.

21. Jackwood MW, Hilt DA, Callison SA, Lee C-W, Plaza H, Wade E. 2001. Spike glycoprotein cleavage recognition site analysis of infectious bronchitis virus. Avian diseases:366-372.

22. Jayakar HR, Jeetendra E, Whitt MA. 2004. Rhabdovirus assembly and budding. Virus research 106:117-132.

23. Kim LM, Suarez DL, Afonso CL. 2008. Detection of a broad range of class I and II Newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. Journal of Veterinary Diagnostic Investigation 20:414-425.

24. Lei J, Shi T, Sun D, Mo K, Yan Y, Jin Y, Liao M, Zhou J. 2017. Development and application of nsp5-ELISA for the detection of antibody to infectious bronchitis virus. Journal of Virological Methods 243:182-189.

25. Mackay IM, Arden KE, Nitsche A. 2002. Real-time PCR in virology. Nucleic acids research 30:1292-1305.

26. Mangor J, Monsma S, Johnson M, Blissard G. 2001. A GP64-null baculovirus pseudotyped with vesicular stomatitis virus G protein. Journal of virology 75:2544-2556.

27. Miller P, Koch G, Suarez D, Mundt E, Jones R, Rautenschlein S. 2013. Newcastle disease, other paramyxoviruses, and avian metapneumovirus infections. Diseases of poultry. 13th ed. Ames, IA: Blackwell Publishing:250-377.

28. Moriya Y, Nakamura T, Okamura N, Sakaeda T, Horinouchi M, Tamura T, Aoyama N, Kasuga M, Okumura K. 2006. Comparison of synthetic DNA templates with authentic cDNA templates in terms of quantification by real-time quantitative reverse transcription polymerase chain reaction. Biological and Pharmaceutical Bulletin 29:535-538.

29. Nordstrom JL, Vickery MC, Blackstone GM, Murray SL, DePaola A. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic Vibrio parahaemolyticus bacteria in oysters. Applied and Environmental Microbiology 73:5840-5847.

30. Oikonomou I, Halatsi K, Kyriacou A. 2008. Selective PCR: a novel internal amplification control strategy for enhanced sensitivity in Salmonella diagnosis. Letters in applied microbiology 46:456-461.

31. Roh H-J, Jordan BJ, Hilt DA, Jackwood MW. 2014. Detection of Infectious Bronchitis Virus with the Use of Real-Time Quantitative Reverse Transcriptase–PCR and Correlation with Virus Detection in Embryonated Eggs. Avian diseases 58:398-403.

32. Shin H-J, Njenga MK, McComb B, Halvorson DA, Nagaraja KV. 2000. Avian pneumovirus (APV) RNA from wild and sentinel birds in the United States has genetic

homology with RNA from APV isolates from domestic turkeys. Journal of clinical microbiology 38:4282-4284.

33. Villegas P. 1998. Viral diseases of the respiratory system. Poultry science 77:1143-1145.

34. Whitt MA. 2010. Generation of VSV pseudotypes using recombinant ΔG-VSV for studies on virus entry, identification of entry inhibitors, and immune responses to vaccines. Journal of virological methods 169:365-374.

35. Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, Kapczynski DR, Spackman E. 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology 42:329-338.

36. Wong ML, Medrano JF. 2005. Real-time PCR for mRNA quantitation. Biotechniques 39:75.

CHAPTER 2

LITERATURE REVIEW

REVIEW OF MAJOR RESPIRATORY VIRUSES IN POULTRY

Infectious Bronchitis. Infectious bronchitis (IB) is a highly contagious disease that affects the upper respiratory tract of chickens caused by avian coronavirus infectious bronchitis virus (IBV) (46, 48, 125). Clinical signs usually appear within 24–48 hours after being exposed to the virus (116). Typical clinical signs in chicks infected with IBV will include nasal discharge, sneezing, rales, and coughing (1, 14). On the onset of infection, IBV infects ciliated and mucosal cells of the upper respiratory tract accompanied by ciliostasis with frequent loss of ciliated epithelial cells (5, 73, 127). Although a respiratory disease, IBV can also affect the female reproductive tract, leading to reduced production and egg quality with some strains causing severe nephritis that results in significant mortality in young birds (48, 63). The nephropathogenic clinical form of IBV includes signs of ruffled feathers, wet droppings, and increased water intake (252).

Morbidity of this disease is typically 100%, and mortality can exceed 50% within a flock when some nephropathogenic strains are paired with opportunistic pathogens such as *Escherichia coli* (125). IBV have a predilection for the epithelium of the upper respiratory tract, which can lead to respiratory distress making the host prone for secondary bacterial airway infections (80, 169). As part of the family *Coronaviridae* genus *Gammacoronavirus* (168), this lipid-enveloped positive-sense single-stranded RNA virus can produce countless genetic and

antigenic variants capable of causing disease due to high mutation rates, rapid replication, and extensive genome recombination results (125, 147, 152, 155).

The morphologic feature of IBV can be described as a round structure that is 100 nm to 160 nm in size with petal-shaped spikes anchored on the viral surface (99). This virus consists of four structural proteins; the helical nucleocapsid (N) surrounding the 27 kilobase pairs (kb) length viral RNA, an integral membrane glycoprotein (M) that spans the lipoprotein envelope three times, small envelope proteins (E) which are present in small amounts on the envelope, and a spike glycoprotein (Spike) located on the surface of the lipoprotein envelope (1, 168, 249). And like other coronaviruses, IBV also encodes a set of accessory protein genes in which the functions have not been fully understood, although several studies including other coronaviruses proved that they are not required for *in vitro* replication (40, 66, 105, 115, 198, 226, 257), and was suggested that they may play a role regarding pathogenesis (198).

The lipoprotein envelope of IBV surrounds the helical nucleocapsid and is anchored with the spike glycoprotein, which is the most significant protein for virus identification as it contains epitopes for serotype-specific antibodies and induces virus-neutralization (1, 38, 44, 63, 125, 137). The spike consists of two subunits: the amino-terminal S1 subunit, which contains serotype specific epitopes and receptor-binding domains (RBD), and S2 carboxyterminal subunit, which anchors the protein to the viral envelope and involves in the fusion of the virus particle and host cell membrane (30, 63). Proteolytic activation of the spike protein is carried out by the furin-like host cell protease that cleaves a highly basic motif generating S1 and S2 subunits resulting in 500 and 600 amino acids in size, respectively (43). As the spike is also involved in host cell attachment, it plays an important role in host specificity (248, 249). The spike mediates virus-cell membrane fusion by being exposed to acidic pH in endosomes, which triggers conformational

changes on the spike itself resulting in fusion with the viral envelope and cell membrane (53). IBV genotyping is generally done based on the nucleotide sequence of the S1 subunit (1, 126) and serotypes have been defined in association with neutralizing-serotype-specific epitopes that mainly exist in this subunit (44, 186, 195). However, there is no clear evidence that the spike protein of IBV itself is directly involved in virulence and disease severity, as replacing the spike gene from an avirulent IBV isolate (Beaudette) with that from a more virulent strain (Massachusetts 41) did not restore virulence (114). Instead, it has been suggested that the replicase-transcriptase gene that makes up two-thirds of the whole genome may be the determinant of pathogenicity (13). The replicase-transcriptase gene, which is encoded in the open reading frame 1a (ORF1a) and 1b (ORF1b) and consists of two polyproteins, pp1a and pp1ab, are cleaved and proteolytically processed by two types of virus-encoded proteinases (papain-like and chymotrypsin-like) resulting in 15 non-structural proteins, nsp2-nsp16 (262). A programmed ribosomal frameshift occurs during the translation of ORF1a when expressing the pp1ab protein (191). Although some of these non-structural proteins are associated with known enzymatic functions such as helicase (nsp3) (122) and endonuclease activity (nsp15) (28), the correlation of these functions with pathogenesis or virulence of the virus still needs to be further elucidated (139, 204, 224).

Being a coronavirus, the hallmark of IBV transcription is the generation of a nested set of subgenome-length mRNAs that contain sequences corresponding to both ends of the genome, in other words, ‘discontinuous transcription’ (218, 265). The replicase-transcriptase proteins, along with other expressed viral, cellular proteins, assemble at the perinuclear region of the host cell to form the replication-transcription complexes (RTC) (219). The RTC then recognizes and binds to the cis-regulatory elements or binding sites located at the 3’ end of the genome, and initiates

synthesis of the minus strand RNA. The RTC then either transcribes the genome continuously into a genome-length template or discontinuously into various subgenome-length minus strand templates. A sequence consisting of around 10 nucleotides known as the transcription-regulating sequences (TRS) regulates the discontinuous transcription process. Starting from the 3' end of the genome, elongation of nascent minus-strand RNA continues until it encounters the first functional TRS motif, which is located in front of each open reading frame (ORF) of the encoded gene. At this point, the RTC would either ignore the TRS motif and continue to synthesize the nascent minus-strand or stop and complete the synthesis process. This 'decision making' process will occur every time the RTC encounters a TRS motif. These nested sets of various length minus strands then are utilized as templates for positive sense genomic and subgenomic mRNA synthesis (199, 217, 218). A leader sequence of around 70-80 nucleotides in length, which is derived from the 5' untranslated region (UTR) of the genome, is fused to the 5' end of the subgenomic mRNA and serves as a primer to produce a nested set of 3' co-terminal subgenomic mRNAs (33). Thus, each subgenomic mRNA contains a 5' leader sequence that corresponds to the 5' end of the genome. The signal mechanism of what causes the RTC to stop or continue minus-strand synthesis when encountering each TRS motif is still vague and needs to be further elucidated.

Countless serotypes of IBV exist around the world and cross-protection between serotypes is poor as the degree of amino acid identity between the S1 proteins of different IBV strains decreases (45, 47, 125). Thus, if lower homology in the sequence of the S1 subunit when comparing a vaccine and a field strain is observed, it may indicate that a greater chance that relevant mutations have occurred, which may result in a lower cross-protection (74). Different serotypes of IBV generally have similarities in the amino acid sequence of the S1 subunit

ranging from 50% to 80 % (151). It is assumed that this great genetic diversity in the S1 subunit results from recombination, mutation, and strong positive selection of a more fitting virus *in vivo* (44, 236). Low fidelity of the RNA-dependent-RNA polymerase paired with limited ability to correct mistakes in the replicated virus genome may also contribute to the extensive genetic diversity of IBV (125). As multiple types of IBV exist that do not cross-protect, control of this disease is highly complicated. Hard evidence of IBV variants consistently circulating and persisting in commercial poultry causing widespread disease outbreaks exists (124). In the United States, Ark, Mass, DE, GA98 and GA08 types are frequently isolated in the field, which are also the commonly used vaccine types in the United States along with two relatively new IBV variants GA13 and DMV1639 (125, 149, 150, 156).

Vaccines play a critical role in the control of IBV in poultry and vaccination against multiple IBV serotypes in commercial poultry operations is routinely practiced (125). Regarding vaccination, live attenuated vaccines are applied in broilers and pullets, whereas a combination of live and killed vaccines are used in layers and breeders (125). Live attenuated vaccines for IBV are usually produced by using virulent viruses as seeds and serially passing them in 10 to 11-day old embryonated chicken eggs, generally more than 50 passages (3, 29, 34, 123). As a consequence, the adaptation of the virus to the embryo occurs, resulting in higher efficiency in replication and higher pathogenicity for the embryo, but being simultaneously attenuated for chickens. Effective control involves detecting and differentiating these various types of IBV within an infected poultry flock accurately in a timely manner, followed by vaccination against that specific type. Standard methodologies for detecting IBV would include virus isolation (VI), reverse transcriptase polymerase chain reaction (RT-PCR) and nowadays, quantitative real-time RT-PCR (qRT-CPR). Tests that focus on detecting serum antibodies would include

haemagglutination inhibition (HI) assay, agar gel precipitation test (AGPT), enzyme-linked immunosorbent assay (ELISA) (38, 73, 212).

Efforts to develop strategies to confer broad protection against various IBV serotypes have been made in the past and continue until today. Although uncommon, some strains of IBV seem to be relatively more effective at providing cross-protection against heterologous serotypes. Such strains are categorized and referred to as protectotypes. Protectotypes have been suggested as a valuable concept for the development of strategies to control IBV (61, 161, 207). Protectotype vaccination is done by administrating two serotypes of IBV vaccine that are capable of providing a certain level of broad cross-protection against heterologous virus types. Combining a vaccine of the Ma5 strain, a Mass serotype and a 4/91 strain variant, provided good protection against various IBV serotypes including the QX strain that is predominant in Asia (61, 234). Whether this is a unique property for just this particular combination or if it applies to other different combinations of IBV is not known, but the same protection levels have not been found in other combinations to date (133). Multi-monovalent vaccination strategies are also commonly used in some parts of the world (126, 133). IBV vaccines against more than one serotype are given one at a time based on a vaccination schedule. This strategy can be customized depending on the type and number of prevalent serotypes that are circulating in the area.

Nowadays, one of the promising novel IBV vaccine candidates is infectious recombinant IBV strains as they can be strategically modified to alter pathogenicity in the laboratory using reverse genetic systems (RGS). The advantage of this system is that it enables rapid creation of an attenuated live vaccine against a newly emerged variant IBV by recombinant techniques, which takes much shorter in time compared to the conventional IBV attenuation method by embryo passage (133). As they are live attenuated viruses, they can be applied by traditional

methods (47). There were several attempts recently to establish an RGS based on several vaccine strains (26, 261).

Infectious laryngotracheitis. Infectious laryngotracheitis (ILT) is an economically important, acute and highly contagious upper respiratory tract disease of poultry that affects the poultry industry worldwide due to decreased egg production, weight losses, and predisposition to other respiratory avian pathogens (19, 93, 174). The etiologic agent infectious laryngotracheitis virus (ILTV), a double-stranded DNA virus, is in the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *iltovirus*, species *gallid herpesvirus 1* (GaHv-1) (68, 175). Natural infections are confined to galliform birds as ILTV exhibits a very narrow host range (87). Besides chickens, the virus can be isolated from pheasants, peafowl and experimentally infected turkeys (19, 64, 92, 253). Apart from direct transmission, ILTV can also be indirectly transmitted by fomites such as improper manure disposal, contaminated equipment, clothing, crates, live haul trucks, and infectious litter (79).

Clinical signs can be observed between 6 to 12 days after natural exposure to the virus (19). Clinical signs such as hemorrhagic conjunctivitis, watery eyes, nasal discharge, respiratory rales, gasping and spewing of bloodstained mucous can occur if the disease is severe (91, 174). Morbidity and mortality vary depending on several factors; virulence of the circulating strain causing disease, amount of virus circulating in the area and whether co-infection with other respiratory diseases is present within the infected flock. Some severe epizootic forms of the disease can elevate morbidity up to 100% and mortality as high as 70% (92). Intense viral replication generally occurs and stays present in tracheal tissues after ingestion of the virus through upper respiratory and ocular routes (110, 210). During the acute infection phase, virus

replication reaches its peak in the tracheal epithelium for up to 2 to 5 days post-infection (145). Although low levels of ILTV load can be sporadically detected up to 10 days post infection, active replication is usually confined to the first week of infection (113, 251). Like other herpes viruses, ILTV establishes latent infections which the virus can be reactivated and re-isolated under various stress factors followed by subsequent virus shedding (18, 251). The main site of latency is known to be the trigeminal ganglion (TRG) (251).

As similar in morphology to the icosahedral structure of herpes simplex virus-1 (HSV-1), the hexagonal nucleocapsid of ILTV containing the double-stranded viral DNA is known to be 80-100 nm in diameter consisting of 162 elongated hollow capsomeres (244). The viral genome of ILTV is approximately 150 kilobase pairs (kb) in length and consists of 79 predicted open reading frames (ORFs), long and short unique regions (UL , US) along with inverted repeats (IR, TR) flanking the short unique (US) region (92, 131, 158). The genome contains two clusters of genes specific for iltovirus, a cluster of five ORFs (A-E) located close to the 5'end, and the UL0, UL(-1) cluster located close to the 3' end of the gnome (87). The nucleocapsid is surrounded with an irregular envelope that is 195- 350 nm in diameter and contains fine projections or viral glycoproteins on its surface (101, 110, 244). As the genetic structure and proteins of ILTV resemble HSV-1, designation of many ORFs and proteins have been adopted from that virus (235). Like other alphaherpesviruses, the surface glycoproteins are responsible for virus mediation, virus egress and stimulating immune responses (228). Among them, five major envelope glycoproteins gB, gC, gD, gK, gX and a unique glycoprotein gp60 were considered as main antigens and first studied in the past (20, 256), and nowadays a total of 12 glycoproteins along with its ORFs (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, gN) are recognized although its functions and interactions are not fully understood (200). Among them, gB, gC, gG, gJ, gM,

gN have been identified at the protein level (86, 143, 146, 205), and was proven that gG, gJ, gM, gN were not essential for virus replication in cell culture (75, 86). Interestingly, sequence analysis of the ILTV glycoprotein B and glycoprotein C revealed that these proteins lacked consensus heparin-binding motifs, indicating that viral attachment of ILTV to its host cell is carried out in a heparin-independent manner, which is quite distinct from other alphaherpesviruses (144). The ILTV gC protein when compared to gC proteins of other alphaherpesviruses, it lacked arginine, lysine-rich sequences, resulting in a deletion of around 100 amino acids, making it shorter than other gC homologs thus lacking the positively charged region in the ectodomain required for heparin interaction (143). As it is commonly known that specific cell surface receptors play a direct role in determining susceptible hosts, the narrow host range of ILTV may be related to its heparin-independent entry pathway.

Along with biosecurity and rapid diagnosis, the primary method for controlling ILT is through vaccination although they are only used in geographic areas where the disease is endemic since live attenuated vaccines can persist in a latent state in the sensory ganglia of birds, thus producing long-term carriers or reservoirs (91, 110, 111, 211). Still, vaccination against an occurring outbreak is capable of both limiting the spread of the virus along with shortening the duration of the disease (110). Live attenuated vaccines and recombinant viral-vectored vaccines for ILTV are mainly used. Traditionally, two types of live attenuated ILT vaccines have been used, chicken embryo origin (CEO) (216) and embryonic tissue culture origin (TCO) (95). Although highly effective, in some cases their use (especially CEO) resulted in lower performance as some strains may carry residual virulence that can further increase during animal passages (87, 110). These types of vaccine strains can readily transmit from vaccinated to unvaccinated birds and may revert to virulent strains after several passages within the flock. The

seed of these vaccines strains was derived from virulent US field strains circulating between the 1950s and 1960s, which were attenuated by serial passages (91). CEO vaccines are generally administrated via drinking water and coarse spray vaccination in addition to the eye-drop route, whereas the TCO vaccine is confined to eye-drop administration (96, 197). Protection conferred by the CEO vaccine is known to be significantly higher than that of the TCO vaccine regarding clearance of the challenge virus and higher replication rates of the vaccine strain (237). However, virulently reverted CEO strains can cause more severe clinical signs compared to TCO revertants at the same passage level (103).

Based on past studies utilizing polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis, it was found that most of the ILTV isolates from commercial poultry in the United States were related to the commercial CEO vaccine strains (90, 138, 140), which didn't seem to have changed through the years according to a relevantly recent study using the same genotyping method (197). Similar studies on ILTV field isolates originating from vaccine strains can also be found outside of the United States (52, 100). In fact, epizootic outbreaks caused by these virulently reverted CEO-derived strains have already occurred worldwide in regions including North and South America, Australia and Europe (174). The TCO strain derived outbreaks, on the other hand, are uncommon (227).

Recombinant vaccines using fowl poxvirus (FPV) or turkey herpes virus (HVT) as viral vectors have been developed as an attempt to prevent CEO derived outbreaks (93). An FPV vector vaccine incorporating glycoprotein B and UL32 genes as antigens was first used in breeders and commercial layers the United States (69) and HVT vectored vaccines utilizing the same glycoprotein B, or glycoprotein I and D were also developed (93). The advantages of using HVT and FPV as vaccine vectors are that they lack bird to bird transmission, and most of all, do

not tend to revert and regain virulence (35, 237). Although it has been proven that such viral vector vaccines are indeed capable of mitigating clinical signs, they are not as effective as traditional live attenuated CEO/TCO vaccines regarding the reduction of virus shedding (237). The technical aspect of proper vaccine administration has been focused on these viral-vectored vaccines, as an inappropriate application can compromise vaccine efficacy eventually resulting in reduced or partial protection (93). Such concerns include technical failure of depositing the vaccines to the intended sites (amniotic cavity, embryo muscle) regarding *in ovo* vaccination or reducing quantities of the vaccine due to economic reasons (16, 79, 250). Recombinant ILTV vaccines based on gene-deletion have also been widely studied, aiming to develop recombinant ILTV strains that don't present growth defects (189, 241). A good example would be a recently developed ORF C gene deleted strain (ΔORF C), as the ORF C deletion didn't affect the fitness of the recombinant strain in vitro, shared the similar growth kinetics and titers comparable to the parental strain, and it's efficacy was comparable to that of a TCO vaccine when given by eye-drop administration, thus having a good potential for being a safe and efficacious vaccine (89).

Newcastle Disease. Newcastle disease (ND), caused by the etiologic agent Newcastle disease virus (NDV) formally known as *avian paramyxovirus 1* (APMV-1) and a member of the genus *avulavirus*, is responsible for severe economic losses throughout the domestic poultry industry (10, 254). This disease can spread rapidly to susceptible chickens, approaching mortality up to 100%, resulting in devastation and trade restraints and embargos on poultry products from areas or countries where outbreaks have occurred (5). However, ND infections are not only confined to poultry as infections have been confirmed in more than 236 avian species (135).

The first described cases of this disease in poultry came from Newcastle-upon-Tyne, England and Java, Indonesia in 1926 although it has been thought that there may have been some earlier outbreaks (11). Since its initial rapid spread in Asia, ND is currently distributed worldwide. At least six of the seven continents of the world have been involved, and ND infections associated with clinical signs were confirmed in many countries from Europe, Asia and the Americas such as Finland, Germany, Japan, Dominican Republic, Belize, and Peru (182). To date, a total of four panzootics have been recognized, causing a negative impact on not only the poultry industry and economy, but even directly affecting human livelihoods due to the reduction in food supplies (4). Between 2013 to 2015, ND outbreaks have been reported in an average of 60 countries (78). Considering unreported cases in Africa and Asia, the global distribution of ND seems to be more extensive.

Clinical signs observed in birds infected with NDV vary widely as they are dependent on factors such as the virulence of the virus itself, the species, immune status and age of host, predisposition with other pathogens, and environmental stress (183). Regarding chickens, pathogenicity and clinical signs of this disease varies significantly among ND strains. ND strains have been classified into five pathotypes based on the characteristic properties of clinical manifestation that can be observed in infected chickens, which are 1) viscerotropic-velogenic, 2) neurotropic-velogenic, 3) mesogenic, 4) lentogenic, 5) asymptomatic (9). When an outbreak of virulent ND occurs, either in a mesogenic or velogenic form, the affected country is obliged to report the case to the OIE (World Organization for Animal Health), and imports of poultry products from that country can be suspended (78). Virulent NDV strains (velogenic and mesogenic NDV) which are classified as select agents in the United States, are not endemic in the American domestic poultry and is often referred to as ‘exotic Newcastle disease (END)’,

although low virulence NDV or lentogenic strains still remain endemic (84, 182). Efficient surveillance and control measures against velogenic and mesogenic NDV are imperative as their existence can severely impact commercial performance and productivity alongside international exports in poultry. Virulent NDV is also capable of mutating, resulting in multiple genotypes (83).

NDV is an enveloped virus that carries a single stranded, negative-sense, non-segmented, RNA genome with a size around 15 kb (67) which contains six genes each encoding their corresponding proteins which are the nucleoprotein (NP), RNA polymerase (L), matrix (M), phosphoprotein (P), fusion (F), and the hemagglutinin-neuraminidase (HN) (9, 51, 148, 213). The HN and the F proteins are required for the virus to attach, fuse and enter the host cell (102). The HN protein binds to the sialic acid receptors, while the F protein mediates membrane fusion between the viral and the host cell membrane. One of the unique features of the F protein is that unlike many other fusion proteins of enveloped viruses, it does not require low or acidic pH to initiate fusion activity, and is likely to be capable of fusing directly at the host cell membrane under neutral pH conditions (109). The F protein is initially expressed as the precursor, F0 (9), which must be proteolytically cleaved by host-cell proteases into disulfide-linked F1 and F2 polypeptides for activation of fusion activity (188, 190). Upon initiation of fusion activity, it is thought that F proteins of paramyxoviruses go through a series of conformational changes that enables insertion of fusional peptides into the host cell membrane, although activation of such conformational changes by the F protein alone is insufficient and requires interaction with the HN protein (120). The HN protein, which mediates the entry process of the virus into the host cell in a receptor-dependent manner (153, 223), also carries specificity determinants or interacting domains for F-protein in the stalk region that involves in complex formation and

oligomerization (102, 172, 173) and is thought to activate refolding of F protein that catalyzes fusion activity. Regarding virulence and pathogenicity, although multiple genes are involved, is it widely known that the fusion or F0 protein cleavage site plays a critical role for significant shifts in virulence (71, 201). The use of reverse genetic systems (RGS) for NDV revealed that phenylalanine at position 117 (F117) along with basic amino acids surrounding glutamine (Q114) is essential for virulence (70, 98). In other words, one or two changes in the amino acid sequence in the F0 cleavage site may result in a non-virulent NDV strain, changing into a virulent strain (70).

NDV is generally classified into two major divisions, represented as class I and class II (21, 142, 182). Class I viruses are known to be non-virulent in chickens and have been isolated from shorebirds and waterfowls or species from the family *Anatidae* (7). Strains from class I possess the most extended genome in length (15,198 bp) among NDV and are further divided into at least nine genotypes originating from the wild bird population and live bird markets from Asia and the United States (67, 142). Class II viruses are comprised of ten genotypes, which includes low virulent vaccine strains (LaSota, B1, VG/GA) and predominant virulent genotypes circulating the world. Genotypes of class II strains are delineated into two categories based on their time of emergence, as the 'early' I, II, III, IV and IX and 'late' V, VI, VII, VIII, and X genotypes (67). The late genotypes of class II only consist of virulent strains and were responsible for various outbreaks in the Eurasian continent, the Americas (21, 203, 243, 254), and Africa (6). Increasing strain isolation and sequencing reports provide hard evidence that various class II genotypes are currently circulating simultaneously worldwide (182).

Like most poultry diseases, vaccination is considered as an effective way to contain ND in conjunction with proper management and strict biosecurity, aided with proper diagnostic tests,

and culling of infected flocks. This also applies in the United States. Although virulent NDVs are not endemic in U.S. domestic poultry, the main reason vaccination is implemented is to prevent and minimize economic losses caused by endemic low virulent NDV (LoNDV) (181). For decades, commercial live and inactivated NDV vaccines contributed in reducing ND outbreaks (130). The most commonly used ND vaccines that are used worldwide were derived from strains isolated from 1940 to 1960s, resulting in the development of LaSota, VG/GA and B1 live vaccines. Among them, the LaSota strain is nearly always used in virulent NDV endemic countries due to high replication rates resulting in higher production of neutralizing antibodies (76, 178). Avirulent strains from class II genotype I group are also widely used as vaccine strains (PHY-LMV42, I2, V4) (94). Inactivated vaccines for NDV have a disadvantage regarding protection as strong cell-mediated responses are not mounted despite high antibody levels, leading to increased shedding of virulent virus compared to live vaccine vaccinated flocks (179, 220). In an attempt to provide safe yet efficacious vaccines, virus vectored vaccines using FPV (32) and HVT (187) were also developed.

In recent years, recurrent infections of virulent NDVs in vaccinated flocks were reported (119), and the number of reported ND strains have increased, involving at least 60 countries, resulting in broadened genetic diversity (77). Currently used vaccine strains are mainly from class II genotype I and II and are known to be genetically distant from contemporary NDV strains that are circulating worldwide, regarding nucleotide distance (77). The high genetic discrepancy between vaccine strains and novel field isolates reduces vaccination efficacy alongside inefficient prevention of virus shedding in vaccinated flocks (180, 181). In other words, antigenic differences between vaccine strains and field isolates are directly correlated to protection efficacy, eventually leading to vaccine failure (184). Innovative approaches for

providing a solution under these circumstances would include the development of antigenically matched engineered vaccines using reverse genetics (78), virus-like particles (VLP) (171), cytokine-expressing vaccines (39) and so on.

Avian metapneumovirus. Avian metapneumoviruses (AMPV) belong to the subfamily *Pneumovirinae* within the family *Paramaxoviridae* and are currently categorized into four different subgroups (A, B, C, D) based on sequence analysis and virus neutralization (VN) patterns (24). Since its first discovery in South Africa, it was thought that the susceptible host was solely limited to turkeys since its first discovery in the 1970s, and was later discovered that infections in chickens are associated with the swollen head syndrome (37, 196, 202). AMPV can also exacerbate secondary bacterial infections such as *Ornithobacterium rhinotracheale* (ORT) and *Mycoplasma gallisepticum* (MG), which is associated with increased mortality (104, 192). In one study, viral priming of AMPV allowed adherence and colonization of ORT in the epithelial cells of the turbinates and trachea, proving synergism between the two respiratory pathogens (167). Although some viral replication occurs in the lungs, AMPV replication is usually limited to the upper respiratory tract and only for a short time. Experimental trials proved that the virus replicates to high loads in nasal and sinus tissues in turkeys for up to only 10 days post-inoculation, and was not isolated in other tissues (59, 240). In chickens, mortality rarely exceeds 2% although morbidity may reach up to 10% and affect egg production in breeders (56).

AMPV is an enveloped virus carrying a negative-sense, non-segmented RNA genome (206). The molecular structure resembles the members of the family *paramyxoviridae.* The size of this pleomorphic, spherical virus is around 150 nm in diameter and is predominantly filamentous (27, 55, 132). The helical nucleocapsid, which is located within the matrix (M)

protein layer, surrounds the 13 kb RNA genome. The nucleocapsid is known to be much smaller than other paramyxoviruses such as NDV (27). The lipid envelope of AMPV contains 3 viral glycoproteins, the G protein that is utilized for host cell attachment, the fusion protein (F) and small hydrophobic (SH) proteins of unknown functions (81). The F and G proteins exist as 10-14 nm spikes in length on the virus surface, and interact with the matrix protein layer located on the internal side of the viral membrane (55). During virus entry, the G proteins interact with heparin-like or glycosaminoglycan receptors on the host cell surface and mediate attachment (85, 106, 160). The F protein then initiates fusion between the viral envelope and host cell membrane in a pH-independent manner, and unpacking of the viral components and replication occurs in the cytoplasm (81). The G protein does not resemble the sequence nor the structure of attachment proteins (HN) of other paramyxoviruses (246). Interestingly, despite the difference in sequence homology between the F proteins of metapneumoviruses and other paramyxoviruses, it has been found that structural similarities are significant (23, 118, 258). Like other paramyxoviruses, the precursor F0 is cleaved into F1 and F2 disulfide-linked subunits by proteases such as furin (263, 264). Nonstructural proteins NS1, and NS2 are unique to animal pneumoviruses, and are expressed abundantly compared to other viral proteins. These relatively small proteins are known to hijack the host cell machinery by inhibiting RNA synthesis and interferon activation (15, 31, 221, 238). Four different subtypes of AMPV (A, B, C, D) exist with having subtype A and B mainly isolated in continental Europe and UK (193, 239), subtype C in North America and D in France (24, 128). Subtypes of AMPV have been differentiated based on nucleotide, amino acid sequence analyses of the G protein (134), and neutralization assays using monoclonal antibodies (54). AMPV is considered a significant threat involving both chickens and turkeys around the world including the United States (60, 222).

Vaccines have been available since the late 1980s in Europe and were capable of controlling the disease (57, 58). However, similar to NDV, disease occasionally has occurred in vaccinated flocks leading to vaccine failure (22, 42). Initially, it was thought that such vaccine failure was caused by poor application technique or administration of vaccines with different subtypes from field strains, which was the occasion in some cases (42, 239). However, it was revealed that using a vaccine strain of the same subtype did not always protect against strains within the same subtype group, suggesting that genetic changes within field isolates may have occurred, especially in the G protein region (22). According to a sequence analysis study on B subtypes that were isolated from 1987 to 2007 in Italy, it was found that consistent mutation of the G attachment protein was present in the newer strains, which was responsible for non-synonymous mutations that altered the amino acid sequence (49). Although it is evident that non-synonymous mutations in the G protein plays a critical role in alteration of AMPV strains, full sequencing revealed that changes in the SH protein were of similar magnitude to the changes seen in the G protein, with other genes remaining relatively unchanged. A correlative study between full genome sequencing data in conjunction with results from experimental vaccination trials suggested that poor protection was due to the significant amino acid changes in the G and SH protein region of the challenge strain (42). Although the precise function of the SH protein is undetermined, a reverse-genetics knockout approach speculated that deletion of the SH gene from the genome had a more significant impact in replication and immunogenicity, as the recombinant strain with a deleted SH gene showed the most significant impairment of fitness in both *in vitro* and *in vivo* experiments (163). As SH proteins are small integral membrane proteins located in the region of the viral membrane (81), it would likely to freely interact with the host immune system, impacting the fitness of AMPV strains.

Consistent shedding of the virus from flocks seems likely the reason for such field virus mutations, as some studies proved that when a live attenuated AMPV vaccine strain persists in the environment, it could sometimes revert to virulent strains (41, 42). Mutation of field strains due to vaccine pressure may occur if they adapt and sustain their fitness under the immune pressure of vaccinated flocks, resulting in numerous generations of mutants shedding in the environment. As several RGSs for AMPV have been utilized in the past (163, 194), development of a non-shedding live attenuated vaccine based on these genetic backbones may work as an alternative, although further understanding of AMPV genetics alongside protein interactions would be essential.

DIAGNOSTIC CONCEPTS OF MAJOR RESPIRATORY VIRUSES IN POULTRY

Detection of respiratory viral diseases in poultry. Major poultry respiratory diseases such as infectious bronchitis (IB), infectious laryngotracheitis (ILT), Newcastle disease (ND) and avian metapneumoviruses (AMPV) are currently distributed worldwide, each capable of causing significant economic losses in the commercial poultry sector. Demonstrating the presence of the virus and identifying the etiologic agent is imperative for control of those diseases. Moreover, it is crucial to differentiate these viruses, which can be similar in the early stages of their pathogenesis (242). Several diagnostic methodologies have been provided for the past decades, including detection or isolation of the viral agent itself, or by aiming at specific antibody responses.

One of the conventional gold standards for routine use is virus isolation (VI). The two most widely utilized methods are propagating the virus in either cell culture or embryonic chicken eggs (136). IBV, NDV and, AMPV mostly use embryonated chicken eggs for primary isolation (8, 62, 73), whereas isolation of ILTV is conducted in both cell culture and embryonated chicken eggs (19, 65). While the inoculation site in embryonated eggs for IBV and NDV is the allantoic cavity (73, 126), AMPV utilizes the yolk sac (170) and ILTV, the chorioallantoic membrane (CAM) (92). Regarding ILTV diagnostics, apart from a direct examination of the CAM focusing on the presence of plaques and thickening of the membrane, CAM samples are often submitted for histological and fluorescent antibody analysis (65). Despite its popular usage, however, several drawbacks exist with this technique. Virus isolation assays heavily rely on optimal sample collection and handling, and moreover, some viruses are not very stable outside of the host *in vivo* environments. Also, it can be quite laborious, time-

consuming and associated with prolonged turnaround times if the virus has to undergo extra in vitro passages (136). The availability of suitable biological systems will also be a concern as not all viruses will grow in the same system. For instance, primary isolation methods by introducing pathological materials directly into conventional monolayer cell cultures systems do not work well for IBV (73), whereas NDV (208), AMPV (62) and, ILTV (19, 65) can be readily adapted to grow in a variety of cell culture systems. Although VI assays have strengths compared to molecular techniques, such as being capable of identifying a new viral agent, as they tend to be less specific, allowing to whatever can grow and propagate well, it remains that they are slow and labor-intensive process. Thus, alternative assays are continually being introduced in the field of viral diagnostics.

Several detection methods focusing on the antibody response to the target virus exist, which will include virus neutralization (VN), hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA). These tests share the same fundamental principles as they function based on the formation of the antigen-antibody complex, which utilizes a known virus or viral protein typically, and samples that contain antibodies, which are usually serum collected from birds. HI tests are widely used for differentiating and serotyping IBV (73) and other paramyxoviruses (8, 17, 112), although in IBV HI tests pre-treatment with neuraminidase is essential so sialic acid residues from the spike protein of the virus envelope can be removed, which is needed to induce hemagglutination (HA) activity (214). Although HI tests can be simple and highly economical, one drawback is that they require a broad panel of antigens of each serotype, which are usually live viruses. And as these whole virus preparations contain conserved viral proteins that can cross-react with antibodies of different serotypes, results can lead to decreased specificity. ELISA has become a popular technique due its functional

simplicity and low costs. A conventional ELISA consists of an array of wells coated with either the target virus as the antigen or a specific viral protein that represents the virus of interest. Should the antibodies in the serum sample bind to the antigens coated in the well, color changes will occur when a secondary antibody labeled with an enzyme, and a substrate is added. Such changes are analyzed by a spectrophotometer and displayed as optical density (OD) signals. ELISAs have been developed and used for detection of IBV (185), NDV (225), AMPV (12, 162) and ILTV (177) in the poultry field, with commercial kits widely available.

Molecular-based methods such as polymerase chain reaction (PCR) or quantitative real-time polymerase chain reaction (qRT-PCR) possess a considerable advantage since such techniques do not rely on the presence of a live virus, but rather a piece of the viral genome, allowing sensitive detection of viral agents. Paired with more refined, standardized techniques of nucleic acid extraction and purification, alongside viral sequence data that are available through various databases, PCR is a technique that is ubiquitously utilized in the field of virus diagnostics regardless of the type of the virus. The discovery of reverse transcriptase (RT), an enzyme capable of generating a complementary template (cDNA) from the RNA template naturally used by retroviruses, allowed PCR reactions to include a reverse transcription step. The cDNA produced based on the viral RNA of the target virus during this step is then used as a template for exponential amplification during the PCR process, thus allowing to identify RNA viruses (154). Real-time PCR belong to a relatively more modern set of diagnostic methodologies, in which amplification of the DNA target template is monitored during the PCR, not after its completion, like conventional end-point PCR. The advancement of new fluorescent DNA labeling techniques has enabled such monitoring and detection of amplified DNA copies in real-time. Real-time PCR is the current golden standard in viral diagnostics, as it provides accurate

quantitation of gene copies without post-PCR processing steps such as the addition of ethidium bromide and densitometric analysis of PCR bands using ultraviolet rays (141), offering advantages in preventing contamination and generating throughput results (108).

Quantitative real-time polymerase chain reaction. Combined with its capacity to detect and quantify accurate amounts of gene copies in a wide range of samples along with its speed, practicality, high sensitivity, and reduced risk of carry-over contamination, quantitative real-time PCR has provided a new criterion for viral diagnostics (166). Compared to conventional PCR, real-time PCR is more reliable and better suited for prompt decision making under clinical situations (164). Real-time monitoring of gene copies or amplicon accumulation has been made possible with the utilization of fluorescent labeling of either primer, probe or the amplicon itself. The introduction of amplifiable hybridization probes regarding gene quantification provided great insight that led to the foundation of this technique (165).

Paired with a primer set for amplifying the target DNA sequence, hydrolysis probes (also known as 5' nuclease probes) are commonly used for real-time PCR assays to monitor amplicon accumulation. Since the first attempt to detect amplicons by tracking the 5'→3' endonuclease activity of the Taq DNA polymerase on oligoprobe-bound target DNA templates (117), based on this concept, fluorophore labeled probes were introduced which are customarily referred to by their proprietary name, TaqMan probes (157). A typical TaqMan probe consists of a short oligonucleotide that contains a fluorescent dye 'reporter' at the 5' end and a quenching dye or 'quencher' at the 3' end. Creation and detection of the fluorescence signal bound to target DNA are performed in two main sequential events; first, during PCR the probe binds to the target-complementary strand (cDNA) and second, the Taq polymerase cleaves the 5' end of the

TaqMan probe by 5'→3' endonuclease activity, spatially releasing the fluorescent reporter from the quencher (82). The real-time PCR instrument then monitors the unquenched emissions of the reporter dye, resulting in fluorescence signals. Moreover, the cleaved fluorescent dye accumulates after each PCR cycle, enabling detection at any time points during the whole thermo-cycling process, in other words, allowing 'real-time' representation of amplification. The signal emission increases proportionally to the quantities of the PCR product that are synthesized during the amplification process.

Certain requirements are needed for the optimal design of TaqMan probes. Their length should be limited to 20-40 nucleotide base pairs, a GC content of at least 40-60%, no repeated sequence motifs and most of all, a melting temperature (TM) at least 5°C higher than that of the primers, to ensure the probes can readily bind to the target template before the primer extension step (176). As Taq polymerase and TaqMan probes require at least 60°C for efficient 5'→3' endonuclease activity, thermo-cycling condition ranges typically between 60°C and 95°C (82).

Data processing is done based on a large amount of raw numerical data collected during the real-time PCR process, by generating a standard curve and evaluating amplification efficiency. A specific guideline, known as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE), was developed to improve real-time PCR based data by increasing uniformity, reliability, and transparency of reporting (36). By this guideline, certain assay performance characteristics must be determined, which the most important would be PCR efficiency. PCR efficiency can be defined as the increase rate of the target DNA template per amplification cycle, simply put, 'amplification efficiency' (215). PCR efficiency must be generated based on calibration or 'standard' curves, as they provide a simple and intuitive indication of the mean PCR efficiency. The standard curve is generally used to

evaluate the overall performance of real-time PCR assays by estimating its efficiency along with the dynamic range, and limit of detection (229). Real-time PCR efficiency is typically determined from the slope of the log-linear portion of the standard curves. To elaborate, the formula used for calculating PCR efficiency is E =[10(−1/slope)] – 1 (36). Generation of the standard curve usually involves a panel of samples with controlled relative quantities of the target template, serially diluted in 10-fold, each analyzed in triplicate (230). These standard samples are analyzed by real-time PCR by measuring the quantification cycle which is represented as CT values (36). CT vs. logarithm of the target template concentrations is plotted, generating a standard curve expected to be linear with a negative slope.

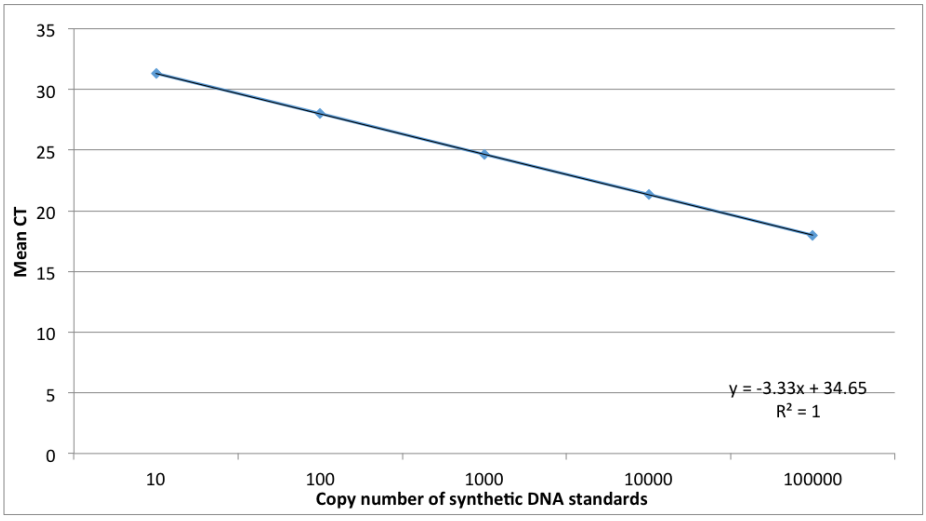


Fig 2-1. A typical standard curve of a real-time PCR assay (E=100%)

In the absence of interfering or inhibitory factors in the sample matrix, a properly designed real-time assay should show efficiencies ranging between 80%-115% (230, 260). If the samples were diluted by 10-fold, the slope of the standard curve should be −3.33 when PCR efficiency is 100%, indicating that a perfect doubling of numbers of the target template has occurred in each amplification cycle. In other words, assays should be designed aiming for standard curves with a -3.33 slope, within ranges between -3.0 (E = 115%) to -3.9 (E = 80%),

although some studies extend it to -2.9 (E=122%) (107). The slope is also directly correlated with the C_T values, as the interval (ΔC_T) between 10-fold diluted target templates at 100% efficiency would be 3.33. However, if the efficiency substantially exceeds 100% (E >115%), it may be indicating PCR inhibition in the sample matrix. If excessive amounts of DNA/RNA or carry-over material (SDS, phenol) are present in a sample matrix, they may function as 'PCR inhibitors' which flattens the efficiency plot, reducing the slope paired with a PCR efficiency of over 115%. As inhibitors are also diluted down along with other materials via serial diluting, the 'inhibited' samples would typically be the most-least diluted samples. It also can be explained based on ΔC_T; if inhibition occurs in the most concentrated (or least diluted) sample, a C_T value higher than the predicted 'earlier' C_T would appear, being the starting point of the standard curve. As inhibition activity is decreased when dilution gets higher in samples, assuming that the efficiency is 100%, the ΔC_T will at some point revert to 3.33. But since the C_T starting point was higher than the predictions and average ΔC_T between the concentrated samples will be smaller than 3.33, the standard curve will 'flatten out' as the slope lowers, which translates to unrealistically higher efficiency. A plausible solution to this would be to omit such 'concentrated' samples when calculating amplification efficiency.

VSV pseudotyping. Pseudotyping is a concept for generating viruses combined with foreign viral envelope proteins. It practically means that single or multiple structural proteins that are harbored by a virus particle are not encoded in the viral genome that carries them. Pseudotyped viruses have been widely utilized to identify proteins that function as host cell receptors such as glycans and lipids to determine virus entry. Typically, they exist as non-pathogenic, replication-deficient particles consisted of a structural core from one virus

incorporating the envelope glycoprotein of another virus on the outer shell. A significant advantage of this technique is that it can facilitate research and diagnostics by allowing researchers to circumvent strict biosafety restrictions (25).

Among several existing pseudotyping systems or vectors, Vesicular stomatitis virus (VSV), a prototypic non-segmented, negative-stranded RNA enveloped virus belonging to the family *Rhabdoviridae*, is a typical example that is used extensively as a versatile tool to study virus entry and replication (247). Paired with its compact structure along with its readily high-titer growth in a broad spectrum of susceptible cells, VSV has become useful in the fields of molecular virology, which was further strengthened and refined as a reliable vector for pseudotyping based on the establishment of the reverse genetics systems for VSV (233). The VSV pseudotyping system is widely used due to its remarkable properties, as they do not require high biosecurity clearance for handling, and that VSV is not particularly fastidious regarding incorporation of exotic or heterologous glycoproteins into the envelope (247).

VSV pseudotyped with heterologous envelope proteins from different viruses were demonstrated in earlier studies by co-infecting or 'phenotypic mixing' of VSV with other viruses in susceptible cells (245, 255, 259). The capability of VSV that makes it suitable for readily generating pseudotype particles is likely due to the well-orchestrated 'budding out' mechanism, and that it does not require its natural glycoprotein G during that process (129, 231). This natural attribute of VSV allows the production of 'spikeless' proteins that could be coated with heterologous envelope proteins by experimental manipulation. The VSV glycoprotein gene G can be deleted from the genome and be replaced with reporter genes such as luciferase or fluorescent reporters such as GFP (50, 72). Therefore it provides a convenient way to monitor the functions of VSV pseudotypes in studies involving viral entry and determining of viral

receptors. When recombinant VSV that is void of the G glycoprotein in its genome (rVSV-ΔG) is infected in susceptible cells, the progenies bud out and incorporates the heterologous envelope proteins that are transiently expressed on the host cell surface, resulting in rVSV-ΔG pseudotype particles coated with foreign glycoproteins (247). Such pseudotypes have been widely used for virus entry study that requires high-level biosafety containments such as ebolavirus (121, 231), SARS (88, 97), hantavirus (159), and recently, VSV particles bearing ebolavirus glycoproteins were developed and tested in clinical phase 1 trials for their use as recombinant subunit vaccines (2, 209).

Apart from the VSV pseudotyped particles that typically undergo a single cycle of replication (247), A more sophisticated approach involving a recombinant VSV virus system encoding a foreign envelope gene instead of the native gene in its genome has been developed (232, 233). As heterologous envelope gene inserted VSV is replication-competent in both in vitro and in vivo environments, it provides a relatively more authentic method for studying virus entry and furthermore, infection processes.

REFERENCES

1. Abdel-Moneim AS. 2017. Coronaviridae: Infectious Bronchitis Virus, p. 133-166, Emerging and Re-emerging Infectious Diseases of Livestock. Springer.

2. Agnandji ST, Huttner A, Zinser ME, Njuguna P, Dahlke C, Fernandes JF, Yerly S, Dayer J-A, Kraehling V, Kasonta R. 2016. Phase 1 trials of rVSV Ebola vaccine in Africa and Europe. New England Journal of Medicine 374:1647-16603.

3. Albanese GA, Lee D-H, Cheng I-HN, Hilt DA, Jackwood MW, Jordan BJJV. 2018. Biological and molecular characterization of ArkGA: A novel Arkansas serotype vaccine that is highly attenuated, efficacious, and protective against homologous challenge.

4. Alders RGJVR. 2014. Making Newcastle disease vaccines available at village level. 174:502-503.

5. Aldous E, Alexander DJAp. 2001. Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). 30:117-128.

6. Aldous E, Mynn J, Banks J, Alexander DJAp. 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. 32:237-255.

7. Alexander D, Campbell G, Manvell R, Collins M, Parsons G, McNulty MJTVR. 1992. Characterisation of an antigenically unusual virus responsible for two outbreaks of Newcastle disease in the Republic of Ireland in 1990. 130:65-68.

8. Alexander D. 2000. Newcastle disease and other avian paramyxoviruses. Revue Scientifique et Technique-Office International des Epizooties 19:443-455.

9. Alexander D, Senne DJDop, 12th ed. Blackwell Publishing, Ames, IA. 2008. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections, p 75–98.

10. Alexander DJ, Aldous EW, Fuller CMJAp. 2012. The long view: a selective review of 40 years of Newcastle disease research. 41:329-335.

11. Alexander DJRSeT-OIdE. 2000. Newcastle disease and other avian paramyxoviruses. 19:443-455.

12. Alvarez R, Njenga MK, Scott M, Seal BS. 2004. Development of a nucleoprotein-based enzyme-linked immunosorbent assay using a synthetic peptide antigen for detection of avian metapneumovirus antibodies in turkey sera. Clinical and diagnostic laboratory immunology 11:245-249.

13. Armesto M, Cavanagh D, Britton P. 2009. The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. PLoS One 4:e7384.

14. Armesto M, Evans S, Cavanagh D, Abu-Median A-B, Keep S, Britton P. 2011. A recombinant avian infectious bronchitis virus expressing a heterologous spike gene belonging to the 4/91 serotype. PLoS One 6:e24352.

15. Atreya PL, Peeples ME, Collins PL. 1998. The NS1 protein of human respiratory syncytial virus is a potent inhibitor of minigenome transcription and RNA replication. Journal of virology 72:1452-1461.

16. Avakian A, Wakenell P, Bryan T, Schaeffer J, Williams C, Whitfill C. 2002. Proceedings of 51º Western Poultry Disease Conference.

17. Awad F, Forrester A, Baylis M, Lemiere S, Jones R, Ganapathy K. 2015. Immune responses and interactions following simultaneous application of live Newcastle disease, infectious bronchitis and avian metapneumovirus vaccines in specific-pathogen-free chicks. Research in veterinary science 98:127-133.

18. Bagust T, Calnek B, Fahey K. 1986. Gallid-1 herpesvirus infection in the chicken. 3. Reinvestigation of the pathogenesis of infectious laryngotracheitis in acute and early post-acute respiratory disease. Avian Diseases:179-190.

19. Bagust T, Jones R, Guy J. 2000. Avian infectious laryngotracheitis. Revue Scientifique et Technique-Office International des Epizooties 19:483-488.

20. Bagust TJ, Johnson MA. 1995. Avian infectious laryngotracheitis: Virus-host interactions in relation to prospects for eradication. Avian Pathology 24:373-391.

21. Ballagi-Pordany A, Wehmann E, Herczeg J, Belak S, Lomniczi BJAov. 1996. Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. 141:243-261.

22. Banet-Noach C, Simanov L, Laham-Karam N, Perk S, Bacharach E. 2009. Longitudinal survey of avian metapneumoviruses in poultry in Israel: infiltration of field strains into vaccinated flocks. Avian diseases 53:184-189.

23. Bastien N, Normand S, Taylor T, Ward D, Peret TC, Boivin G, Anderson LJ, Li Y. 2003. Sequence analysis of the N, P, M and F genes of Canadian human metapneumovirus strains. Virus research 93:51-62.

24. Bäyon-Auboyer M-H, Arnauld C, Toquin D, Eterradossi NJJoGV. 2000. Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. 81:2723-2733.

25. Bentley EM, Mather ST, Temperton NJ. 2015. The use of pseudotypes to study viruses, virus sero-epidemiology and vaccination. Vaccine 33:2955-2962.

26. Berends AJ, Krämer-Kühl A, Spekreijse D, Mundt E, Chénard G, Philipp H-C, Verheije MH, Rottier PJ, van Beurden SJ. 2017. A reverse genetics system for avian coronavirus infectious bronchitis virus based on targeted RNA recombination. Virology journal 14:109.

27. Berthiaume L, Joncas J, Pavilanis V. 1974. Comparative structure, morphogenesis and biological characteristics of the respiratory syncytial (RS) virus and the pneumonia virus of mice (PVM). Archiv für die gesamte Virusforschung 45:39-51.

28. Bhardwaj K, Palaninathan S, Alcantara JMO, Yi LL, Guarino L, Sacchettini JC, Kao CCJJoBC. 2008. Structural and functional analyses of the severe acute respiratory syndrome coronavirus endoribonuclease Nsp15. 283:3655-3664.

29. Bijlenga G, Cook JK, Gelb J, Jack, Wit JdJAP. 2004. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. 33:550-557.

30. Bosch BJ, van der Zee R, de Haan CA, Rottier PJJJov. 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. 77:8801-8811.

31. Bossert B, Conzelmann K-K. 2002. Respiratory syncytial virus (RSV) nonstructural (NS) proteins as host range determinants: a chimeric bovine RSV with NS genes from human RSV is attenuated in interferon-competent bovine cells. Journal of virology 76:4287-4293.

32. Boursnell M, Green P, Campbell J, Deuter A, Peters R, Tomley F, Samson A, Chambers P, Emmerson P, Binns MJJoGV. 1990. Insertion of the fusion gene from Newcastle disease virus into a non-essential region in the terminal repeats of fowlpox virus and demonstration of protective immunity induced by the recombinant. 71:621-628.

33. Bredenbeek P, Noten J, Lenstra J, Horzinek M, van der Zeijst B, Spaan WJNar. 1986. The nucleotide sequence of the extreme 5'end of the avian coronavirus genome; implications for the discontinuous mRNA synthesis. 14:7806.

34. Britton P, Cavanagh DJCM, Biology C. 2007. Avian coronavirus diseases and infectious bronchitis virus vaccine development.161-181.

35. Bublot M, Pritchard N, Swayne DE, Selleck P, Karaca K, Suarez DL, AUDONNET JC, Mickle TRJAotNYAoS. 2006. Development and use of fowlpox vectored vaccines for avian influenza. 1081:193-201.

36. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry 55:611-622.

37. Buys SJT. 1980. A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. 28:36.

38. Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, Jackwood MW. 2006. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. Journal of virological methods 138:60-65.

39. Cardenas-Garcia S, Dunwoody RP, Marcano V, Diel DG, Williams RJ, Gogal Jr RM, Brown CC, Miller PJ, Afonso CLJPo. 2016. Effects of chicken interferon gamma on newcastle disease virus vaccine immunogenicity. 11:e0159153.

40. Casais R, Davies M, Cavanagh D, Britton P. 2005. Gene 5 of the avian coronavirus infectious bronchitis virus is not essential for replication. Journal of virology 79:8065-8078.

41. Catelli E, Cecchinato M, Savage CE, Jones RC, Naylor CJ. 2006. Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. Vaccine 24:6476-6482.

42. Catelli E, Lupini C, Cecchinato M, Ricchizzi E, Brown P, Naylor CJ. 2010. Field avian metapneumovirus evolution avoiding vaccine induced immunity. Vaccine 28:916-921.

43. Cavanagh D. 1983. Coronavirus IBV glycopolypeptides: size of their polypeptide moieties and nature of their oligosaccharides. Journal of General Virology 64:1187-1191.

44. Cavanagh D, Davis PJ, Mockett AA. 1988. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus research 11:141-150.

45. Cavanagh D, Elus M, Cook J. 1997. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathology 26:63-74.

46. Cavanagh D, Naqi S. 2003. Infectious bronchitis. Diseases of poultry 11:101-119.

47. Cavanagh D. 2007. Coronavirus avian infectious bronchitis virus. Veterinary research 38:281-297.

48. Cavanagh D, and J. Gelb 2008. Infectious bronchitis, Diseases of Poultry 12th Edition, Blackwell Publishing, Ames, IA.

49. Cecchinato M, Catelli E, Lupini C, Ricchizzi E, Clubbe J, Naylor C. 2009, p 278-284. VI International Symposium on Avian Corona-and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany, 14-17 June 2009.

50. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. 1994. Green fluorescent protein as a marker for gene expression. Science 263:802-805.

51. Chambers P, Millar NS, Bingham RW, Emmerson PTJJogv. 1986. Molecular cloning of complementary DNA to Newcastle disease virus, and nucleotide sequence analysis of the junction between the genes encoding the haemagglutinin-neuraminidase and the large protein. 67:475-486.

52. Chang P-C, Lee Y-L, Shien J-H, Shieh HK. 1997. Rapid differentiation of vaccine strains and field isolates of infectious laryngotracheitis virus by restriction fragment length polymorphism of PCR products. Journal of virological methods 66:179-186.

53. Chu VC, McElroy LJ, Chu V, Bauman BE, Whittaker GR. 2006. The avian coronavirus infectious bronchitis virus undergoes direct low-pH-dependent fusion activation during entry into host cells. Journal of virology 80:3180-3188.

54. Collins M, Gough R, Alexander D. 1993. Antigenic differentiation of avian pneumovirus isolates using polyclonal antisera and mouse monoclonal antibodies. Avian Pathology 22:469-479.

55. Compans RW, Harter DH, Choppin PW. 1967. STUDIES ON PNEUMONIA VIRUS OF MICE (PVM) IN CELL CULTURE: II. STRUCTURE AND MORPHOGENESIS OF THE VIRUS PARTICLE. Journal of Experimental Medicine 126:267-276.

56. Cook JJTVJ. 2000. Avian pneumovirus infections of turkeys and chickens. 160:118-125.

57. Cook JK, Ellis MM, Dolby CA, Holmes HC, Finney PM, Huggins MB. 1989. A live attenuated turkey rhinotracheitis virus vaccine. 1. Stability of the attenuated strain. Avian Pathology 18:511-522.

58. Cook JK, Holmes H, Finney P, Dolby CA, Ellis MM, Huggins M. 1989. A live attenuated turkey rhinotracheitis virus vaccine. 2. The use of the attenuated strain as an experimental vaccine. Avian Pathology 18:523-534.

59. Cook JK, Ellis MM, Huggins MB. 1991. The pathogenesis of turkey rhinotracheitis virus in turkey poults inoculated with the virus alone or together with two strains of bacteria. Avian Pathology 20:155-166.

60. Cook JK, Huggins MB, Orbell SJ, Senne DA. 1999. Preliminary antigenic characterization of an avian pneumovirus isolated from commercial turkeys in Colorado, USA. Avian Pathology 28:607-617.

61. 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 Pathology 28:477-485.

62. Cook JK, Cavanagh D. 2002. Detection and differentiation of avian pneumoviruses (metapneumoviruses). Avian Pathology 31:117-132.

63. Cook JK, Jackwood M, Jones R. 2012. The long view: 40 years of infectious bronchitis research. Avian Pathology 41:239-250.

64. Crawshaw GJ, Boycott BR. 1982. Infectious laryngotracheitis in peafowl and pheasants. Avian diseases:397-401.

65. Crespo R, Woolcock PR, Chin R, Shivaprasad H, García M. 2007. Comparison of diagnostics techniques in an outbreak of infectious laryngotracheitis from meat chickens. Avian diseases 51:858-862.

66. Curtis KM, Yount B, Baric RSJJov. 2002. Heterologous gene expression from transmissible gastroenteritis virus replicon particles. 76:1422-1434.

67. Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, Lomniczi BJVr. 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. 120:36-48.

68. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E. 2009. The order herpesvirales. Archives of virology 154:171-177.

69. Davison S, Gingerich EN, Casavant S, Eckroade RJJAd. 2006. Evaluation of the efficacy of a live fowlpox-vectored infectious laryngotracheitis/avian encephalomyelitis vaccine against ILT viral challenge. 50:50-54.

70. de Leeuw OS, Hartog L, Koch G, Peeters BPJJoGV. 2003. Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus: non-virulent cleavage site mutants revert to virulence after one passage in chicken brain. 84:475-484.

71. de Leeuw OS, Koch G, Hartog L, Ravenshorst N, Peeters BPJJoGV. 2005. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin–neuraminidase protein. 86:1759-1769.

72. De Wet JR, Wood K, DeLuca M, Helinski DR, Subramani S. 1987. Firefly luciferase gene: structure and expression in mammalian cells. Molecular and cellular biology 7:725-737.

73. De Wit J. 2000. Detection of infectious bronchitis virus. Avian pathology 29:71-93.

74. De Wit J, Cook JK, Van Der Heijden HMJAP. 2011. Infectious bronchitis virus variants: a review of the history, current situation and control measures. 40:223-235.

75. Devlin J, Browning G, Hartley C, Kirkpatrick N, Mahmoudian A, Noormohammadi A, Gilkerson J. 2006. Glycoprotein G is a virulence factor in infectious laryngotracheitis virus. Journal of General Virology 87:2839-2847.

76. Diel DG, Susta L, Garcia SC, Killian ML, Brown CC, Miller PJ, Afonso CLJJocm. 2012. Complete genome and clinicopathological characterization of a virulent Newcastle disease virus isolate from South America. 50:378-387.

77. Dimitrov KM, Ramey AM, Qiu X, Bahl J, Afonso CLJI, genetics, evolution. 2016. Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). 39:22-34.

78. Dimitrov KM, Afonso CL, Yu Q, Miller PJJVm. 2017. Newcastle disease vaccines—a solved problem or a continuous challenge? 206:126-136.

79. Dufour-Zavala LJAd. 2008. Epizootiology of infectious laryngotracheitis and presentation of an industry control program. 52:1-7.

80. Dwars RM, Matthijs MG, Daemen AJ, van Eck JH, Vervelde L, Landman WJ. 2009. Progression of lesions in the respiratory tract of broilers after single infection with Escherichia coli compared to superinfection with E. coli after infection with infectious bronchitis virus. Veterinary immunology and immunopathology 127:65-76.

81. Easton AJ, Domachowske JB, Rosenberg HF. 2004. Animal pneumoviruses: molecular genetics and pathogenesis. Clinical Microbiology Reviews 17:390-412.

82. Espy M, Uhl J, Sloan L, Buckwalter S, Jones M, Vetter E, Yao J, Wengenack N, Rosenblatt J, Cockerill F. 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clinical microbiology reviews 19:165-256.

83. Ewies SS, Ali A, Tamam SM, Madbouly HMJB-SUJoB, Sciences A. 2017. Molecular characterization of Newcastle disease virus (genotype VII) from broiler chickens in Egypt. 6:232-237.

84. Falcon MD. 2004, p 79-85. Seminars in avian and exotic pet medicine.

85. Feldman SA, Audet S, Beeler JA. 2000. The fusion glycoprotein of human respiratory syncytial virus facilitates virus attachment and infectivity via an interaction with cellular heparan sulfate. Journal of virology 74:6442-6447.

86. Fuchs W, Wiesner D, Veits J, Teifke JP, Mettenleiter TC. 2005. In vitro and in vivo relevance of infectious laryngotracheitis virus gJ proteins that are expressed from spliced and nonspliced mRNAs. Journal of virology 79:705-716.

87. Fuchs W, Veits J, Helferich D, Granzow H, Teifke JP, Mettenleiter TC. 2007. Molecular biology of avian infectious laryngotracheitis virus. Veterinary research 38:261-279.

88. Fukushi S, Watanabe R, Taguchi F. 2008. Pseudotyped vesicular stomatitis virus for analysis of virus entry mediated by SARS coronavirus spike proteins, p. 331-338, SARS-and Other Coronaviruses. Springer.

89. Garcia M, Spatz S, Cheng Y, Riblet S, Volkening J, Schneiders GJJoGV. 2016. Attenuation and protection efficacy of ORF C gene-deleted recombinant of infectious laryngotracheitis virus. 97:2352-2362.

90. García M, Riblet SM. 2001. Characterization of infectious laryngotracheitis virus isolates: demonstration of viral subpopulations within vaccine preparations. Avian diseases:558-566.

91. García M, Volkening J, Riblet S, Spatz S. 2013. Genomic sequence analysis of the United States infectious laryngotracheitis vaccine strains chicken embryo origin (CEO) and tissue culture origin (TCO). Virology 440:64-74.

92. García M, Spatz S, Guy JS. 2013. Infectious laryngotracheitis. Diseases of poultry:161-179.

93. García M. 2017. Current and future vaccines and vaccination strategies against infectious laryngotracheitis (ILT) respiratory disease of poultry. Veterinary microbiology 206:157-162.

94. Garcia SC, Lopez RN, Morales R, Olvera MA, Marquez MA, Merino R, Miller PJ, Afonso CLJA, microbiology e. 2013. Molecular epidemiology of Newcastle disease in Mexico: potential spillover of viruses from poultry into wild bird species.AEM. 00993-00913.

95. Gelenczei E, Marty E. 1964. Studies on a tissue-culture-modified infectious laryngotracheitis virus. Avian Diseases 8:105-122.

96. Gelenczei E, Marty EJAD. 1964. Studies on a tissue-culture-modified infectious laryngotracheitis virus. 8:105-122.

97. Glende J, Schwegmann-Wessels C, Al-Falah M, Pfefferle S, Qu X, Deng H, Drosten C, Naim HY, Herrler G. 2008. Importance of cholesterol-rich membrane microdomains in the interaction of the S protein of SARS-coronavirus with the cellular receptor angiotensin-converting enzyme 2. Virology 381:215-221.

98. Glickman RL, Syddall RJ, Iorio RM, Sheehan JP, Bratt MAJJoV. 1988. Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. 62:354-356.

99. Gonzalez J, Gomez-Puertas P, Cavanagh D, Gorbalenya A, Enjuanes L. 2003. A comparative sequence analysis to revise the current taxonomy of the family Coronaviridae. Archives of virology 148:2207-2235.

100. Graham D, McLaren I, Calvert V, Torrens D, Meehan B. 2000. RFLP analysis of recent Northern Ireland isolates of infectious laryngotracheitis virus: comparison with vaccine virus and field isolates from England, Scotland and the Republic of Ireland. Avian Pathology 29:57-62.

101. Granzow H, Klupp BG, Fuchs W, Veits J, Osterrieder N, Mettenleiter TC. 2001. Egress of alphaherpesviruses: comparative ultrastructural study. Journal of virology 75:3675-3684.

102. Gravel KA, Morrison TGJJov. 2003. Interacting domains of the HN and F proteins of Newcastle disease virus. 77:11040-11049.

103. Guy JS, Barnes HJ, Smith L. 1991. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian diseases:348-355.

104. Hafez H. 1998, p 138-145. Proceedings of 1th International Symposium on Turkey Disease.

105. Haijema BJ, Volders H, Rottier PJJJov. 2004. Live, attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. 78:3863-3871.

106. Hallak LK, Collins PL, Knudson W, Peeples ME. 2000. Iduronic acid-containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection. Virology 271:264-275.

107. Hamalainen H, Tubman J, Vikman S, Kyrölä T, Ylikoski E, Warrington J, Lahesmaa R. 2001. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. Analytical biochemistry 299:63-70.

108. Heid CA, Stevens J, Livak KJ, Williams PM. 1996. Real time quantitative PCR. Genome research 6:986-994.

109. Hernandez L, Hoffman L, Wolfsberg T, White JJAroc, biology d. 1996. Virus-cell and cell-cell fusion. 12:627-661.

110. Hidalgo H. 2003. Infectious laryngotracheitis: a review. Revista Brasileira de Ciência Avícola 5:157-168.

111. Hilbink F, Oei H, Van Roozelaar D. 1987. Virulence of five live vaccines against avian infectious laryngotracheitis and their immunogenicity and spread after eyedrop or spray application. Veterinary Quarterly 9:215-225.

112. Hinshaw V, Webster R, Turner B. 1980. The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. Canadian Journal of Microbiology 26:622-629.

113. Hitchner S, Fabricant J, Bagust TJAd. 1977. A fluorescent-antibody study of the pathogenesis of infectious laryngotracheitis.185-194.

114. Hodgson T, Casais R, Dove B, Britton P, Cavanagh DJJov. 2004. Recombinant infectious bronchitis coronavirus Beaudette with the spike protein gene of the pathogenic M41 strain remains attenuated but induces protective immunity. 78:13804-13811.

115. Hodgson T, Britton P, Cavanagh DJJov. 2006. Neither the RNA nor the proteins of open reading frames 3a and 3b of the coronavirus infectious bronchitis virus are essential for replication. 80:296-305.

116. Hofstad M, Yoder HW. 1966. Avian infectious bronchitis: Virus distribution in tissues of chicks. Avian diseases 10:230-239.

117. Holland PM, Abramson RD, Watson R, Gelfand DH. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3'exonuclease activity of Thermus aquaticus DNA polymerase. Proceedings of the National Academy of Sciences 88:7276-7280.

118. Hoogen B, Bestebroer TM, Osterhaus AD, Fouchier RA. 2002. Analysis of the genomic sequence of a human metapneumovirus (hMPV). Virology 295:119-132.

119. Hu S, Ma H, Wu Y, Liu W, Wang X, Liu Y, Liu XJV. 2009. A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. 27:904-910.

120. Hu X, Ray R, Compans RJJov. 1992. Functional interactions between the fusion protein and hemagglutinin-neuraminidase of human parainfluenza viruses. 66:1528-1534.

121. Ito H, Watanabe S, Sanchez A, Whitt MA, Kawaoka Y. 1999. Mutational analysis of the putative fusion domain of Ebola virus glycoprotein. Journal of virology 73:8907-8912.

122. Ivanov KA, Thiel V, Dobbe JC, van der Meer Y, Snijder EJ, Ziebuhr JJJov. 2004. Multiple enzymatic activities associated with severe acute respiratory syndrome coronavirus helicase. 78:5619-5632.

123. Jackwood MW, Hilt DA, Brown TPJAd. 2003. Attenuation, safety, and efficacy of an infectious bronchitis virus GA98 serotype vaccine. 47:627-632.

124. Jackwood MW, Hilt DA, Lee C-W, Kwon HM, Callison SA, Moore KM, Moscoso H, Sellers H, Thayer S. 2005. Data from 11 years of molecular typing infectious bronchitis virus field isolates. Avian diseases 49:614-618.

125. Jackwood MW. 2012. Review of infectious bronchitis virus around the world. Avian diseases 56:634-641.

126. Jackwood MW, de Wit S. 2013. Infectious bronchitis. Diseases of poultry:139-159.

127. Jackwood MW, Jordan BJ, Roh H-J, Hilt DA, Williams SM. 2015. Evaluating protection against infectious bronchitis virus by clinical signs, ciliostasis, challenge virus detection, and histopathology. Avian diseases 59:368-374.

128. Jardine C, Parmley E, Buchanan T, Nituch L, Ojkic D. 2018. Avian metapneumovirus subtype C in Wild Waterfowl in Ontario, Canada. Transboundary and emerging diseases.

129. Jayakar HR, Jeetendra E, Whitt MA. 2004. Rhabdovirus assembly and budding. Virus research 106:117-132.

130. Ji Y, Liu T, Du Y, Cui X, Yu Q, Wang Z, Zhang J, Li Y, Zhu QJVm. 2018. A novel genotype VII Newcastle disease virus vaccine candidate generated by mutation in the L and F genes confers improved protection in chickens. 216:99-106.

131. Johnson M, Prideaux C, Kongsuwan K, Sheppard M, Fahey K. 1991. Gallid herpesvirus 1 (infectious laryngotracheitis virus): cloning and physical maps of the SA-2 strain. Archives of virology 119:181-198.

132. Joncas J, Berthiaume L, Pavilanis V. 1969. The structure of the respiratory syncytial virus. Virology 38:493.

133. Jordan B. 2017. Vaccination against infectious bronchitis virus: a continuous challenge. Veterinary microbiology.

134. Juhasz K, Easton A. 1994. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. Journal of General Virology 75:2873-2880.

135. Kaleta EF, Baldauf C. 1988. Newcastle disease in free-living and pet birds, p. 197-246, Newcastle disease. Springer.

136. Kangro HO, Mahy BW. 1996. Virology methods manual. Elsevier.

137. Kant A, Koch G, Van Roozelaar D, Kusters J, Poelwijk F, Van der Zeijst B. 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. Journal of General Virology 73:591-596.

138. Keeler Jr CL, Hazel JW, Hastings JE, Rosenberger JK. 1993. Restriction endonuclease analysis of Delmarva field isolates of infectious laryngotracheitis virus. Avian diseases:418-426.

139. Keep S, Bickerton E, Armesto M, Britton PJJoGV. 2018. The ADRP domain from a virulent strain of infectious bronchitis virus is not sufficient to confer a pathogenic phenotype to the attenuated Beaudette strain.

140. Keller LH, Benson CE, Davison S, Eckroade RJ. 1992. Differences among restriction endonuclease DNA fingerprints of Pennsylvania field isolates, vaccine strains, and challenge strains of infectious laryngotracheitis virus. Avian diseases:575-581.

141. Kidd IM, Clark DA, Emery VC. 2000. A non-radioisotopic quantitative competitive polymerase chain reaction method: application in measurement of human herpesvirus 7 load. Journal of virological methods 87:177-181.

142. Kim LM, King DJ, Curry PE, Suarez DL, Swayne DE, Stallknecht DE, Slemons RD, Pedersen JC, Senne DA, Winker KJJov. 2007. Phylogenetic diversity among low-virulence Newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates. 81:12641-12653.

143. Kingsley DH, Hazel JW, Keeler Jr CL. 1994. Identification and characterization of the infectious laryngotracheitis virus glycoprotein C gene. Virology 203:336-343.

144. Kingsley DH, Keeler Jr CLJV. 1999. Infectious laryngotracheitis virus, an alpha herpesvirus that does not interact with cell surface heparan sulfate. 256:213-219.

145. Kirkpatrick NC, Mahmoudian A, Colson CA, Devlin JM, Noormohammadi AHJAP. 2006. Relationship between mortality, clinical signs and tracheal pathology in infectious laryngotracheitis. 35:449-453.

146. Kongsuwan K, Johnson MA, Prideaux CT, Sheppard M. 1993. Identification of an infectious laryngotracheitis virus gene encoding an immunogenic protein with a predicted Mr of 32 kilodaltons. Virus research 29:125-140.

147. Kottier SA, Cavanagh D, Britton P. 1995. Experimental evidence of recombination in coronavirus infectious bronchitis virus. Virology 213:569-580.

148. Krishnamurthy S, Samal SKJJoGV. 1998. Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. 79:2419-2424.

149. Kulkarni A. 2016, p 139. WESTERN POULTRY DISEASE CONFERENCE.

150. Kulkarni AB, Resurreccion RS. 2010. Genotyping of newly isolated infectious bronchitis virus isolates from northeastern Georgia. Avian diseases 54:1144-1151.

151. Kusters J, Niesters H, Lenstra J, Horzinek M, Van Der Zeijst B. 1989. Phylogeny of antigenic variants of avian coronavirus IBV. Virology 169:217-221.

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

153. Lamb RA, Jardetzky TSJCoisb. 2007. Structural basis of viral invasion: lessons from paramyxovirus F. 17:427-436.

154. Lanciotti RS, Calisher CH, Gubler DJ, Chang G-J, Vorndam AV. 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. Journal of clinical microbiology 30:545-551.

155. Lee C-W, Jackwood M. 2000. Evidence of genetic diversity generated by recombination among avian coronavirus IBV. Archives of virology 145:2135-2148.

156. Lee C-W, Jackwood MW. 2001. Origin and evolution of Georgia 98 (GA98), a new serotype of avian infectious bronchitis virus. Virus research 80:33-39.

157. Lee LG, Connell CR, Bloch W. 1993. Allelic discrimination by nick-translation PCR with fluorgenic probes. Nucleic acids research 21:3761-3766.

158. Leib D, Bradbury JM, Hart C, McCarthy K. 1987. Genome isomerism in two alphaherpesviruses: herpesvirus saimiri-1 (herpesvirus tamarinus) and avian infectious laryngotracheitis virus. Archives of virology 93:287-294.

159. Levanov L, Iheozor-Ejiofor RP, Lundkvist Å, Vapalahti O, Plyusnin A. 2018. Defining of MAbs-neutralizing sites on the surface glycoproteins Gn and Gc of a hantavirus using vesicular stomatitis virus pseudotypes and site-directed mutagenesis. bioRxiv:307975.

160. Levine S, Klaiber-Franco R, Paradiso P. 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. Journal of General Virology 68:2521-2524.

161. Lim T-H, Kim M-S, Jang J-H, Lee D-H, Park J-K, Youn H-N, Lee J-B, Park S-Y, Choi I-S, Song C-S. 2012. Live attenuated nephropathogenic infectious bronchitis virus vaccine provides broad cross protection against new variant strains. Poultry science 91:89-94.

162. Liman M, Rautenschlein S. 2007. Induction of local and systemic immune reactions following infection of turkeys with avian Metapneumovirus (aMPV) subtypes A and B. Veterinary immunology and immunopathology 115:273-285.

163. Ling R, Sinkovic S, Toquin D, Guionie O, Eterradossi N, Easton AJ. 2008. Deletion of the SH gene from avian metapneumovirus has a greater impact on virus production and immunogenicity in turkeys than deletion of the G gene or M2-2 open reading frame. Journal of general virology 89:525-533.

164. Locatelli G, Santoro F, Veglia F, Gobbi A, Lusso P, Malnati MS. 2000. Real-time quantitative PCR for human herpesvirus 6 DNA. Journal of clinical microbiology 38:4042-4048.

165. Lomeli H, Tyagi S, Pritchard C, Lizardi P, Kramer FR. 1989. Quantitative assays based on the use of replicatable hybridization probes. Clinical chemistry 35:1826-1831.

166. Mackay IM, Arden KE, Nitsche A. 2002. Real-time PCR in virology. Nucleic acids research 30:1292-1305.

167. Marien M, Decostere A, Martel A, Chiers K, Froyman R, Nauwynck H. 2005. Synergy between avian pneumovirus and Ornithobacterium rhinotracheale in turkeys. Avian pathology 34:204-211.

168. Masters PS. 2006. The molecular biology of coronaviruses. Advances in virus research 66:193-292.

169. Matthijs M, Van Eck J, Landman W, Stegeman J. 2003. Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: a comparison between vaccine and virulent field virus. Avian Pathology 32:473-481.

170. McDougall J, Cook J. 1986. Turkey rhinotracheitis: preliminary investigations. Veterinary Record 118:206-207.

171. McGinnes LW, Pantua H, Laliberte JP, Gravel KA, Jain S, Morrison TGJJov. 2010. Assembly and biological and immunological properties of Newcastle disease virus-like particles. 84:4513-4523.

172. Melanson VR, Iorio RMJJov. 2004. Amino acid substitutions in the F-specific domain in the stalk of the Newcastle disease virus HN protein modulate fusion and interfere with its interaction with the F protein. 78:13053-13061.

173. Melanson VR, Iorio RMJJov. 2006. Addition of N-glycans in the stalk of the Newcastle disease virus HN protein blocks its interaction with the F protein and prevents fusion. 80:623-633.

174. Menendez KR, García M, Spatz S, Tablante NL. 2014. Molecular epidemiology of infectious laryngotracheitis: a review. Avian pathology 43:108-117.

175. Mettenleiter TC, Klupp BG, Granzow H. 2009. Herpesvirus assembly: an update. Virus research 143:222-234.

176. Meuer S, Wittwer C, Nakagawara K-I. 2012. Rapid cycle real-time PCR: methods and applications. Springer Science & Business Media.

177. Meulemans G, Halen P. 1982. Enzyme-linked immunosorbent assay (ELISA) for detecting infectious laryngotracheitis viral antibodies in chicken serum. Avian Pathology 11:361-368.

178. Meulemans G. 1988. Control by vaccination, p. 318-332, Newcastle disease. Springer.

179. Miller P, Koch G, Suarez D, Mundt E, Jones R, Rautenschlein S. 2013. Newcastle disease, other paramyxoviruses, and avian metapneumovirus infections. Diseases of poultry. 13th ed. Ames, IA: Blackwell Publishing:250-377.

180. Miller PJ, King DJ, Afonso CL, Suarez DLJV. 2007. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. 25:7238-7246.

181. Miller PJ, Estevez C, Yu Q, Suarez DL, King DJJAd. 2009. Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. 53:39-49.

182. Miller PJ, Decanini EL, Afonso CLJI, genetics, evolution. 2010. Newcastle disease: evolution of genotypes and the related diagnostic challenges. 10:26-35.

183. Miller PJ, Koch GJDop. 2013. Newcastle disease. 13:89-138.

184. Miller PJ, Afonso CL, El Attrache J, Dorsey KM, Courtney SC, Guo Z, Kapczynski DRJD, Immunology C. 2013. Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. 41:505-513.

185. Mockett A, Darbyshire J. 1981. Comparative studies with an enzyme-linked immunosorbent assay (ELISA) for antibodies to avian infectious bronchitis virus. Avian Pathology 10:1-10.

186. Mockett AA, Cavanagh D, Brown TDK. 1984. Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis coronavirus strain Massachusetts M41. Journal of General Virology 65:2281-2286.

187. Morgan RW, Gelb Jr J, Schreurs CS, Lütticken D, Rosenberger JK, Sondermeijer PJJAd. 1992. Protection of chickens from Newcastle and Marek's diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein.858-870.

188. Morrison T, Ward L, Semerjian AJJov. 1985. Intracellular processing of the Newcastle disease virus fusion glycoprotein. 53:851-857.

189. Mundt A, Mundt E, Hogan RJ, García MJJogv. 2011. Glycoprotein J of infectious laryngotracheitis virus is required for efficient egress of infectious virions from cells. 92:2586-2589.

190. Nagai Y, Klenk H-D, Rott RJV. 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. 72:494-508.

191. Namy O, Moran SJ, Stuart DI, Gilbert RJ, Brierley IJN. 2006. A mechanical explanation of RNA pseudoknot function in programmed ribosomal frameshifting. 441:244.

192. Naylor C, Al-Ankari A, Al-Afaleq A, Bradbury J, Jones R. 1992. Exacerbation of Mycoplasma gallisepticum infection in turkeys by rhinotracheitis virus. Avian Pathology 21:295-305.

193. Naylor C, Shaw K, Britton P, Cavanagh D. 1997. Appearance of type B avian pneumovirus in Great Britain. Avian Pathology 26:327-338.

194. Naylor CJ, Brown PA, Edworthy N, Ling R, Jones RC, Savage CE, Easton AJ. 2004. Development of a reverse-genetics system for Avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability. Journal of general virology 85:3219-3227.

195. Niesters HG, Bleumink-Pluym NM, Osterhaus AD, Horzinek MC, Van Der Zeijst BA. 1987. Epitopes on the peplomer protein of infectious bronchitis virus strain M41 as defined by monoclonal antibodies. Virology 161:511-519.

196. O'Brien JJVR. 1985. Swollen head syndrome in broiler breeders. 117:619-620.

197. Oldoni I, García M. 2007. Characterization of infectious laryngotracheitis virus isolates from the US by polymerase chain reaction and restriction fragment length polymorphism of multiple genome regions. Avian Pathology 36:167-176.

198. Ortego J, Sola I, Almazán F, Ceriani JE, Riquelme C, Balasch M, Plana J, Enjuanes LJV. 2003. Transmissible gastroenteritis coronavirus gene 7 is not essential but influences in vivo virus replication and virulence. 308:13-22.

199. Pasternak AO, Spaan WJ, Snijder EJJJogv. 2006. Nidovirus transcription: how to make sense…? 87:1403-1421.

200. Pavlova SP, Veits J, Blohm U, Maresch C, Mettenleiter TC, Fuchs W. 2010. In vitro and in vivo characterization of glycoprotein C-deleted infectious laryngotracheitis virus. Journal of general virology 91:847-857.

201. Peeters BP, de Leeuw OS, Koch G, Gielkens ALJJov. 1999. Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. 73:5001-5009.

202. Perelman B, Meroz M, Samberg YJVR. 1988. 'Swollen head syndrome'in broiler breeders in Israel. 123.

203. Perozo F, Merino R, Afonso C, Villegas P, Calderon NJAd. 2008. Biological and phylogenetic characterization of virulent Newcastle disease virus circulating in Mexico. 52:472-479.

204. Phillips J, Jackwood M, McKinley E, Thor S, Hilt D, Acevedol N, Williams S, Kissinger J, Paterson A, Robertson JJVg. 2012. Changes in nonstructural protein 3 are associated with attenuation in avian coronavirus infectious bronchitis virus. 44:63-74.

205. Poulsen DJ, Keeler Jr CL. 1997. Characterization of the assembly and processing of infectious laryngotracheitis virus glycoprotein B. Journal of general virology 78:2945-2951.

206. Pringle C. 1998. Virus taxonomy–San Diego 1998. Archives of virology 143:1449-1459.

207. Raj GD, Jones R. 1996. Protectotypic differentiation of avian infectious bronchitis viruses using an in vitro challenge model. Veterinary microbiology 53:239-252.

208. Reddy G, Srinivasan V. 1992. Use of BHK cell culture-adapted Newcastle disease virus for immunization of chicks. Vaccine 10:164-166.

209. Regules JA, Beigel JH, Paolino KM, Voell J, Castellano AR, Hu Z, Muñoz P, Moon JE, Ruck RC, Bennett JW. 2017. A recombinant vesicular stomatitis virus Ebola vaccine. New England Journal of Medicine 376:330-341.

210. Robertson G, Egerton J. 1981. Replication of infectious laryngotracheitis virus in chickens following vaccination. Australian Veterinary Journal 57:119-123.

211. Rodriguez-Avila A, Oldoni I, Riblet S, García M. 2008. Evaluation of the protection elicited by direct and indirect exposure to live attenuated infectious laryngotracheitis virus vaccines against a recent challenge strain from the United States. Avian pathology 37:287-292.

212. Roh H-J, Jordan BJ, Hilt DA, Jackwood MW. 2014. Detection of Infectious Bronchitis Virus with the Use of Real-Time Quantitative Reverse Transcriptase–PCR and Correlation with Virus Detection in Embryonated Eggs. Avian diseases 58:398-403.

213. Römer-Oberdörfer A, Mundt E, Mebatsion T, Buchholz UJ, Mettenleiter TCJJoGV. 1999. Generation of recombinant lentogenic Newcastle disease virus from cDNA. 80:2987-2995.

214. Ruano M, El-Attrache J, Villegas P. 2000. A rapid-plate hemagglutination assay for the detection of infectious bronchitis virus. Avian diseases:99-104.

215. Ruijter J, Ramakers C, Hoogaars W, Karlen Y, Bakker O, Van den Hoff M, Moorman A. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic acids research 37:e45-e45.

216. Samberg Y, Cuperstein E, Bendheim U, Aronovici I. 1971. The development of a vaccine against avian infectious laryngotracheitis IV. Immunization of chickens with a modified laryngotracheitis vaccine in the drinking water. Avian diseases:413-417.

217. Sawicki S, Sawicki D. 2005. Coronavirus transcription: a perspective, p. 31-55, Coronavirus replication and reverse genetics. Springer.

218. Sawicki SG, Sawicki DL. 1995. Coronaviruses use discontinuous extension for synthesis of subgenome-length negative strands, p. 499-506, Corona-and Related Viruses. Springer.

219. Sawicki SG, Sawicki DL, Siddell SGJJov. 2007. A contemporary view of coronavirus transcription. 81:20-29.

220. Schijns VE, van de Zande S, Lupiani B, Reddy SM. 2014. Practical aspects of poultry vaccination, p. 345-362, Avian Immunology (Second Edition). Elsevier.

221. Schlender J, Bossert B, Buchholz U, Conzelmann K-K. 2000. Bovine respiratory syncytial virus nonstructural proteins NS1 and NS2 cooperatively antagonize alpha/beta interferon-induced antiviral response. Journal of virology 74:8234-8242.

222. Seal BS. 2000. Avian pneumoviruses and emergence of a new type in the United States of America. Animal Health Research Reviews 1:67-72.

223. Smith EC, Popa A, Chang A, Masante C, Dutch REJTFj. 2009. Viral entry mechanisms: the increasing diversity of paramyxovirus entry. 276:7217-7227.

224. Snijder EJ, Bredenbeek PJ, Dobbe JC, Thiel V, Ziebuhr J, Poon LL, Guan Y, Rozanov M, Spaan WJ, Gorbalenya AEJJomb. 2003. Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. 331:991-1004.

225. Snyder D, Marquardt W, Mallinson E, Russek E. 1983. Rapid serological profiling by enzyme-linked immunosorbent assay. I. Measurement of antibody activity titer against Newcastle disease virus in a single serum dilution. Avian diseases:161-170.

226. Sola I, Alonso S, Zúñiga S, Balasch M, Plana-Durán J, Enjuanes LJJov. 2003. Engineering the transmissible gastroenteritis virus genome as an expression vector inducing lactogenic immunity. 77:4357-4369.

227. Spatz S, Volkening J, Keeler C, Kutish G, Riblet S, Boettger C, Clark K, Zsak L, Afonso C, Mundt E. 2012. Comparative full genome analysis of four infectious laryngotracheitis virus (Gallid herpesvirus-1) virulent isolates from the United States. Virus Genes 44:273-285.

228. Spear P. 1993, p 167-180. Seminars in virology.

229. Stolovitzky G, Cecchi G. 1996. Efficiency of DNA replication in the polymerase chain reaction. Proceedings of the National Academy of Sciences 93:12947-12952.

230. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. 2015. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomolecular detection and quantification 3:9-16.

231. Takada A, Robison C, Goto H, Sanchez A, Murti KG, Whitt MA, Kawaoka Y. 1997. A system for functional analysis of Ebola virus glycoprotein. Proceedings of the National Academy of Sciences 94:14764-14769.

232. Tani H, Komoda Y, Matsuo E, Suzuki K, Hamamoto I, Yamashita T, Moriishi K, Fujiyama K, Kanto T, Hayashi N. 2007. Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins. Journal of virology 81:8601-8612.

233. Tani H, Morikawa S, Matsuura Y. 2012. Development and applications of VSV vectors based on cell tropism. Frontiers in microbiology 2:272.

234. Terregino C, Toffan A, Serena Beato M, De Nardi R, Vascellari M, Meini A, Ortali G, Mancin M, Capua I. 2008. Pathogenicity of a QX strain of infectious bronchitis virus in specific

pathogen free and commercial broiler chickens, and evaluation of protection induced by a vaccination programme based on the Ma5 and 4/91 serotypes. Avian Pathology 37:487-493.

235. Thureen DR, Keeler CL. 2006. Psittacid herpesvirus 1 and infectious laryngotracheitis virus: comparative genome sequence analysis of two avian alphaherpesviruses. Journal of virology 80:7863-7872.

236. Toro H, Pennington D, Gallardo RA, Van Santen VL, Van Ginkel FW, Zhang J, Joiner KS. 2012. Infectious bronchitis virus subpopulations in vaccinated chickens after challenge. Avian diseases 56:501-508.

237. Vagnozzi A, Zavala G, Riblet SM, Mundt A, García M. 2012. Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens. Avian pathology 41:21-31.

238. Valarcher J-F, Furze J, Wyld S, Cook R, Conzelmann K-K, Taylor G. 2003. Role of alpha/beta interferons in the attenuation and immunogenicity of recombinant bovine respiratory syncytial viruses lacking NS proteins. Journal of virology 77:8426-8439.

239. Van de Zande S, Nauwynck H, Cavanagh D, Pensaert M. 1998. Infections and reinfections with avian pneumovirus subtype A and B on Belgian turkey farms and relation to respiratory problems. Journal of Veterinary Medicine, Series B 45:621-626.

240. Van de Zande S, Nauwynck H, De Jonghe S, Pensaert M. 1999. Comparative pathogenesis of a subtype A with a subtype B avian pneumovirus in turkeys. Avian Pathology 28:239-244.

241. Veits J, Mettenleiter TC, Fuchs WJJogv. 2003. Five unique open reading frames of infectious laryngotracheitis virus are expressed during infection but are dispensable for virus replication in cell culture. 84:1415-1425.

242. Villegas P. 1998. Viral diseases of the respiratory system. Poultry science 77:1143-1145.

243. Wang Z, Liu H, Xu J, Bao J, Zheng D, Sun C, Wei R, Song C, Chen JJAotNYAoS. 2006. Genotyping of Newcastle disease viruses isolated from 2002 to 2004 in China. 1081:228-239.

244. Watrach A, Hanson L, Watrach M. 1963. The structure of infectious laryngotracheitis virus. Virology 21:601-608.

245. Weiss RA, Boettiger D, Murphy HM. 1977. Pseudotypes of avian sarcoma viruses with the envelope properties of vesicular stomatitis virus. Virology 76:808-825.

246. Wertz GW, Collins PL, Huang Y, Gruber C, Levine S, Ball LA. 1985. Nucleotide sequence of the G protein gene of human respiratory syncytial virus reveals an unusual type of viral membrane protein. Proceedings of the National Academy of Sciences 82:4075-4079.

247. Whitt MA. 2010. Generation of VSV pseudotypes using recombinant ΔG-VSV for studies on virus entry, identification of entry inhibitors, and immune responses to vaccines. Journal of virological methods 169:365-374.

248. Wickramasinghe IA, de Vries R, Gröne A, De Haan C, Verheije M. 2011. Binding of avian coronavirus spike proteins to host factors reflects virus tropism and pathogenicity. Journal of virology:JVI. 05112-05111.

249. Wickramasinghe IA, van Beurden S, Weerts E, Verheije M. 2014. The avian coronavirus spike protein. Virus research 194:37-48.

250. Williams C, Zedek AJPs. 2010. Comparative field evaluations of in ovo applied technology. 89:189-193.

251. Williams R, Bennett M, Bradbury J, Gaskell R, Jones R, Jordan F. 1992. Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain reaction. Journal of General Virology 73:2415-2420.

252. Winterfield R, Hitchner S. 1962. Etiology of an infectious nephritis-nephrosis syndrome of chickens. American Journal of Veterinary Research 23:1273-1279.

253. Winterfield R, So IG. 1968. Susceptibility of turkeys to infectious laryngotracheitis. Avian diseases 12:191-202.

254. Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, Kapczynski DR, Spackman E. 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology 42:329-338.

255. Witte ON, Baltimore D. 1977. Mechanism of formation of pseudotypes between vesicular stomatitis virus and murine leukemia virus. Cell 11:505-511.

256. York JJ, Sonza S, Brandon M, Fahey K. 1990. Antigens of infectious laryngotracheitis herpesvirus defined by monoclonal antibodies. Archives of virology 115:147-162.

257. Yount B, Roberts RS, Sims AC, Deming D, Frieman MB, Sparks J, Denison MR, Davis N, Baric RSJJov. 2005. Severe acute respiratory syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice. 79:14909-14922.

258. Yu Q, Davis P, Barrett T, Binns M, Boursnell M, Cavanagh D. 1991. Deduced amino acid sequence of the fusion glycoprotein of turkey rhinotracheitis virus has greater identity with that of human respiratory syncytial virus, a pneumovirus, than that of paramyxoviruses and morbilliviruses. Journal of general virology 72:75-81.

259. Zavada J, Rosenbergova M. 1972. Phenotypic mixing of vesicular stomatitis virus with fowl plague virus. Acta virologica.

260. Zhang AN, Mao Y, Zhang T. 2016. Development of Quantitative Real-time PCR Assays for Different Clades of “Candidatus Accumulibacter”. Scientific reports 6:23993.

261. Zhou YS, Zhang Y, Wang HN, Fan WQ, Yang X, Zhang AY, Zeng FY, Zhang ZK, Cao HP, Zeng C. 2013. Establishment of reverse genetics system for infectious bronchitis virus attenuated vaccine strain H120. Veterinary microbiology 162:53-61.

262. Ziebuhr J, Snijder EJ, Gorbalenya AEJJoGV. 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. 81:853-879.

263. Zimmer G, Budz L, Herrler G. 2001. Proteolytic activation of respiratory syncytial virus fusion protein cleavage at two furin consensus sequences. Journal of Biological Chemistry 276:31642-31650.

264. Zimmer G, Conzelmann K-K, Herrler G. 2002. Cleavage at the furin consensus sequence RAR/KR109 and presence of the intervening peptide of the respiratory syncytial virus fusion protein are dispensable for virus replication in cell culture. Journal of virology 76:9218-9224.

265. Zúñiga S, Sola I, Alonso S, Enjuanes LJJov. 2004. Sequence motifs involved in the regulation of discontinuous coronavirus subgenomic RNA synthesis. 78:980-994

CHAPTER 3

DEVELOPMENT OF SPECIFIC REAL-TIME QUANTITATIVE RT-PCR ASSAY PANEL FOR INFECTIOUS BRONCHITIS USING SYNTHETIC DNA STANDARDS AND CLINICAL SPECIMENS[1]

[1]J. Mo, M. Angelichio, L. Gow, V. Leathers, M. W. Jackwood. To be submitted to *Journal of virological methods*

ABSTRACT

Infectious bronchitis 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. We developed six TaqMan™-based quantitative RT-PCR assays (Universal, Ark, Mass, DE/GA98, GA07, GA08) 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 the 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.

Key words: infectious bronchitis virus, quantitative real time PCR, Ark, Mass, DE, GA98, GA07, GA08 serotype

Abbreviations: Ark = Arkansas; IBV = infectious bronchitis virus; qRT-PCR = quantitative real-time reverse-transcriptase polymerase chain reaction; Mass = Massachusetts; DE = Delaware; GA = Georgia, C_T = cycle threshold; IPC = internal positive control

INTRODUCTION

Infectious bronchitis is a highly contagious upper respiratory tract disease of chickens that is caused by avian coronavirus infectious bronchitis virus and constitutes a significant cause of economic loss in the industry (7). Although a respiratory disease, IBV can also affect the female reproductive tract, leading to poor production and egg quality. Some strains can even cause severe nephritis that results in significant mortality in young birds (10).

IBV is a lipid-enveloped positive-sense single-stranded RNA virus of the family *Coronaviridae* genus *gammacoronavirus* (14, 19). 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 (9, 14). 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 (5, 8, 14). This fact makes constant worldwide surveillance and identification of IBV types fundamental.

Vaccines play a critical role in the control of IBV in poultry (11), and vaccination against multiple IBV serotypes in commercial poultry operations is routinely practiced. Among the various types of IBV, Ark, Mass, DE and GA98 types are frequently isolated in the field, which are also the commonly used vaccine types in the United States (14). And during the recent years, 2 new IBV variants GA07, GA08 are also emerging (15, 16). Therefore, it is imperative to detect and differentiate these serotypes 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 is insensitive, laborious and time-consuming to be applicable in clinical detection.

To aid accurate and rapid diagnosis of IBV in the field we wanted to develop real time RT-PCR assays that would quickly identify specific IBV types and could be conducted on clinical samples. Real-time polymerase chain reaction has nowadays become one of the most common methods of gene quantitation due to its broad dynamic range, high sensitivity, and high sequence-specificity (26) in addition to functional simplicity and short run times. Real-time PCR has been very useful for detecting viral agents of infectious diseases (18). In this study, TaqMan™-based quantitative RT-PCR (qRT-PCR) methods for rapidly detecting and serotyping infectious bronchitis virus were evaluated using synthetic DNA templates that represented 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 (1, 20). An internal positive control (IPC) was also designed to verify the integrity of the universal test, and its effect on the test performance itself was evaluated. 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 (22), thus preventing false-negative reporting due to PCR inhibition (13, 21). 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 (2).

MATERIALS AND METHODS

Design of primers, probes. Primers and probes for each test are listed in (Table 3-1). IBV universal and IBV type-specific primers and probes previously designed by our laboratory (4, 23, 24) each targeting the 5'- UTR region and the hyper-variable region in the S1 gene subunit of IBV were used in this study as well as newly designed primers and probe sets. The IBV types involved 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, GA08 (14-16, 24). All hydrolysis, minor groove binding (MGB) probes used in this study was labeled at the 5' end with the reporter dye 6-carboxyfluorescein and MBG quencher at the 3' end (Applied Biosystems, Foster City, CA, USA). 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 commonly exists in avian originated samples for usage as internal positive controls (IPC) were also designed for the universal assay. The probe for the IPC was tagged with VIC dye at the 5' end and black hole quencher (BHQ1) at the 3' end (Biosearch Technologies, Novato, CA, USA).

Preparation of synthetic DNA standards. Double-stranded synthetic DNA standards were designed and synthesized based on the sequence of the hyper-variable 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 less than 200 base pairs, synthetic DNA standards were designed accordingly. The synthetic DNA standards were serially diluted 10-fold at a 5 $\log_{10}$ range. The starting point of synthetic DNA standards regarding copy numbers was 10^5 and was diluted down to 10 copies which was our goal regarding analytical sensitivity or limit of detection (LOD). We added 5 µl of each diluted synthetic DNA standard to the qRT-PCR reactions. Then, 10-fold serial dilutions of all synthetic DNA standards ranging from 10 to 10^5 copies per reaction were made and tested in triplicate. The qRT-PCR reactions with a final volume of 25 µl were prepared as follows: 5 µl of synthetic DNA standards, 10 µl of RealPCR™ RNA master mix (IDEXX Laboratories, Westbrook, ME, USA), forward primer with a final concentration of 0.4 µM in reaction, reverse primer with a final concentration of 0.4 µM in reaction, probe with a final concentration of 0.1 µM in reaction and RNase/DNase free water (Integrated DNA Technologies, Coralville, IA, USA) added to the final reaction volume of 25 µl. This reaction combination was the same for all IBV type-specific tests. Because there is a genetic variant of the GA08 type IBV, that assay included an additional forward primer (0.4 µM in reaction) targeting that genetic variant (Table 3-1). The IBV universal assay included an extra pair of primers and probe targeting endogenous avian RNA for use as an internal positive control (IPC) in avian originated samples. As biological avian samples were not used in this step, a separate IPC template mimicking the target sequence of the specific endogenous avian RNA was also added to 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. Each assay included positive and negative controls.

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, GA08 type IBVs for a thorough validation. Clinical samples refer to swabs that were collected from live infected birds whereas biological samples were consisted of virus stocks and organic tissue samples (Table 3-2). All available samples used in this study were from our laboratory archives. Briefly, viral nucleic acid was extracted from samples with the use of MagMax96 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 of it was subsequently used for the qRT-PCR assays. The 25 µl qRT-PCR reaction mixture was the same as when testing synthetic DNA standards except that synthetic DNA was replaced with viral nucleic acid extracted from clinical and biological samples, and that 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.

Thermocycling conditions for qRT-PCR. Amplification and detection were conducted in an Applied Biosystems® 7500 fast Real-Time PCR system (Applied Biosystems, Foster City,

CA, USA) under the following conditions: Reverse Transcription (RT) at 50 °C for 15 min and 95 °C for 1 min. RT was followed by 45 cycles of 95 °C for 15 sec and 60 °C for 30 sec with optics on. Thermocycling conditions for all assays were identical.

Statistical Analyses. 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 (3, 12, 17), the standard curve of each test was generated by plotting C_T values against relative input copy numbers. 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.

RESULTS

Primer and probe design. Specific primer and probes for universal assays as well as Ark, Mass, DE, GA98 types were previously designed by our laboratory (4, 23, 24). Type-specific primers and probes for GA07 and GA08 IBV types were newly designed from alignments of currently available IBV sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank). For the GA08 type assay, a forward primer targeting a variant type GA08 was also added. This primer shared the same reverse primer and probe with the original GA08 assay to amplify the target sequence of GA08 variant strains. And as the nucleotide sequences in the S1 subunit region were very similar in DE/GA98 type, they shared a single set of primers and probe (24).

Sensitivity of universal and type-specific assays using synthetic DNA standards. To determine the dynamic range, LOD, and quantitative capabilities of the developed IBV universal and type-specific assays, standard curves were generated using synthetic DNA standards that were designed identically to the target sequence on 5'- UTR region or the hyper-variable region in the S1 gene subunit. The specificity of the primers and probe designed for each assay was examined in detail *in silico* and was found that they were specific for their intended usage. 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 99% (Table 3-2). 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 3-2. All type-specific assays retained linearity for 5 orders of magnitude at a LOD of ≤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 of 80%-115% (25, 27, 28, 30) as it is generally recommended that qRT-PCR assays should always aim for $R^2 \geq 0.95$ for standard slopes between −3.0 and −3.9 with corresponding amplification efficiencies between 80% and 115% (29). The R^2 of all type-specific assays (Fig. 1) were ≥ 0.99, which indicates that results are highly reproducible.

Retrospective validation of universal and type-specific assays using clinical and biological specimens. To further verify the qRT-PCR assays whether they were suitable for diagnostic applications, 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 3-4). Specificity of the universal assay was verified stringently by testing 60 samples (Table 3-2) that were positive of 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 3-4, 3-5). The endogenous avian RNA IPCs in the samples were successfully co-amplified with the target sequence in the universal assay and did not seem to show any interference regarding performance (data not shown). The retrospective validation results of type-specific assays using clinical specimens are also presented in table 3-4. 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 94% (Table 3-4). No cross detection was observed with the non-target serotypes in the negative samples, indicating a high specificity of the developed type-specific assays (Table 3-5).

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 (6). For that purpose, we created 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 statistically measurable amplification efficiency compared to authentic cDNA templates (20). Using such material provided a more stringent validation criteria for evaluation of LOD and analytical specificity.

All developed IBV universal and type-specific assays performed equivalently when testing synthetic DNA templates, as the detection was linear over 5 $\log_{10}$ steps with amplification efficiencies ranging from 91.9% to 113.3% (Table 3-2, Fig 1). All assays showed LOD of $\leq$10 copy numbers per reaction with an R^2 $\geq$0.99 indicating high reproducibility. Regarding verification of sensitivity and specificity using clinical and biological samples, 100% 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. As the

endogenous avian RNA IPC did not seem to interfere with the universal assay in terms of performance, there were clear signs of interference when incorporating the IPC in the type-specific assays (data not shown), which was the reason why the IPC was only used in the universal assay. 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). One of the achievements of this study was that all developed qRT-PCR assays shared the same conditions concerning thermocycling parameters, thus allowing to test for various types of IBV in a single session depending on the sample size.

In summary, a panel of highly sensitive and specific qRT-PCR assays for universal detection and serotyping of IBV were developed which were validated with synthetic DNA standards, clinical and biological specimens. The provision of these assays will facilitate IBV diagnostics in the field in terms of accuracy, functional simplicity and rapidity.

REFERENCES

1. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, Tanaka S, Yoshiba M, Kohara M. 1999. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. Journal of Clinical Microbiology 37:2899-2903.

2. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry 55:611-622.

3. Callison S, Riblet S, Oldoni I, Sun S, Zavala G, Williams S, Resurreccion R, Spackman E, Garcia M. 2007. Development and validation of a real-time Taqman® PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. Journal of virological methods 139:31-38.

4. Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, Jackwood MW. 2006. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. Journal of virological methods 138:60-65.

5. Cavanagh D, Elus M, Cook J. 1997. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathology 26:63-74.

6. Cavanagh D. 2003. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian pathology 32:567-582.

7. Cavanagh D, Naqi S. 2003. Infectious bronchitis. Diseases of poultry 11:101-119.

8. Cavanagh D. 2007. Coronavirus avian infectious bronchitis virus. Veterinary research 38:281-297.

9. Cavanagh D, and J. Gelb 2008. Infectious bronchitis, Diseases of Poultry 12th Edition, Blackwell Publishing, Ames, IA.

10. Cook JK, Jackwood M, Jones R. 2012. The long view: 40 years of infectious bronchitis research. Avian Pathology 41:239-250.

11. Devlin JM, Vaz PK, Coppo MJ, Browning GF. 2016. Impacts of poultry vaccination on viruses of wild bird. Current Opinion in Virology 19:23-29.

12. Faye O, Faye O, Diallo D, Diallo M, Weidmann M. 2013. Quantitative real-time PCR detection of Zika virus and evaluation with field-caught mosquitoes. Virology journal 10:311.

13. Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. Journal of clinical microbiology 42:1863-1868.

14. Jackwood MW. 2012. Review of infectious bronchitis virus around the world. Avian diseases 56:634-641.

15. Kulkarni A. 2016, p 139. WESTERN POULTRY DISEASE CONFERENCE.

16. Kulkarni AB, Resurreccion RS. 2010. Genotyping of newly isolated infectious bronchitis virus isolates from northeastern Georgia. Avian diseases 54:1144-1151.

17. Larionov A, Krause A, Miller W. 2005. A standard curve based method for relative real time PCR data processing. BMC bioinformatics 6:62.

18. Mackay IM, Arden KE, Nitsche A. 2002. Real-time PCR in virology. Nucleic acids research 30:1292-1305.

19. Masters PS. 2006. The molecular biology of coronaviruses. Advances in virus research 66:193-292.

20. Moriya Y, Nakamura T, Okamura N, Sakaeda T, Horinouchi M, Tamura T, Aoyama N, Kasuga M, Okumura K. 2006. Comparison of synthetic DNA templates with authentic cDNA templates in terms of quantification by real-time quantitative reverse transcription polymerase chain reaction. Biological and Pharmaceutical Bulletin 29:535-538.

21. Nordstrom JL, Vickery MC, Blackstone GM, Murray SL, DePaola A. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic Vibrio parahaemolyticus bacteria in oysters. Applied and Environmental Microbiology 73:5840-5847.

22. Oikonomou I, Halatsi K, Kyriacou A. 2008. Selective PCR: a novel internal amplification control strategy for enhanced sensitivity in Salmonella diagnosis. Letters in applied microbiology 46:456-461.

23. Roh H-J, Hilt DA, Williams SM, Jackwood MW. 2013. Evaluation of infectious bronchitis virus Arkansas-type vaccine failure in commercial broilers. Avian diseases 57:248-259.

24. Roh H-J, Jordan BJ, Hilt DA, Jackwood MW. 2014. Detection of infectious bronchitis virus with the use of real-time quantitative reverse transcriptase–PCR and correlation with virus detection in embryonated eggs. Avian diseases 58:398-403.

25. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. 2015. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomolecular detection and quantification 3:9-16.

26. Wong ML, Medrano JF. 2005. Real-time PCR for mRNA quantitation. Biotechniques 39:75.

27. Zhang AN, Mao Y, Zhang T. 2016. Development of Quantitative Real-time PCR Assays for Different Clades of “Candidatus Accumulibacter”. Scientific reports 6:23993.

28. Zhang T, Fang HH. 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Applied Microbiology and Biotechnology 70:281-289.

29. Zhang T, Fang HHP. 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Applied Microbiology and Biotechnology 70:281-289.

30. Zheng F, Sun J, Cheng C, Rui Y. 2013. The establishment of a duplex real-time PCR assay for rapid and simultaneous detection of bla NDM and bla KPC genes in bacteria. Annals of clinical microbiology and antimicrobials 12:30.

TABLES

Table 3-1. Primers and probe used in this study.

Primers/Probes	**Target**	**Sequences(5'–3')**	**Reference**
IBV 59 GU391		GCTTTTGAGCCTAGCGTT	
IBV 59 GL533	Universal	GCCATGTTGTCACTGTCTATT	(4)
IBV 59 G Probe		FAM-CACCACCAGAACCTGTCACCTC-MGBNFQ	
Ark-F'		GGTGAAGTCACTGTTTCTA	
Ark-R'	Arkansas	AGCACTCTGGTAGTAATAC	(23)
Ark-Probe		FAM-TRTATGACAACGAATC-MGBNFQ	
Mass-F'		CGTKTACTACTAYCAAAGTGC	
Mass-R'	Massachusetts	CCATGAATARTACCAACARTACAC	(24)
Mass-Probe		FAM-AGGTGAAGAGCCTGCATTATTAGATTC-MGBNFQ	
DE/GA98-F'		AGGCGTTTGTACTGYATA	
DE/GA98-R'	Delaware/Georgia 98	GCCATGCCTTAAAATTTG	(24)
DE/GA98-Probe		FAM-ACTATGCAAYTATGACCRGTTCCACCAC-MGBNFQ	

Primers/Probes	Target	Sequences(5'–3')	Reference
GA07-F'		ACAAGGGGGTGCGTATGC	
GA07-R'	Georgia 07	TGCGTAACAAACACAGTAAAGTCT	This study
GA07-Probe		FAM-TGCATCAGTATGTACT-MGBNFQ	
GA08-F'		GCAGGCTCCTCATCTTCTTG	
GA08-V-F'[a]		GCAGGTACTGCCCAAAGTTG	This study
GA08-R'	Georgia 08	CAGGCCCACTACCGTTTTG	
GA08-Probe		FAM-TAAGTCAGGTGCCAAGGA-MGBNFQ	

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

Table 3-2. 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

Table 3-3. Efficiency of IBV Real-time RT 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	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

Table 3-4. Sensitivity of the IBV universal and type-specific assays using clinical and biological samples

	Known positive samples		
Assay type	**No. positive**	**No. negative**	**Sensitivity (%)[c]**
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	94 (28/30)

[a] Known positive sample group for the universal assay was consisted of Mass, Ark, DE, GA98, GA07, GA08, CONN[b], GA13 type IBV

[b] Connecticut type IBV

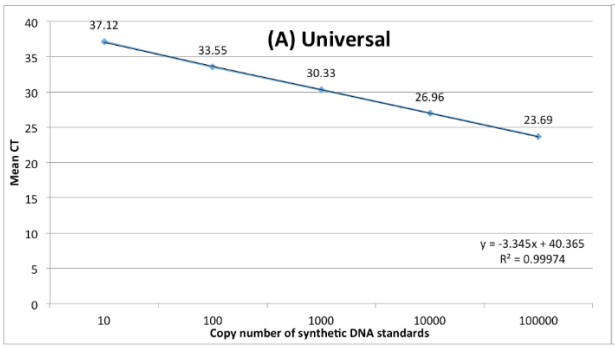
[c] Percentage of positive samples within a given subset

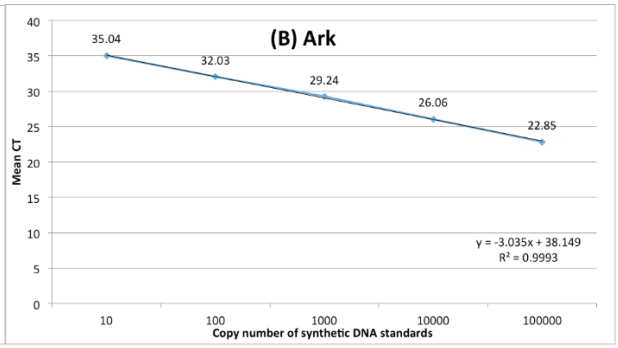
Table 3-5. Specificity of the IBV universal and type-specific assays using clinical and biological samples

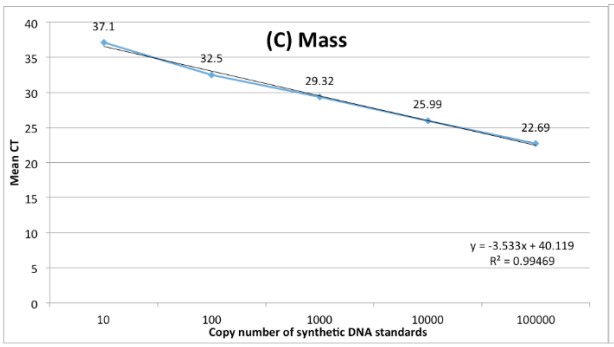
		Specific IBV qRT-PCR assay					
Virus	**Type**	**Universal**	**Ark**	**Mass**	**DE/GA98**	**GA07**	**GA08**
IBV	**Ark**	+	+	-	-	-	-
	Mass	+	-	+	-	-	-
	DE	+	-	-	+	-	-
	GA98	+	-	-	+	-	-
	GA07	+	-	-	-	+	-
	GA08	+	-	-	-	-	+
	GA13	+	-	-	-	-	-
	CONN	+	-	-	-	-	-
NDV[a]	**Lasota**	-	-	-	-	-	-

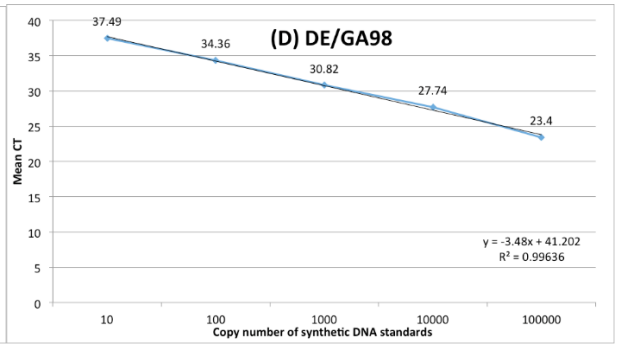
[a] New castle disease virus

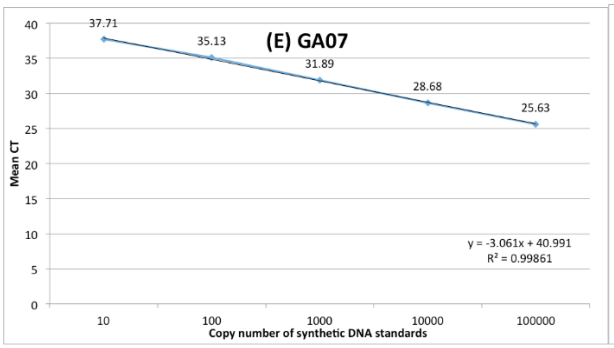
FIGURES

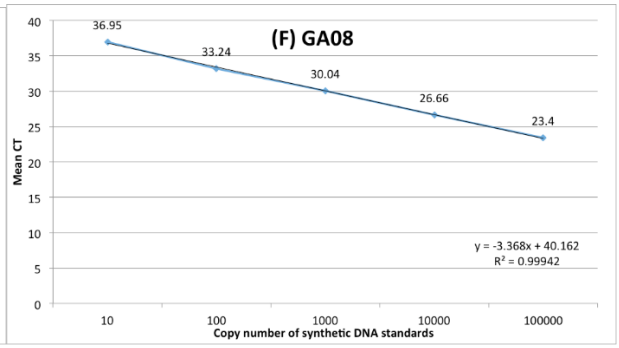

(A) Universal
37.12
33.55
30.33
26.96
23.69
y = -3.345x + 40.365
R² = 0.99974
Mean CT
Copy number of synthetic DNA standards
10
100
1000
10000
100000


(B) Ark
35.04
32.03
29.24
26.06
22.85
y = -3.035x + 38.149
R² = 0.9993
Mean CT
Copy number of synthetic DNA standards
10
100
1000
10000
100000


(C) Mass
37.1
32.5
29.32
25.99
22.69
y = -3.533x + 40.119
R² = 0.99469
Mean CT
Copy number of synthetic DNA standards
10
100
1000
10000
100000


(D) DE/GA98
37.49
34.36
30.82
27.74
23.4
y = -3.48x + 41.202
R² = 0.99636
Mean CT
Copy number of synthetic DNA standards
10
100
1000
10000
100000


(E) GA07
37.71
35.13
31.89
28.68
25.63
y = -3.061x + 40.991
R² = 0.99861
Mean CT
Copy number of synthetic DNA standards
10
100
1000
10000
100000


(F) GA08
36.95
33.24
30.04
26.66
23.4
y = -3.368x + 40.162
R² = 0.99942
Mean CT
Copy number of synthetic DNA standards
10
100
1000
10000
100000

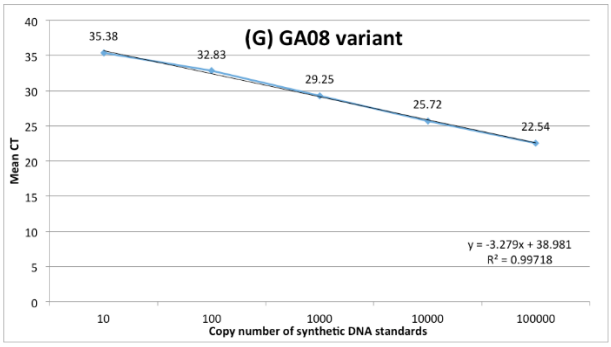

(G) GA08 variant
35.38
32.83
29.25
25.72
22.54
y = -3.279x + 38.981
R² = 0.99718
Mean CT
Copy number of synthetic DNA standards
10
100
1000
10000
100000

Figure 3-1. *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 ≤10 copies per reaction

CHAPTER 4

DEVELOPMENT OF SPECIFIC REAL-TIME QUANTITATIVE RT-PCR ASSAY PANELS FOR INFECTIOUS LARYNGOTRACHEITIS, NEWCASTLE DISEASE AND AVIAN METAPNEUMOVIRUS USING SYNTHETIC DNA STANDARDS, INTERNAL POSITIVE CONTROLS AND CLINICAL SPECIMENS[1]

[1]J. Mo, M. Angelichio, L. Gow, V. Leathers, M. W. Jackwood. To be submitted to *Journal of virological methods*

ABSTRACT

Respiratory viral diseases are common in the poultry, and diseases such as Newcastle disease, infectious laryngotracheitis and avian metapneumovirus can severely affect the respiratory tract of chickens, which can cause significant economic losses in the industry. Therefore it is crucial to rapidly detect and differentiate the etiologic agent within an infected poultry flock so that countermeasures against those viral diseases can be promptly implemented. We created a panel of qRT-PCR assays for these viruses that would be suitable for rapid and type specific diagnostic purposes. Analytical sensitivity was evaluated using synthetic DNA standards that mimicked the target sequence and specificity was verified using clinical and tissue specimens. All developed assays performed equivalently as it achieved linearity over a 5 $\log_{10}$ dynamic range with a reproducible (R^2 $\geq$0.99) limit of detection of $\leq$10 target copies per reaction, paired with high calculated amplification efficiencies ranging between 86.8%-98.2%. Further validation of specificity using clinical and biological specimens was also successful.

Key words: Newcastle disease, infectious laryngotracheitis, avian metapneumovirus, real-time polymerase chain reaction

Abbreviations: NDV = Newcastle disease virus; ILTV = infectious laryngotracheitis; AMPV = avian metapneumovirus; qRT-PCR = quantitative real-time reverse-transcriptase polymerase chain reaction; C_T = cycle threshold; IPC = internal positive control

INTRODUCTION

Respiratory viral diseases are frequent in poultry and constitute an important cause of economic loss in the industry. Among such viral diseases, Newcastle disease, infectious laryngotracheitis and avian metapneumovirus are the viruses that frequently affect the respiratory tract of chickens (23). Infectious laryngotracheitis (ILTV) is an upper-respiratory disease of poultry from the family *Herpesviridae* that causes sporadic cases of disease around the world associated with acute respiratory signs (2, 6). Newcastle disease virus (NDV), which was formally known as *avian paramyxovirus 1* (APMV-1) can lead to results in trade embargoes and quarantine depending on the type and virulence of the strain (24). And avian metapneumovirus (AMPV) a member of *Paramyxoviridae*, is the etiologic agent for severe rhinotracheitis in turkeys, causing increased mortality in conjunction with secondary bacterial infections (12, 21). Like most poultry diseases, it is crucial to rapidly detect and differentiate these viruses within an infected poultry flock so that appropriate measures against those diseases can be implemented promptly. Vaccines play a critical role in preventing such viral diseases in commercial poultry (1, 8, 10, 17). Therefore, identification of the etiologic agent should be done accurately and rapidly so effective vaccination programs can be implemented. However, diagnosis of respiratory virus infections using traditional methods like virus isolation and conventional serology is too insensitive and laborious to be applicable in clinical practice (3, 5, 7, 11, 24).

Real-time PCR (qRT-PCR) has nowadays become the golden standard of gene quantitation due to its large dynamic range, high sensitivity, high sequence-specificity in addition to short run times (25). Quantitative RT-PCR has proven to be useful for detecting viral agents of infectious diseases as they are capable of producing quantitative data in a short time with greater

precision (16). In this study, TaqMan™-based quantitative RT-PCR methods for rapidly detecting and typing a set of common avian respiratory viruses found in the field were developed and evaluated using synthetic DNA templates. Double-stranded oligonucleotide DNA templates were synthesized identically with the target sequences found in ILTV, NDV, and AMPV and utilized as standards for evaluating the analytical sensitivity or limit of detection (LOD) of the developed assays. Synthetic DNA templates mimicking the biological target sequence can be used for high-quality standardization of assay performance due to higher stability and amplification efficiency compared to biological cDNA. (18). An internal positive control (IPC) was incorporated to verify the integrity of all the developed assays and to rule out potential false negative results due to PCR inhibition by the sample matrix. The IPC is a non-target template present in the same well as the sample, which is co-amplified simultaneously with the target sequence of interest (13, 19, 20). Lastly, clinical specimens obtained from experimentally infected birds from our lab were processed for verifying specificity and sensitivity of these assays. Thermocycling conditions were synchronized for all assays to offer convenient diagnostic testing. In accordance with the MIQE guidelines, the performance of these assays was evaluated (4).

MATERIALS AND METHODS

Design of primers, probes. Primers and probes for each test are listed in Table 4-1. ILTV (5) and NDV (14) specific primers and probes sequences that were widely used for diagnostics were selected based on other literature, with AMPV-A and B primer, probes newly designed for this study. The primers and probe sets for each virus targeted the gC gene of ILTV (5), M and L gene of NDV (14, 24) and the N gene of AMPV-A and B respectively. All hydrolysis probes except the AMPV probe used in this study were labeled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM) and the minor groove binder non-florescent quencher (MGBNFQ) at the 3' end (Thermo Fisher Scientific, Waltham, MA, USA). The AMPV-A probe was tagged with cyanine 5 (Cy5) at the 5' end and quenched with BHQ1 at the 3' end. Specificity of the primers and probes for AMPV was validated by an in-depth *in silico* examination with the use of the BLAST search tool at National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov).

A set of primers and probe targeting endogenous avian RNA that naturally exists in avian originated samples for usage as internal positive controls (RNA-IPC) were also designed for the RNA virus AMPV-A/B, NDV assays. The probe for the RNA IPC was tagged with Cal Fluor® Orange 560 (CF560) dye at the 5' end and black hole quencher (BHQ1) at the 3' end (Biosearch Technologies, Novato, CA, USA). For the DNA virus ILTV assay, another set of primers and probe targeting the DNA-IPC was also designed. The DNA-IPC was a synthesized template that was artificially added to the sample matrix. The probe for the DNA IPC was tagged with VIC dye at the 5' end and black hole quencher (BHQ1) at the 3' end (Biosearch Technologies, Novato, CA, USA).

Preparation of synthetic DNA standards. Double-stranded synthetic DNA standards were designed and synthesized based on the target sequence in the gC gene of ILTV, M and L gene of NDV and the N gene of AMPV-A and B respectively (Integrated DNA Technologies, Coralville, IA, USA) after an in-depth *in silico* search 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 100 base pairs based on the target gene, synthetic DNA standards were designed accordingly. The synthetic DNA standards were serially diluted 10-fold spanning a 5 $\log_{10}$ range. The starting point of synthetic DNA standards regarding copy numbers was 10^5 and was diluted down to 10 copies, which was our goal LOD. 5 µl of each diluted synthetic DNA standard was added to the qRT-PCR reactions. Then, 10-fold serial dilutions of all synthetic DNA standards ranging from 10 to 10^5 copies were made and tested in triplicate. An extra pair of primers and probe focusing on endogenous avian RNA for use as an IPC in avian originated samples were added to the RNA virus NDV and AMPV assays. As biological avian samples were not included in this step, a separate IPC template mimicking the target sequence of the specific endogenous avian RNA was also added to the sample matrix. Primers and probes targeting the DNA template IPC, along with the IPC itself were added in DNA virus ILTV runs. Unlike RNA-IPC, DNA-IPC templates were designed as external templates to be added artificially in the sample matrix. The qRT-PCR reactions with a final volume of 25 µl were prepared as follows;

(a) NDV assay: 5 µl of synthetic DNA standards (NDV M or L gene) 10 µl of RealPCR™ RNA master mix (IDEXX Laboratories, Westbrook, ME, USA), forward M+4100-F primer (0.4 µM in reaction), reverse M-4220-R primer (0.4 µM in reaction), M+4169-P probe

(0.1 µM in reaction), forward L+8738-F primer (0.4 µM in reaction), reverse L-8847-R primer (0.4 µM in reaction), L+8762-P probe (0.1 µM in reaction), RNA-IPC (Internal positive control) forward primer (0.4 µM in reaction), RNA-IPC reverse primer (0.4 µM in reaction) ,RNA-IPC probe (0.1 µM in reaction), 2 µl of the RNA-IPC (10^3 target copy numbers) and RNase/DNase free water (Integrated DNA Technologies, Coralville, IA, USA) added to the final reaction volume of 25 µl. Synthetic DNA standards mimicking M and L gene were tested separately.

(b) ILTV assay: 5 µl of synthetic DNA standards (gC gene) 10 µl of RealPCR™ DNA master mix (IDEXX Laboratories, Westbrook, ME, USA), forward ILTVgCU771 primer (0.4 µM in reaction), reverse ILTVgCL873 primer (0.4 µM in reaction), ILTVprobe817 probe (0.1 µM in reaction), DNA-IPC forward primer (0.4 µM in reaction), DNA-IPC reverse primer (0.4 µM in reaction), DNA-IPC probe (0.1 µM in reaction), 2 µl of the DNA-IPC (10^3 target copy numbers) and RNase/DNase free water (Integrated DNA Technologies, Coralville, IA, USA) added to the final reaction volume of 25 µl.

(c) AMPV-A assay: 5 µl of synthetic DNA standards (gC gene) 10 µl of RealPCR™ DNA master mix (IDEXX Laboratories, Westbrook, ME, USA), forward AMPV-A primer (0.4 µM in reaction), reverse AMPV-A primer (0.4 µM in reaction), AMPV-A probe (0.1 µM in reaction), RNA-IPC forward primer (0.4 µM in reaction), RNA-IPC reverse primer (0.4 µM in reaction), RNA-IPC probe (0.1 µM in reaction), 2 µl of the RNA-IPC (10^3 target copy numbers) and RNase/DNase free water (Integrated DNA Technologies, Coralville, IA, USA) added to the final reaction volume of 25 µl.

(d) AMPV-B assay: 5 µl of synthetic DNA standards (gC gene) 10 µl of RealPCR™ DNA master mix (IDEXX Laboratories, Westbrook, ME, USA), forward AMPV-B primer (0.4 µM in reaction), reverse AMPV-B primer (0.4 µM in reaction), AMPV-B probe (0.1 µM in

reaction), DNA-IPC forward primer (0.4 µM in reaction), RNA-IPC reverse primer (0.4 µM in reaction), RNA-IPC probe (0.1 µM in reaction), 2 µl of the RNA-IPC (10^3 target copy numbers) and RNase/DNase free water (Integrated DNA Technologies, Coralville, IA, USA) added to the final reaction volume of 25 µl. Materials and reagents regarding the IPC were provided by IDEXX laboratories. Each assay included positive and negative controls.

Preparation and processing of clinical and biological samples. Panels of 30 clinical and biological samples that were positive for each virus were prepared and processed for verification of the type-specific assays as well as 30 known negative samples that included non-target avian respiratory viruses. Clinical samples were consisted of swabs that were collected from live infected birds whereas biological samples referred to organic tissue samples (Table 4-2). All samples used in this study were from our laboratory bird experiments. As we did not have the clearance to handle clinical samples of AMPV-A and AMPV-B, these assays were excluded from this step.

Briefly, viral RNA was extracted from NDV samples with the use of MagMax96 total RNA isolation kit (Ambion, Inc., Austin, TX) and the MagMAX™ Express 96 automated nucleic acid purification machine (Applied Biosystems, Foster City, CA, USA) based on the manufacturers' protocols. Viral DNA from ILTV samples was purified with the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) by the manufacturer's instructions. The extracted nucleic acid was suspended in 50 µl of elution buffer, and 5 µl of it was used for the qRT-PCR assays. The composition of the 25 µl qRT-PCR reaction mixture was identical as when testing synthetic DNA standards except that synthetic DNA was replaced with viral nucleic acid extracted from clinical and tissue samples, and that the RNA-IPC template

mimicking the target sequence of the specific endogenous avian RNA was excluded from at this step. 10^3 copies of DNA-IPC for the ILTV assays were spiked in the reaction. Each assay included positive and negative controls.

Thermocycling conditions for qRT-PCR. Assays were conducted in an Applied Biosystems® 7500 fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) by the following conditions: Reverse Transcription (RT) at 50 °C for 15 min and 95 °C for 1 min. RT step was followed by 45 cycles of 95 °C for 15 sec and 60 °C for 30 sec with optics on. Test duration was 81 minutes, and thermocycling conditions for all assays were synchronized.

Statistical Analyses. The amplification efficiency was determined using the 7500 Fast Software v2.0.6 (Applied Biosystems, Foster City, CA, USA). As PCR efficiency is generally calculated by the standard calibration curve method (5, 9, 15), the standard curve of each test was established by plotting C_T values against relative input copy numbers. The amplification efficiency of each assay was determined by the slope of the log-linear portion of the standard curve. The coefficient of determination (R^2) was determined for each assay.

RESULTS

Primer and probe design. Widely used ILTV (5) and NDV (14, 24) specific primers and probes sequences each targeting the ILTV gC gene and NDV M/L gene were selected for this study, with the newly designed AMPV-A and B primers, probes targeting the N gene. Type-specific primers and probes for AMPV A and AMPV B types were designed from alignments of currently available AMPV sequences in GenBank.

Sensitivity qRT-PCR assays using synthetic DNA standards. To determine the dynamic range, LOD and quantitative capabilities of the developed ILTV, NDV, AMPV-A, AMPV-B assays, standard curves were generated using synthetic DNA standards that were designed identically to the target sequence of each assay. The specificity of the primers and probe designed for each assay was examined in detail *in-silico* and was found that they were specific for only amplifying the target sequence. The dynamic range of all developed assays spanned 5 $\log_{10}$ units from 10 to 10^5 copies per reaction with a slope ranging from -3.37 to -3.68 and R^2 values $\geq$ 0.99, at a LOD of $\leq$10 copy numbers and with amplifications efficiencies of 86.8%-98.2%. The assays were able to detect 10 copy numbers in all triplicate runs. The artificially added IPC templates were successfully amplified and did not show any interference with the amplification of the target template regarding all assays (data not shown). The mean C_T values, slopes of the standard curve and R^2 values are presented in Table 3. The amplification efficiency was calculated by inserting the slope to the linear equation $E = [10(-1/\text{slope})] - 1$ (4). The average efficiencies of all assays stayed within the acceptable amplification range of 80%-115% (22, 26, 27), as it is recommended that functional qRT-PCR assays should always have a

$R^2 \geq 0.95$, with standard slopes between −3.0 and −3.9 and amplification efficiencies within 80% and 115%. The R^2 of all type-specific assays in this study (Table 4-3, Fig 4-1) were over 0.99, which indicates high reproducibility of the results.

Retrospective validation of universal and type-specific assays using clinical and biological specimen. To further verify the qRT-PCR assays whether they were applicable for diagnostic purposes, panels of 30 positive samples corresponding to NDV and ILTV were tested, as well as 30 known negative samples that included non-target sequences of other avian respiratory viruses. AMPV-A, B assays were excluded from this step due to limited access to clinical and tissue samples that were positive for AMPV. Samples consisted of tracheal and choanal swabs, and tissue samples collected from experimentally infected birds. Sensitivity was determined by the percentage of positive samples detected retrospectively within a group of known positive samples. C_T values over 40 were considered as negative. The retrospective validation results of NDV, ILTV assays using clinical specimens are presented in table 4-4. Both assays successfully detected their target virus in all known positive samples, and none of the non-target avian respiratory viruses were detected. The endogenous avian RNA IPCs in the NDV assay and the artificially spiked DNA-IPCs in the ILTV assay were successfully co-amplified with the target, and did not interfere the performance of the tests (data not shown). NDV, ILTV specific assays successfully detected the target in 100% of known positive samples, and no cross detection was observed with the non-target viruses in the negative samples, indicating a high specificity of the developed assays (Table 4-5).

DISCUSSION

Respiratory viral diseases are common in poultry and diseases such as Newcastle disease, infectious laryngotracheitis and avian metapneumovirus can severely affect the respiratory tract of chickens, which can cause significant economic losses in the industry (23). Like the majority of poultry diseases, it is crucial to rapidly detect and differentiate these viruses within an infected poultry flock so that countermeasures against those diseases can be implemented promptly. To aid that pursuit we created a panel of qRT-PCR assays for these viruses that would be suitable for rapid and type specific diagnostic purposes. Synthetic DNA templates mimicking target sequences in the NDV M/L gene, ILTV gG gene, and AMPV-A/B N gene were utilized for standardization and verification of the developed assays, as they hold advantages over cDNA templates that are prepared from biological samples since they present statistically measurable amplification efficiency compared to authentic cDNA templates (18). The usage of these synthetics provided a more rigorous verification criteria for evaluation of LOD and analytical specificity. Regarding NDV assays, to detect low virulence class I NDV along with class II NDV isolates from wild birds and live bird markets, primer and probe sets targeting for the M gene of class I (24) and L gene of class II (14) were selected based on previous publications.

All developed assays performed equivalently when testing synthetic DNA templates, as the dynamic range spanned over 5 $\log_{10}$ steps paired with amplification efficiencies ranging from 86.8%-98.2% (Table 3, Fig 1), and by presenting a LOD of ≤10 copy numbers per reaction with an R^2 ≥0.99 indicating high reproducibility of data. Regarding sensitivity and specificity validation steps using clinical and biological samples, 100% of the known positive samples were determined as positive by NDV and ILTV assays (Table 4-4). However, the retrospective sample verification

step for AMPV-A and B was excluded due limited access to clinical samples. An internal positive control (IPC) was designed and incorporated to prevent false negative reports due to PCR inhibition. The endogenous avian RNA-IPC in NDV and AMPV-A/B assays and the artificially spiked DNA-IPC in ILTV assays did not seem to interfere concerning performance. The RNA-IPC was designed to generate a mean C_T value of 27-29 and the DNA-IPC was designed to generate a mean C_T value of 30 with 103 copy numbers under non-inhibitory conditions (Data not shown). The thermocycling conditions for all developed assays were synchronized, thus allowing to test for several types of avian respiratory viruses in a single session depending on the sample size.

In summary, a panel of highly sensitive and specific qRT-PCR assays paired with internal positive controls for major avian respiratory viruses were developed which were verified with synthetic DNA standards, clinical and biological tissue specimens. The provision of these assays will aid field diagnostics to readily detect avian respiratory viruses accurately and rapidly.

REFERENCES

1. Alexander D. 2000. Newcastle disease and other avian paramyxoviruses. Revue Scientifique et Technique-Office International des Epizooties 19:443-455.

2. Bagust T, Jones R, Guy J. 2000. Avian infectious laryngotracheitis. Revue Scientifique et Technique-Office International des Epizooties 19:483-488.

3. Brittain-Long R, Nord S, Olofsson S, Westin J, Anderson L-M, Lindh M. 2008. Multiplex real-time PCR for detection of respiratory tract infections. Journal of Clinical Virology 41:53-56.

4. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry 55:611-622.

5. Callison S, Riblet S, Oldoni I, Sun S, Zavala G, Williams S, Resurreccion R, Spackman E, Garcia M. 2007. Development and validation of a real-time Taqman® PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. Journal of virological methods 139:31-38.

6. Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, Jackwood MW. 2006. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. Journal of virological methods 138:60-65.

7. Cook JK, Cavanagh D. 2002. Detection and differentiation of avian pneumoviruses (metapneumoviruses). Avian Pathology 31:117-132.

8. Devlin JM, Vaz PK, Coppo MJ, Browning GF. 2016. Impacts of poultry vaccination on viruses of wild bird. Current Opinion in Virology 19:23-29.

9. Faye O, Faye O, Diallo D, Diallo M, Weidmann M. 2013. Quantitative real-time PCR detection of Zika virus and evaluation with field-caught mosquitoes. Virology journal 10:311.

10. García M. 2017. Current and future vaccines and vaccination strategies against infectious laryngotracheitis (ILT) respiratory disease of poultry. Veterinary microbiology 206:157-162.

11. Gunson R, Collins T, Carman W. 2005. Real-time RT-PCR detection of 12 respiratory viral infections in four triplex reactions. Journal of clinical virology 33:341-344.

12. Ho Lee E, Song M-S, Shin J-Y, Lee Y-M, Kim C-J, Lee YS, Kim H, Choi YK. 2007. Genetic characterization of avian metapneumovirus subtype C isolated from pheasants in a live bird market. Virus research 128:18-25.

13. Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. Journal of clinical microbiology 42:1863-1868.

14. Kim LM, Suarez DL, Afonso CL. 2008. Detection of a broad range of class I and II Newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. Journal of Veterinary Diagnostic Investigation 20:414-425.

15. Larionov A, Krause A, Miller W. 2005. A standard curve based method for relative real time PCR data processing. BMC bioinformatics 6:62.

16. Mackay IM, Arden KE, Nitsche A. 2002. Real-time PCR in virology. Nucleic acids research 30:1292-1305.

17. Miller P, Koch G, Suarez D, Mundt E, Jones R, Rautenschlein S. 2013. Newcastle disease, other paramyxoviruses, and avian metapneumovirus infections. Diseases of poultry. 13th ed. Ames, IA: Blackwell Publishing:250-377.

18. Moriya Y, Nakamura T, Okamura N, Sakaeda T, Horinouchi M, Tamura T, Aoyama N, Kasuga M, Okumura K. 2006. Comparison of synthetic DNA templates with authentic cDNA templates in terms of quantification by real-time quantitative reverse transcription polymerase chain reaction. Biological and Pharmaceutical Bulletin 29:535-538.

19. Nordstrom JL, Vickery MC, Blackstone GM, Murray SL, DePaola A. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic Vibrio parahaemolyticus bacteria in oysters. Applied and Environmental Microbiology 73:5840-5847.

20. Renault T, Arzul I, Lipart C. 2004. Development and use of an internal standard for oyster herpesvirus 1 detection by PCR. Journal of virological methods 121:17-23.

21. Shin H-J, Njenga MK, McComb B, Halvorson DA, Nagaraja KV. 2000. Avian pneumovirus (APV) RNA from wild and sentinel birds in the United States has genetic homology with RNA from APV isolates from domestic turkeys. Journal of clinical microbiology 38:4282-4284.

22. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. 2015. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomolecular detection and quantification 3:9-16.

23. Villegas P. 1998. Viral diseases of the respiratory system. Poultry science 77:1143-1145.

24. Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, Kapczynski DR, Spackman E. 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology 42:329-338.

25. Wong ML, Medrano JF. 2005. Real-time PCR for mRNA quantitation. Biotechniques 39:75.

26. Zhang AN, Mao Y, Zhang T. 2016. Development of Quantitative Real-time PCR Assays for Different Clades of “Candidatus Accumulibacter”. Scientific reports 6:23993.

27. Zhang T, Fang HH. 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Applied Microbiology and Biotechnology 70:281-289.

TABLES

Table 4-1. Primers and probe used in this study

Primers/Probes	Target	Sequences (5'–3')	Reference
NDV M+4100	Matrix (M)	AGTGATGTGCTCGGACCTTC	(24)
NDV M-4220		CCTGAGGAGAGGCATTTGCTA	
NDV M+4169 probe		FAM-TTCTCTAGCAGTGGGACAGCCTGC-MGBNFQ	
NDV L+8738	Polymerase (L)	TGTTGAAAAGAAGCTGCTAGGC	(14)
NDV L-8847		TGGACCATGAAGAGTGGAACC	
NDV L+8762 probe		FAM-TGCCTGGTCACACAAGATCCGCCG-MGBNFQ	
ILTVgCU771	Glycoprotein (gC)	CCTTGCGTTTGAATTTTTCTGT	(5)
ILTVgCL873		TTCGTGGGTTAGAGGTCTGT	
ILTVprobe817		FAM-CAGCTCGGTGACCCCATTCTA-MGBNFQ	
AMPV-A-F	Nucleoprotein (N)	GGGAGCAATGGTTAGGGATAAA	This study
AMPV-A -R		TGAGGGCACCAATGCATAATA	
AMPV-A– probe		Cy5-AATAACGGGAGCATCCAAGGCAGA-BHQ1	

Primers/Probes	Target	Sequences (5'–3')	Reference
AMPV-B-F	Nucleoprotein (N)	CAAGCATGCAATCCTTGATGA	This study
AMPV-B-R		GTGGATACCTTTGGCTGTAGTT	
AMPV-B-probe		FAM-GGGTGTGATAGCAGTTGTAGCACCA-MGBNFQ	

Table 4-2. Known positive clinical and biological tissue samples used for this study

Virus	Clinical samples		Biological tissue samples
	Choanal swabs	**Tracheal swabs**	**CALT[a]**
NDV	30	0	0
ILTV	0	20	10

[a] Conjunctiva-associated lymphoid tissue

Table 4-3. Efficiency of NDV-M/L, ILTV, AMPV-A, AMPV-B Real-time RT 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			
NDV-M	22.05± 0.02	25.37± 0.05	28.80 ± 0.10	31.82± 1.47	36.27± 0.71	-3.49	93.4	0.99
NDV-L	23.36 ± 0.02	26.73± 0.08	30.04 ± 0.19	33.85± 0.14	38.17± 0.71	−3.68	86.8	0.99
ILTV	22.78± 0.02	26.15± 0.02	29.45 ± 0.04	32.92± 0.19	36.23± 0.32	-3.37	98.2	0.99
AMPV-A	23.30± 0.03	26.57± 0.09	30.13 ± 0.09	34.05± 0.14	36.45± 2.24	−3.38	97.1	0.99
AMPV-B	23.82± 0.11	27.08± 0.02	30.71 ± 0.14	34.45± 0.45	37.82± 0.21	−3.53	91.8	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

[*] All assays were co-amplified with internal positive controls

Table 4-4. Sensitivity of NDV, ILTV Real-time RT PCR assays using clinical and biological samples.

	Known positive samples		
Assay type	**No. positive**	**No. negative**	**Sensitivity (%)** [*]
NDV	30	0	100 (30/30)
ILTV	30	0	100 (30/30)

[*] Percentage of positive samples within a given subset

Table 4-5. Specificity of NDV, ILTV Real-time RT PCR assays using clinical and biological samples

	Target viruses		
Assay	**NDV**	**ILTV**	**IBV**
NDV	+	-	-
ILTV	-	+	-

+ = Positive, - = Negative

FIGURES

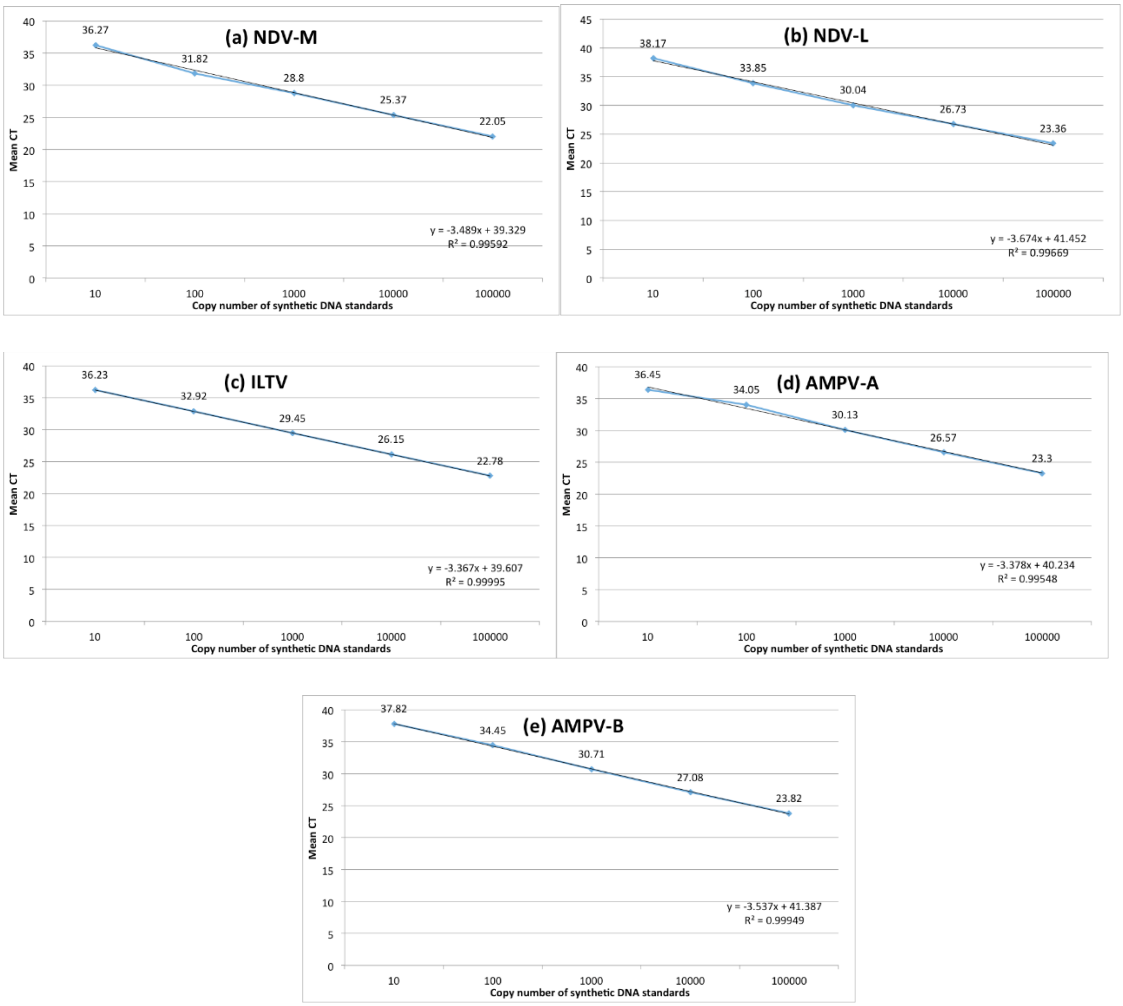


Figure 4-1. *Analytical sensitivity of qRT-PCR assays*. Standard curves for (a) NDV-M, (b) NDV-L, (c) ILTV, (d) AMPV-A, (e) AMPV-B assays presenting the mean C_T plotted against the relative input copy numbers (log_{10}) of synthetic DNA standards. Synthetic DNA standards were serially diluted by 10-fold at a 5 log_{10} range, starting from 10^5 copies down to ≤10 copies per reaction

CHAPTER 5

THE POTENTIAL OF PSEUDOTYPED VESICULAR STOMATITIS VIRUS (VSV) PARTICLES EXPRESSING IBV SPIKE AS SEROTYPING ELISA ANTIGENS[1]

[1]J. Mo, C. L. Leyson, B. J. Jordan, M. W. Jackwood to be submitted to *Journal of virological methods*

ABSTRACT

Infectious bronchitis (IB) is a highly contagious disease that affects the upper respiratory tract of chickens caused by avian coronavirus infectious bronchitis virus. Various serotypes of this virus are distributed worldwide, and cross-protection between serotypes is poor, making constant worldwide surveillance of IBV types important. The primary objective of this study was to address the need for a diagnostic test that could distinguish serotype-specific antibodies against various IBV types. Vesicular stomatitis virus (VSV) has been used as a versatile tool in research due to its capability of readily producing pseudotype progenies bearing foreign envelope proteins. Genes of the IBV spike protein were incorporated into the outer shell of the viral envelope of VSV and the functionality of these VSV particles carrying different IBV spikes (Massachusetts, Arkansas) as serotyping ELISA antigens was studied and evaluated. This proof of concept study proved some potential of the IBV spike pseudotyped VSV particles as serotyping antigens.

Keywords: infectious bronchitis, spike protein, glycoprotein, vesicular stomatitis virus, pseudotyping

Abbreviations: IBV = infectious bronchitis virus; Ark = Arkansas; Mass = Massachusetts; VSV = vesicular stomatitis virus; ELISA = Enzyme-linked immunosorbent assay

INTRODUCTION

Infectious bronchitis (IB) is a highly contagious disease that affects the upper respiratory tract of chickens caused by avian coronavirus infectious bronchitis virus (IBV) (4, 11). Although a respiratory disease, IBV can also affect the female reproductive tract; leading to reduced production and egg quality with some strains cause severe nephritis that results in high mortality in young birds (4, 7). IBV is currently distributed worldwide and is capable of causing significant economic losses in the commercial poultry sector (6). As a lipid-enveloped positive-sense single-stranded RNA virus of the family *Coronaviridae* genus *gammacoronavirus* (11, 18), the primary determinant of IBV serotype specificity is the spike protein. Because it contains epitopes for serotype-specific antibodies, spike protein is focused as the main protein for IBV identification (11). Various serotypes exist across the globe, and cross-protection between serotypes is poor, making constant worldwide surveillance and identification of IBV types fundamental (4, 5). For that purpose, enzyme-linked immunosorbent assay (ELISA) is the standard test that is widely used to detect and quantify IBV antibody titers in flocks due to its high sensitivity (10, 13). Although they are the primary method of choice for serological monitoring for IBV as they can facilitate large-scale monitoring of flocks, commercial ELISA kits focus on detecting group-specific antibodies rather than type-specific antibodies, thus cannot distinguish among different serotypes (7). Although it seems obvious that the development of serotyping ELISAs for IBV to detect serotype-specific antibodies would provide benefits, the reproduction of authentic serotype-specific spike proteins under laboratory conditions has proven to be difficult since the S2 portion of the spike has to be in an anchored form on the viral envelope for proper protein folding in the S1 antibody binding region to occur (3).

Vesicular stomatitis virus (VSV) has been widely used in research to produce pseudotyped virus particles, which contain the envelope glycoproteins of heterologous viruses (1, 15, 24, 25, 30). The concept of pseudotyping is that single or multiple structural proteins are harbored by a virus particle that is not encoded in the viral genome that carries them (2, 21). The capability of VSV that makes it suitable for readily generating pseudotype particles is likely due to the well-orchestrated 'budding out' mechanism, which does not require its natural glycoprotein G during that process (12). When recombinant VSV, that is void of the G glycoprotein in its genome, is infected in susceptible cells, the progenies bud out and incorporate the heterologous envelope proteins that are transiently expressed on the host cell surface, resulting in VSV pseudotype particles coated with foreign glycoproteins. Since VSV is not particularly selective in regards to the type of membrane protein that can be incorporated into the viral envelope (30), it is possible to generate a pseudotype that has the envelope protein of the IBV spike protein. Another advantage of pseudotyping IBV spikes is that it only requires knowledge of the nucleotide sequence of the spike gene, which can be synthesized and cloned for expression. Spike proteins of Mass and Ark serotypes, which are frequently isolated in the field, and also commonly used vaccine types in the United States were selected for this study (11). These newly prepared antigens will only contain the serotype determinant spike, thus having a good potential for serotype-specific ELISA antigens.

Thus, the overall goal of this study is to address the need for a diagnostic test that can distinguish serotype-specific antibodies against various IBV types, and that can be rapidly developed to include antigens from new and emerging IBV variants in the field.

MATERIALS AND METHODS

Cell lines and plasmids. HEK293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA). Chemically competent *E. coli* cells (One Shot™ TOP10) (Invitrogen, Waltham, MA, USA) were used for transformation of cloned plasmids. The envelope gene-deficient VSV (VSV-ΔG) and the high efficient mammalian expression vector pCAGGS were kindly provided by Dr. Melinda Brindley (The University of Georgia, College of veterinary medicine).

Construction of expression plasmids encoding IBV Mass & Ark whole spike genes. The whole spike glycoprotein sequences of Mass (accession number GQ504725) and Ark IBV (accession number GQ504721) were obtained from the database of the National Center for Bioinformatics (https://www.ncbi.nlm.nih.gov) and codon optimized. Plasmids (pUC57) containing the codon-optimized sequence for Mass and Ark whole spike (Mass-FL-S, Ark-FL-S) were commercially synthesized (Genewiz Inc., South Plainfield, NJ). EcoRI and NotI restrictions sites were appended at the 5' and 3' ends of Mass-FL-S and Ark-FL-S, respectively and the PCR products were cleaned up using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). The pUC57 plasmids containing Mass-FL-S and Ark-FL-S were double digested with EcoRI and NotI. The digested DNAs were electrophoresed in 0.8% agarose gel, and the target bands (3500 bp) were excised and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). To ligate Mass-FL-S and Ark-FL-S genes into the pCAGGS vector (9), digested fragments were combined at a 3:1 (insert: vector) ratio and mixed with T4 ligase (Thermo Scientific, Waltham, MA, USA). The ligation mixture

was incubated at 4°C overnight and subsequently transformed into chemically competent TOP10 *E. coli* cells using the heat shock method described by the manufacturer (Invitrogen, Waltham, MA, USA). The transformation reaction mix was plated on Luria-Bertani (LB) agar plates supplemented with 100 μg/mL carbenicillin for colony selection. Positive bacterial colonies were obtained and cultured overnight. Plasmid extraction was performed using the GeneJet plasmid miniprep kit (Thermo Scientific, Waltham, MA, USA). To identify positive transformants containing the right genes, plasmid minipreps from each bacterial colony was digested with EcoRI and subsequently, sequenced at the Georgia Genomics Facility (University of Georgia) for full validation. Large stocks of Mass-FL-S and Ark-FL-S plasmids planning to be transfected into HEK 293T cells were prepared using the GeneJet Maxiprep kit (Thermo Scientific, Waltham, MA, USA) as per the manufacturer's instructions.

Expression of IBV Mass & Ark whole spike protein. Plasmids coding for whole Mass and Ark IBV spike were transfected separately in different batches of HEK 293T cells using Turbofect (Thermo Scientific, Waltham, MA) transfection reagent following the manufacturer's instructions. HEK 293T cells were seeded in T75 flasks at a density of 2.1 x 10^6 cells and were grown up to 70% confluency. Plasmid DNA was diluted in OptiMEM (Gibco, Waltham, MA) and incubated in room temperature with Turbofect for 20 minutes. The DNA: OptiMEM: Turbofect ratio used for the transfection mixture was 18 μg: 2.4 mL: 30 μL for cells seeded in a T75 flask around 70% confluency. The transfection mixture was added to the T75 flask cell media (DMEM, 5% FBS) and incubated for 24 hours at 37°C, 5% CO2. After 24 hours, VSV-ΔG virus prep (Kerafast, Boston, MA, USA) was added to each T75 flask at a multiplicity of infection (MOI) of 3-5 (30). The 5% FBS DMEM was replaced with maintenance media

(DMEM, 1% FBS) the volume of which was reduced by half prior to VSV-ΔG infection as an attempt to concentrate Mass-FL-S and Ark-FL-S incorporated VSV pseudotypes. At 20 hours post infection, the supernatants containing VSV particles pseudotyped with Mass and Ark whole spike (VSV-Mass-FL-S, VSV-Ark-FL-S) were collected, centrifuged at 4°C, 1320 x g for 10 minutes and the supernatant was filtered using 0.22 µm filtered syringes and stored in aliquots at -80°C.

Validation and quantification of VSV-Mass-FL-S and VSV-Ark-FL-S. To quantify and validate if the IBV spikes coated on the surface of VSV-Mass-FL-S and VSV-Ark-FL-S were authentically expressed, hemagglutination assays (HA) were performed. One set of supernatants containing VSV-Mass-FL-S and VSV-Ark-FL-S was treated with neuraminidase to remove sialic acid residues on the spike protein to induce HA (20) and was incubated for 30 minutes at 37°C, and 5 minutes at 4°C. Another set of supernatants received no neuraminidase treatment. After incubation, 50 µl of supernatants from both sets were added to 96 well plates and serially diluted by 2-fold in phosphate-buffered saline (PBS). Chicken red blood cells (RBC) were added subsequently to these plates and HA titers were determined, following basic laboratory protocols (8, 14, 20).

Normalization of IBV primary antibodies for use in VSV-Mass-FL-S and VSV-Ark-FL-S coated ELISAs. The Ark serum was commercially obtained from Charles River Laboratories (Wilmington, MA, USA) and the Mass serum was produced in our lab. IBV serum stocks were titrated and normalized prior to use as primary antibodies for VSV-Mass-FL-S, VSV-Ark-FL-S coated ELISAs. As we wanted to assure that the same amount of antibodies from each serum

were equally administrated to the IBV spike pseudotyped VSV coated ELISAs, three types of assays were performed for serum normalization; Virus neutralization (VN), hemagglutination inhibition (HI) and normalization by using commercial IBV ELISA coated with whole inactivated IBV (IDEXX Laboratories, Westbrook, ME, USA). The homologous VN, HI titers and ELISA OD (optical density) signals were compared between serially 2-fold diluted Mass and Ark serum. VN and HI assays were performed accordingly with 'A laboratory manual for the isolation, identification, and characterization of avian pathogens'(8), and ELISA was performed as per manufacturer's instructions. Based on the results, we decided to normalize the sera based on commercial ELISA OD signals. Consequently, the Ark serum had to be diluted down 32 times more (dilution factor of 1:32) to obtain the same OD signal for Mass serum (Figure 3). These tests were repeated at least twice.

VSV-Mass-FL-S, VSV-Ark-FL-S coated ELISA assays using normalized IBV primary antibodies. Procedures for making direct ELISA assays were based on 'ELISA technical guide and protocols' (26). Briefly, 125 µl of 16 HA units of VSV-Mass-FL-S and 8 HA units of VSV-Ark-FL-S were serially diluted in bicarbonate buffer (100 mM, pH 9.6) by 2-fold spanning 10 log2 ranges and were coated on separate 96 well ELISA microplates respectively (Fisherbrand, Waltham, MA, USA), and incubated at 4°C overnight. In order to coat as much antigen as possible, this step was repeated twice, resulting in coating a total of 250 µl of antigen in each well. Wells coated with these diluted antigens were washed with PBST twice (Phosphate buffered saline with Tween-20). Blocking of the remaining protein-binding sites in the coated wells was done by adding 200 µl of 5% non-fat dry milk/PBST as blocking buffer and incubating at 4°C overnight. After repeating washing steps, normalized Mass and Ark serum

were diluted with 5% non-fat dry milk/PBST at 1:32 and 1:1024 respectively (Fig 3) and added to the wells followed by a 4°C overnight incubation. Mass and Ark serum were each added as a homologous and a heterologous serum to VSV-Mass-FL-S coated plates (one plate with Mass serum, one plate with Ark serum), and VSV-Ark-FL-S coated plates (one plate with Ark serum, one plate with Mass serum), respectively. After the wells were washed with PBST for five times, rabbit-anti chicken IgY tagged with horseradish peroxidase (HRP) (Invitrogen, Waltham, MA, USA) was diluted at 1:1000 and added to the wells as secondary antibodies. After incubating for 2 hours at room temperature and repeating the 5X PBST washing step, 100ul of substrate (IDEXX Laboratories, Westbrook, ME, USA) was dispensed in the wells, and after color development was observed, a stop solution (IDEXX Laboratories, Westbrook, ME, USA) was subsequently added. The plates were analyzed at a primary wavelength of 650 nm by an ELx800 ELISA spectrophotometer (Biotek, Winooski, VT, USA). For positive controls, antibodies against the matrix protein of VSV (VSV-M) (Kerafast, Boston, MA, USA) were diluted 1:100 per manufacturer's protocols and added to the wells coated with the same antigens.

Statistical analysis of OD signals obtained from VSV-Mass-FL-S, VSV-Ark-FL-S coated ELISA. Statistical analysis of ELISA data was done by Prism 6 software (GraphPad Software, La Jolla, CA, USA). To determine the specificity of the VSV-Mass-FL-S, VSV-Ark-FL-S coated ELISAs, or to see if there were significantly specific differences in OD signals when adding homologous serum against heterologous serum of different serotypes, the dilution linearity (17) was analyzed. The demonstration of dilution linearity of the serially diluted VSV spike pseudotypes containing the antibody of interest was critical to validating the specificity

and accuracy of the given ELISA results. For that purpose, the OD signal data sets were normalized and converted to percentage (%), and were plotted on a non-linear regression prediction curve based on an exponential decrease function (19, 29) that could exhibit the signal decrease followed by 2-fold serial dilutions. The generated curves were then compared with an *in-silico* reference curve that represented a theoretically ideal 2-fold exponential decrease of signals (Fig 5-4, 5-5).

RESULTS

Validation and quantification of VSV-Mass-FL-S and VSV-Ark-FL-S. The HA titers of VSV-Mass-FL-S and VSV-Ark-FL-S are shown in table 5-1. As HA activity becomes inducible or enhanced when neuraminidase is treated in wild IBV spikes (22, 23), we wanted to observe if recombinant spike proteins expressed on the outer shell of a heterologous virus would share the same properties. Based on the results of HA assays, it seemed evident that HA activity was either induced or enhanced when recombinant Mass and Ark spikes coated on VSV received neuraminidase treatment, thus resembling the intrinsic properties of wild IBV spike protein. The given results were convincing to assure that the confirmations of the spike proteins harbored by VSV were intact and adequately expressed.

Normalization of IBV primary antibodies for use in VSV-Mass-FL-S and VSV-Ark-FL-S coated ELISAs. To ensure that the same quantities of antibodies from each serum were equally administrated to the IBV spike pseudotyped VSV coated ELISAs; normalization was done based on serological methodologies; VN, HI, and commercial IBV ELISA assays. The assays were repeated at least twice and the results are displayed in figures 5-1, 5-2, and 5-3 respectively. Discrepancies in antibody titers were observed in the three different tests. The homologous VN titer of the Mass serum was much higher compared to Ark serum (Fig. 5-1) but exhibited significantly low ELISA titers (OD signals) than Ark serum when titrated with inactivated whole IBV (Fig. 5-3), whereas the homologous HI titers of both antisera were identical (Fig 5-2). Eventually, the primary serum for VSV-IBV-Spike coated ELISAs were normalized based on commercial ELISA titers or OD signals.

VSV-Mass-FL-S, VSV-Ark-FL-S coated ELISA assays using normalized IBV primary antibodies. To determine the specificity of the VSV-Mass-FL-S, VSV-Ark-FL-S coated ELISAs against homologous and heterologous serum; the dilution linearity was demonstrated by normalizing the raw OD data and converting them to percentage (%) for relative comparison between dilutions. Theoretically, if the starting point or the lowest dilution point was set to 100%, then followed by each serial 2-fold dilution of the antigens, the signals produced by binding of antibodies will decline exponentially starting from 100%, to 50%, to 25% and so on. Based on this rationale, an *in-silico* reference curve that could represent an expected ideal 2-fold dilution was generated by doing nonlinear regression prediction analysis based on an exponential decrease function provided by the prism 6 software. The same plotting and analysis were done with the obtained experimental data (Fig. 4, 5). Should a given experimental dataset exhibit a specific 2-fold decrease in signals that correlate with the 2-fold antigen dilutions, it would try to fit in the *in-silico* reference curve or at least will be more inclined towards it.

An exponential decay constant (λ) (18) was provided when generating each curve. In this relative comparison between curves, if the constant λ of a curve is closer to the reference curve (λ = 0.0645), it signifies that the two curves share similarities, particularly; the curve is displaying a specific 2-fold exponential dilution effect similar to the reference curve. The closer the constants are, more likely the curve would be inclined towards the reference curve. When comparing the curves between adding homologous Ark serum and heterologous Mass serum to VSV-Ark-FL-S ELISA, the constant of the Ark serum curve was 0.03972 (λ = 0.03972) which was closer to the reference curve (λ = 0.0645), than the Mass serum curve (λ = 0.0240), indicating a more specific 2-fold dilution effect compared to the heterologous Mass serum. Results were similar for VSV-Mass-FL-S ELISA, as the exponential decay constant of the

curve when adding homologous Mass serum ($\lambda = 0.01364$) was closer to the reference curve ($\lambda = 0.0645$) compared to adding heterologous Ark serum (0.006810). Conclusively, the 2-fold dilution effect of OD signals followed by serial dilutions of the IBV spike pseudotyped VSV antigens was manifested clearer when adding the right homologous serum, although dilution effects were also observed in signals produced with the heterologous serum.

DISCUSSION

Vesicular stomatitis virus (VSV) has been used as a versatile tool in research due to its capability of readily producing pseudotype progenies bearing foreign envelope proteins based on a well-orchestrated budding out mechanism (25, 30). As VSV is not particularly selective in regards to the type of membrane protein that can be coated on the outer shell of the viral envelope, genes of the IBV spike protein were incorporated, and the potential of these IBV spike pseudotypes as serotyping ELISA antigens was studied. As the reproduction of authentic whole spike proteins under laboratory conditions was one of our primary focuses, we wanted to see if the spike was adequately expressed and anchored on the surface of these particles. Although western blot is the commonly used analytical technique to demonstrate the presence of a specific protein by immunoblotting (16), it involves a denaturation step followed by gel electrophoresis that alters the complex dimensional structure in their native state. So an alternative yet informative approach was to quantify and confirm the intact native conformation of the spike proteins by hemagglutination (HA) assay, since the recombinant spike proteins incorporated into the viral envelope of the VSV pseudotype would have to be very similar with the native structure of wild IBV spikes to be capable of HA activity. And as the induction or enhancement of viral HA activity by neuraminidase treatment is one of the unique properties of the IBV spike protein (22), we also focused on to see if changes in the HA titers were present before and after neuraminidase treatment of the incorporated spikes. Consequently, increase or induction of HA titers were observed among the pseudotyped spikes, strongly indicating that they were accurately expressed (Table 1).

To assure that equal quantities of primary antibodies contained in each IBV serum were administrated to the IBV spike pseudotyped VSV coated ELISAs, antibodies were normalized by VN, HI assays and commercial IBV ELISA coated with whole inactivated IBV with the IBV sera.

Unexpectedly, discrepancies between results from the three assays were apparent, each exhibiting different antibody titers (Figures 5-1, 5-2 and 5-3). The underlying reason for this phenomenon seemed accountable to the difference of antibody-binding epitopes due to conformational alterations of the diagnostic IBV viral antigens used for each different assay. In other words, it may be relevant to the state of IBV that was used for each assay, as the IBV used for VN assays was untreated wild-type IBV, whereas the IBV used for HI was neuraminidase treated and for commercial ELISAs, inactivated with chemicals. Such treatments may have altered the conformation of the spike proteins in some way, changing the antibody-binding site, thus giving different results in titers. After some contemplation, we decided to normalize the serum based on the OD signals obtained from commercial ELISA (Fig 5-3), as this study was directly involved in producing ELISAs.

To determine the specificity of the VSV-Mass-FL-S, VSV-Ark-FL-S coated ELISAs against homologous and heterologous serum, the dilution linearity of each ELISA assay correlating with the serial dilution of IBV spike pseudotyped VSV antigens was demonstrated and compared with a reference curve that represented an ideally predicted 2-fold dilution. Although it turned out that the dilution linearity was relevantly clearer when applying homologous serum to the VSV that harbored the spike of that same serotype (Fig 5-4, 5-5), dilution effects were also apparent in the heterologous serum added ELISAs, which seemed due to cross-reactivity. This was not unexpected, as it is known that the N-terminal portion of the conserved S2 domain of the spike protein contains immune-dominant regions including a neutralizing epitope, although not as much as S1 (27), and that there are also some conserved regions among the genetically diverse S1 region (28). As an attempt to reduce cross-reactions, constructing a chimeric spike protein by replacing the S2 domain with that of a non-IBV coronavirus might help, although strict prediction modeling

would be imperative, as the introduction of an exotic S2 domain would be unpredictable regarding conformational changes.

In summary, the potential of VSV particles expressing IBV spike proteins as serotyping ELISA antigens was examined by a proof of concept study. Further optimization of this concept would help for the development of IBV serotyping ELISA that can be facilitated in the field.

REFERENCES

1. Anderson DB, Laquerre S, Goins WF, Cohen JB, Glorioso JC. 2000. Pseudotyping of glycoprotein D-deficient herpes simplex virus type 1 with vesicular stomatitis virus glycoprotein G enables mutant virus attachment and entry. Journal of virology 74:2481-2487.

2. Bentley EM, Mather ST, Temperton NJ. 2015. The use of pseudotypes to study viruses, virus sero-epidemiology and vaccination. Vaccine 33:2955-2962.

3. Callison SA, Jackwood MW, Hilt DA. 1999. Infectious bronchitis virus S2 gene sequence variability may affect S1 subunit specific antibody binding. Virus Genes 19:143-151.

4. Cavanagh D. 2007. Coronavirus avian infectious bronchitis virus. Veterinary research 38:281-297.

5. Cavanagh D, Elus M, Cook J. 1997. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathology 26:63-74.

6. Cavanagh D, Naqi S. 2003. Infectious bronchitis. Diseases of poultry 11:101-119.

7. Cook JK, Jackwood M, Jones R. 2012. The long view: 40 years of infectious bronchitis research. Avian Pathology 41:239-250.

8. Dufour-Zavala L. 2008. A laboratory manual for the isolation, identification, and characterization of avian pathogens. American Association of Avian Pathologists.

9. Hitoshi N, Ken-ichi Y, Jun-ichi M. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193-199.

10. Ignjatovic EJ, Ashton F. 1996. Detection and differentiation of avian infectious bronchitis viruses using a monoclonal antibody-based. Avian Pathology 25:721-736.

11. Jackwood MW. 2012. Review of infectious bronchitis virus around the world. Avian diseases 56:634-641.

12. Jayakar HR, Jeetendra E, Whitt MA. 2004. Rhabdovirus assembly and budding. Virus research 106:117-132.

13. Karaca K, Syed N. 1993. A monoclonal antibody blocking ELISA to detect serotype-specific infectious bronchitis virus antibodies. Veterinary microbiology 34:249-257.

14. Killian ML. 2008. Hemagglutination assay for the avian influenza virus, p. 47-52, Avian influenza virus. Springer.

15. Levanov L, Iheozor-Ejiofor RP, Lundkvist Å, Vapalahti O, Plyusnin A. 2018. Defining of MAbs-neutralizing sites on the surface glycoproteins Gn and Gc of a hantavirus using vesicular stomatitis virus pseudotypes and site-directed mutagenesis. bioRxiv:307975.

16. Mahmood T, Yang P-C. 2012. Western blot: technique, theory, and trouble shooting. North American journal of medical sciences 4:429.

17. Malvano R, Boniolo A, Dovis M, Zannino M. 1982. ELISA for antibody measurement: aspects related to data expression. Journal of immunological methods 48:51-60.

18. Masters PS. 2006. The molecular biology of coronaviruses. Advances in virus research 66:193-292.

19. Prism G. 6.0 software GraphPad Software, Inc.

20. Ruano M, El-Attrache J, Villegas P. 2000. A rapid-plate hemagglutination assay for the detection of infectious bronchitis virus. Avian diseases:99-104.

21. Sanders DA. 2002. No false start for novel pseudotyped vectors. Current opinion in biotechnology 13:437-442.

22. Schultze B, Cavanagh D, Herrler G. 1992. Neuraminidase treatment of avian infectious bronchitis coronavirus reveals a hemagglutinating activity that is dependent on sialic acid-containing receptors on erythrocytes. Virology 189:792-794.

23. Schultze B, Enjuanes L, Cavanagh D, Herrler G. 1994. N-acetylneuraminic acid plays a critical role for the haemagglutinating activity of avian infectious bronchitis virus and porcine transmissible gastroenteritis virus, p. 305-310, Coronaviruses. Springer.

24. Takada A, Robison C, Goto H, Sanchez A, Murti KG, Whitt MA, Kawaoka Y. 1997. A system for functional analysis of Ebola virus glycoprotein. Proceedings of the National Academy of Sciences 94:14764-14769.

25. Tani H, Morikawa S, Matsuura Y. 2012. Development and applications of VSV vectors based on cell tropism. Frontiers in microbiology 2:272.

26. TIP T. ELISA technical guide and protocols.

27. Toro H, Zhao W, Breedlove C, Zhang Z, Van Santen V, Yu Q. 2013. Infectious bronchitis virus S2 expressed from recombinant virus confers broad protection against challenge. Avian diseases 58:83-89.

28. Wang L, Parr R, King D, Collisson E. 1995. A highly conserved epitope on the spike protein of infectious bronchitis virus. Archives of virology 140:2201-2213.

29. Weisstein EW. 2002. Eric Weisstein's World of Mathematics: mathworld.

30. Whitt MA. 2010. Generation of VSV pseudotypes using recombinant ΔG-VSV for studies on virus entry, identification of entry inhibitors, and immune responses to vaccines. Journal of virological methods 169:365-374.

TABLES

Table 5-1. HA titers of VSV-IBV-spike treated or not treated with neuraminidase

VSV-IBV-Spike/Experimental conditions	**HA titers of VSV-Ark-FL-S**	**HA titers of VSV-Mass-FL-S**
Not treated with Neuraminidase	0	4
Treated with Neuraminidase	8	16

FIGURES

Fig 5-1. *Homologous VN titer of serotype-specific IBV sera.*

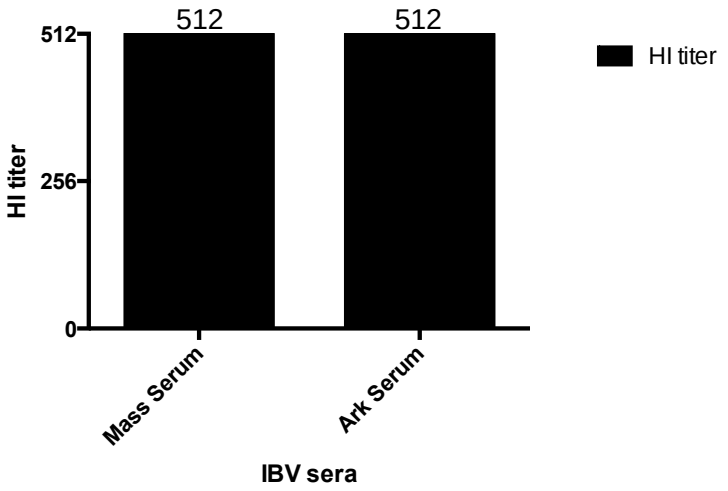


Fig 5-2. *Homologous HI titer of serotype-specific IBV sera.*

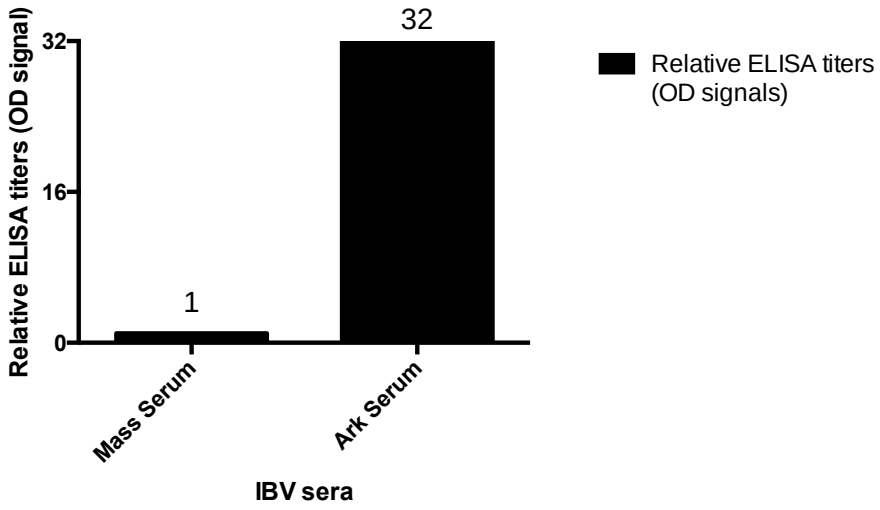


Fig 5-3. *Commercial ELISA* titers of serotype-specific IBV sera.*

* Antigens for commercial ELISA were inactivated whole IBV

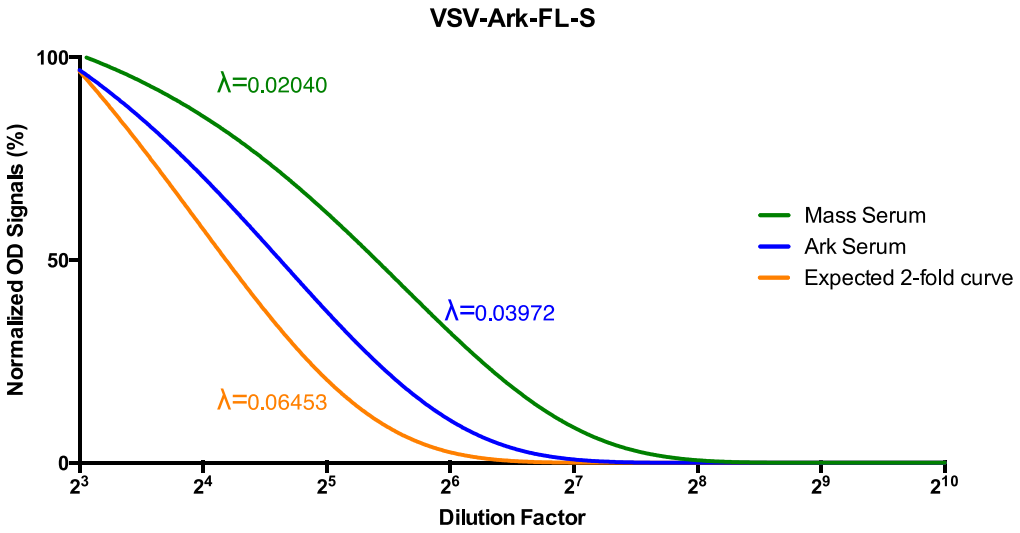


Fig 5-4. *Comparison of dilution linearity of VSV-Ark-FL-S coated ELISA.*

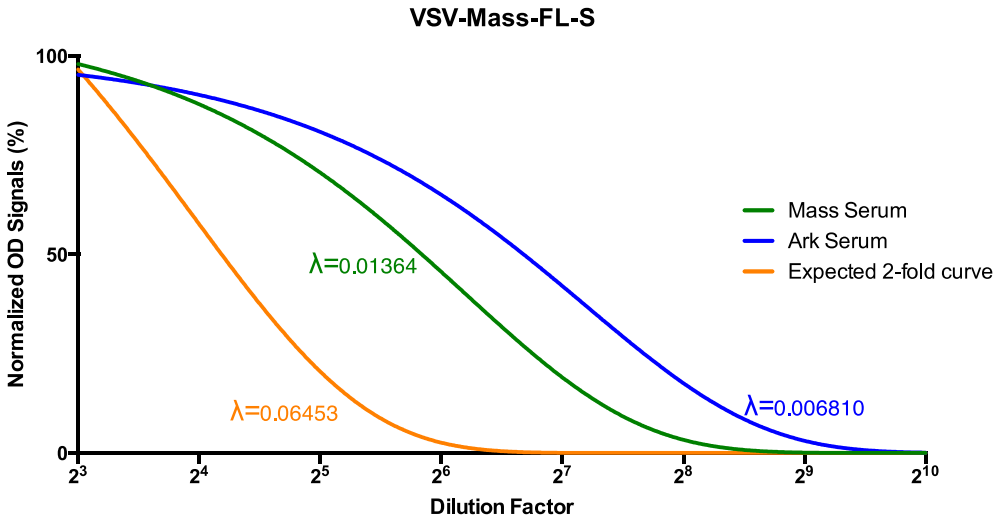


Fig 5. *Comparison of dilution linearity of VSV-Mass-FL-S coated ELISA.*

CHAPTER 6

SUMMARY AND CONCLUSIONS

Poultry respiratory disease viruses are currently distributed worldwide affecting the poultry industry and among them, major diseases such as infectious bronchitis (IB), infectious laryngotracheitis (ILT), Newcastle disease (ND) and avian metapneumoviruses (AMPV) are known to cause significant economic losses (20). It is imperative to rapidly detect and differentiate these viruses within an infected poultry flock so that appropriate countermeasures can be implemented promptly. As it is well known that vaccines can play a critical role in the containment of such viral diseases (7), demonstrating the presence of the virus and identifying the etiologic agent along with differentiating between possible candidates, which can be similar in the early stages of their pathogenesis is essential (4, 5, 20). To serve that purpose, researchers have introduced various diagnostic techniques for the past decades, including detection or isolation of the viral agent itself, or by aiming at specific antibody responses. Although conventional detection methods like virus isolation (VI) or virus neutralization (VN) have been used widely and are reliable, despite their widespread usage, several drawbacks like slow and labor-intensive processes existed (9). Since the introduction of molecular concepts, alternative assays are continually being introduced in the field of viral diagnostics, which confer several advantages; they are more sensitive in the detection of viral agents, less time-consuming and require less labor. Nowadays, the commonly used methods for detecting poultry respiratory virus and its antibodies are quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-

linked immunosorbent assay (ELISA) (1, 13-15, 17). The main goal of this research was to develop rapid and accurate diagnostic tools that can be applied for clinical detection of major poultry respiratory disease viruses and serotyping. Specifically, this research aims (1) to develop quantitative real-time RT-PCR assays for IBV, NDV, ILTV and AMPV and rigorously evaluate their sensitivity & specificity by using synthetic DNA templates, internal positive controls, and clinical samples, (2) generate pseudotyped VSV particles that harbor IBV spike proteins using an in vitro culture system and characterize produced particles for the incorporation of IBV spike proteins for potential use as serotyping ELISA antigens.

Development of specific real-time quantitative RT-PCR assay panel for Infectious Bronchitis using synthetic DNA standards and clinical specimens. The aim of this research was to develop six TaqMan™-based quantitative RT-PCR assays (Universal, Ark, Mass, DE072/GA98, GA07, GA08) using the same thermo-cycling conditions that can aid in surveillance and devising vaccination strategies for IBV. Assays were developed targeting the hyper-variable region in the S1 gene subunit of the IBV genome that represented the specific serotype (6, 8, 10), and was evaluated using synthetic DNA standards that were identical with the target sequence and specificity was further validated using clinical and biological tissue specimens. All developed IBV universal and type-specific assays performed equivalently when testing synthetic DNA standards, as the detection was linear over 5 $\log_{10}$ steps with amplification efficiencies ranging from 91.9% to 113.3%, paired with high sensitivity as all assays showed LOD of ≤10 copy numbers per reaction with an R^2 ≥0.99 indicating high reproducibility. Regarding verification using clinical and biological samples, 100% 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 successfully incorporated in the universal assay without hindering its performance. Regarding analytical sensitivity and specificity, all developed assays met the initial qualifications.

Development of specific real-time quantitative RT-PCR assay panels for infectious laryngotracheitis, Newcastle disease and avian metapneumovirus using synthetic DNA standards, internal positive controls and clinical specimens. The aim of this research was to develop TaqMan™-based quantitative RT-PCR assays for detecting ILTV, NDV and AMPV-A/B. Assays were developed targeting the gC gene of ILTV (4), M (22), L gene (11) of NDV and the N gene of AMPV-A and B respectively. Analytical sensitivity was evaluated by using synthetic DNA standards that were designed and synthesized based on the target sequence in that gene. Specificity of the assays was further validated using clinical and biological tissue specimens. The clinical sample verification step for AMPV-A and B assays was excluded due to limited access to clinical samples. All developed assays performed equivalently when testing synthetic DNA standards, as the detection was linear over 5 $\log_{10}$ steps with amplification efficiencies ranging from 86.8% to 98.2%, paired with high sensitivity as all assays showed LOD of ≤10 copy numbers per reaction with an R^2 ≥0.99 indicating high reproducibility. Regarding verification using clinical and biological samples, 100% of the known positive samples were determined as positive by the NDV, ILTV assays. An internal positive control (IPC) was successfully incorporated in all the assays without any performance issues. Conclusively, all developed assays met the initial qualifications regarding analytical sensitivity and specificity.

The potential of pseudotyped vesicular stomatitis virus (VSV) particles expressing IBV spike as serotyping ELISA antigens. Vesicular stomatitis virus (VSV) has been widely used in research to produce pseudotyped virus particles, which contain the envelope glycoproteins of heterologous viruses (2, 12, 18, 19, 21). The concept of pseudotyping is that single or multiple structural proteins are harbored by a virus particle that is not encoded in the viral genome that carries them (3, 16). The primary objective of this study was to address the need for a diagnostic test that could distinguish serotype-specific antibodies against various IBV types by using VSV pseudotyping technique. Full IBV spike proteins were coated on the outer shell of the viral envelope of VSV and the potential functionality of these VSV particles carrying different IBV spikes (Mass, Ark) as serotyping ELISA antigens was studied and evaluated. The induction and enhancement of HA titers were observed among the pseudotyped IBV spikes, strongly indicating that they were accurately expressed on the surface of VSV. To determine the specificity of the VSV-Mass-FL-S, VSV-Ark-FL-S coated ELISAs against homologous and heterologous serum; the dilution linearity correlating with the serial dilution of IBV spike pseudotyped VSV antigens was demonstrated. This proof of concept study did prove some potential of the IBV spike pseudotyped VSV particles as serotyping antigens, although further optimization would be helpful to introduce this concept for poultry diagnostics.

The primary focus of this whole study was the development and provision of a board panel of diagnostic assays and practical concepts that could aid in identifying the viral etiologic agents that are distributed worldwide and are capable of causing severe implications to the poultry industry resulting in economic devastation and even trade restraints and embargos on poultry products. We are confident that this study will help facilitate avian respiratory viral

disease diagnostics in the field regarding accuracy; functional simplicity and rapidity so that countermeasures like vaccination can be promptly implemented.

REFERENCES

1. Alvarez R, Njenga MK, Scott M, Seal BS. 2004. Development of a nucleoprotein-based enzyme-linked immunosorbent assay using a synthetic peptide antigen for detection of avian metapneumovirus antibodies in turkey sera. Clinical and diagnostic laboratory immunology 11:245-249.

2. Anderson DB, Laquerre S, Goins WF, Cohen JB, Glorioso JC. 2000. Pseudotyping of glycoprotein D-deficient herpes simplex virus type 1 with vesicular stomatitis virus glycoprotein G enables mutant virus attachment and entry. Journal of virology 74:2481-2487.

3. Bentley EM, Mather ST, Temperton NJ. 2015. The use of pseudotypes to study viruses, virus sero-epidemiology and vaccination. Vaccine 33:2955-2962.

4. Callison S, Riblet S, Oldoni I, Sun S, Zavala G, Williams S, Resurreccion R, Spackman E, Garcia M. 2007. Development and validation of a real-time Taqman® PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. Journal of virological methods 139:31-38.

5. Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, Jackwood MW. 2006. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. Journal of virological methods 138:60-65.

6. Cavanagh D, Elus M, Cook J. 1997. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathology 26:63-74.

7. Devlin JM, Vaz PK, Coppo MJ, Browning GF. 2016. Impacts of poultry vaccination on viruses of wild bird. Current Opinion in Virology 19:23-29.

8. Ignjatovic J, Galli L. 1994. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. Archives of virology 138:117-134.

9. Kangro HO, Mahy BW. 1996. Virology methods manual. Elsevier.

10. Kant A, Koch G, Van Roozelaar D, Kusters J, Poelwijk F, Van der Zeijst B. 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. Journal of General Virology 73:591-596.

11. Kim LM, Suarez DL, Afonso CL. 2008. Detection of a broad range of class I and II Newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. Journal of Veterinary Diagnostic Investigation 20:414-425.

12. Levanov L, Iheozor-Ejiofor RP, Lundkvist Å, Vapalahti O, Plyusnin A. 2018. Defining of MAbs-neutralizing sites on the surface glycoproteins Gn and Gc of a hantavirus using vesicular stomatitis virus pseudotypes and site-directed mutagenesis. bioRxiv:307975.

13. Mackay IM, Arden KE, Nitsche A. 2002. Real-time PCR in virology. Nucleic acids research 30:1292-1305.

14. Meulemans G, Halen P. 1982. Enzyme-linked immunosorbent assay (ELISA) for detecting infectious laryngotracheitis viral antibodies in chicken serum. Avian Pathology 11:361-368.

15. Mockett A, Darbyshire J. 1981. Comparative studies with an enzyme-linked immunosorbent assay (ELISA) for antibodies to avian infectious bronchitis virus. Avian Pathology 10:1-10.

16. Sanders DA. 2002. No false start for novel pseudotyped vectors. Current opinion in biotechnology 13:437-442.

17. Snyder D, Marquardt W, Mallinson E, Russek E. 1983. Rapid serological profiling by enzyme-linked immunosorbent assay. I. Measurement of antibody activity titer against Newcastle disease virus in a single serum dilution. Avian diseases:161-170.

18. Takada A, Robison C, Goto H, Sanchez A, Murti KG, Whitt MA, Kawaoka Y. 1997. A system for functional analysis of Ebola virus glycoprotein. Proceedings of the National Academy of Sciences 94:14764-14769.

19. Tani H, Morikawa S, Matsuura Y. 2012. Development and applications of VSV vectors based on cell tropism. Frontiers in microbiology 2:272.

20. Villegas P. 1998. Viral diseases of the respiratory system. Poultry science 77:1143-1145.

21. Whitt MA. 2010. Generation of VSV pseudotypes using recombinant ΔG-VSV for studies on virus entry, identification of entry inhibitors, and immune responses to vaccines. Journal of virological methods 169:365-374.

22. Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, Kapczynski DR, Spackman E. 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology 42:329-33