Development of a Quadriplex Fluorescent Microsphere Immunoassay (FMIA) for the Detection of Antibody Responses to Influenza A Viruses and Newcastle Disease Virus

By

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Thesis Abstract

Surveillance of domestic poultry flocks for antibodies against avian influenza and Newcastle disease to detect and differentiate between these diseases is very important. The ability to determine if the detected influenza virus antibodies belong to one of the reportable H5 or H7 subtypes is imperative. These two major viruses are continually responsible for economic loss in poultry industries all over the world. Current serological methods of detection are an effective means of detecting antibody responses to these viruses, however continually investigating improved methods of surveillance is important.

Development of a serological assay using Luminex technology which involves the use of recombinantly generated influenza A nucleoprotein, hemagglutinin H5, hemagglutinin H7, and Newcastle disease nucleocapsid proteins bound to Magplex beads allowed for the simultaneous detection of antibodies against these proteins that matches the efficiency of past methods while maintaining high levels of specificity and overall accuracy.

Assay development took the form of two connected projects beginning with construction of an assay that operated in duplex, detecting antibodies against influenza nucleoprotein (AIV-NP) and Newcastle disease nucleocapsid protein (APMV-1-NC). Once optimized, the second half of development involved expansion of the assay to include detection of H5 (AIV-H5) and H7 (AIV-H7) subtypes, as well as the addition of internal assay quality controls to monitor assay performance over time.

Assay thresholds and overall performance of both of these functional assays were evaluated using large quantities of field and experimental sera from chickens and turkeys to maximize specificity and overall accuracy.

Forward

This thesis was written in the style of a manuscript format which is composed of two manuscripts. The first manuscript details the Duplex format of the FMIA assay and was published in the Journal of Immunological Methods, and is formatted according to the journal guidelines. The results of the Duplex manuscript were presented at the American Association of Avian Pathologists conference in July 2013 in Chicago, IL. The second manuscript describes the Quadriplex format of the FMIA assay (Q-FMIA) and has not been submitted to a journal yet. The authors of both manuscripts are Mathieu Pinette, Yohannes Berhane, John Pasick, Davor Ojkic, Marsha Leith, Matthew Suderman, and Juan-Carlos Rodriguez-Lecompte.

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Table of Contents

Development of a Quadriplex Fluorescent Microsphere Immunoassay (FMIA) for the Detection of Antibody Responses to Influenza A Viruses and Newcastle Disease Virus

List of Tables

List of Figures

List of Abbreviations

AIV	Avian Influenza Virus
AIV-NP	Luminex Bead bound to Recombinant Avian Influenza Nucleoprotein
AIV-H5	Luminex Bead bound to Recombinant Avian Influenza H5 Hemagglutinin Protein
AIV-H7	Luminex Bead bound to Recombinant Avian Influenza H7 Hemagglutinin Protein
AI	Avian Influenza
AGID	Agarose Gel Immunodiffusion Assay
APMV	Avian Paramyxovirus
APMV-1	Avian Paramyxovirus Type 1
ASe	Analytical Sensitivity
ASp	Analytical Specificity
CK IgY	Chicken Immunoglobulin Y
CPE	Cytopathic Effect
DK anti CK IgY	Secondary Antibody used in the assay; Donkey anti Chicken Immunoglobulin Y
DSe	Diagnostic Sensitivity
DSp	Diagnostic Specificity
EDC	1-ethyl-3-3-dimethylaminopropyl carbodiimide
ELISA	Enzyme-Linked Immunosorbent Assay

HA	Hemagglutinin Protein
HI	Hemagglutination Inhibition Assay
HIS-tag	6X Poly-histidine motif for protein purification
HPAI	High Pathogenic Avian Influenza
IVPI	Intravenous Pathogenicity Index
LPAI	Low Pathogenic Avian Influenza
OIE	World Organization of Animal Health
PBS	Phosphate Buffered Saline
MFI	Median Fluorescent Intensity
Fl	Median Fluorescent Intensity
NC	Nucleocapsid Protein
NDV	Newcastle Disease Virus
ND	Newcastle Disease
NP	Nucleoprotein
PCR	Polymerase Chain Reaction
Q-FMIA	Quadriplex Fluorescent Microsphere Immunoassay
ROC	Receiver Operating Characteristic Analysis
RT-PCR	Real-Time Polymerase Chain Reaction
SA-PE	Streptavidin – Phycoerythrin Fluorescent Dye

Sf9	*Spodoptera frugiperda* insect cells
Sulfo-NHS	N-hydroxysulfosuccinate
VNT	Virus Neutralization Assay

1.0 General Introduction

Surveillance and screening assays are important tools for monitoring large flocks of domestic poultry to allow for quick responses to outbreaks of dangerous diseases before they become out of control (Christensen *et al.* 2011l; Desvaux *et al.* 2012; Katz *et al.* 2011; Pawar *et al.* 2012). Serological assays function optimally when the results are used to evaluate the condition of the entire flock, and highlight the importance of early detection (Berhane *et al.* 2014). There are a variety of different serological assays currently in use today but most can be separated into two major groups being "direct" or "indirect" methods of detection (Schipper *et al.* 1973). Direct serologic testing uses known antibodies or chicken red blood cells in the case of the HI assay to directly interact with, and identify an unknown antigen. Indirect serologic testing is detecting antibodies produced against a specific antigen within the sample sera using a known antigen. It is difficult to accurately compare results between assays with different methods of detection. In this study, antibody responses to avian influenza and Newcastle disease virus infections were evaluated using chicken and turkey sera from both field and experimentally infected birds.

The effectiveness of serological assays depend on the immunogenicity of the proteins selected to bind with antibodies in the test sera for production of a strong immune response (Singh *et al.* 2010; Rao *et al.* 2010; Zhirnov *et al.* 2007; Morrison 2010; McGinnes *et al.* 2010). For this reason, the influenza virus nucleoprotein, H5 and H7, and the Newcastle disease virus nucleocapsid proteins were chosen because they are proteins produced by the viruses in high quantities and induce strong immune responses in poultry. The hemagglutinin proteins found on the surface of the influenza virus are not only immunogenic, but are specific to virus sub-type they are associated with and can determine the viral sub-type when detected.

Luminex technology is a relatively recent improvement in serological assay technology with the primary benefit being that samples can be tested for multiple analytes simultaneously while using less reagents and sample than traditional serological tests.

Assay development according to the guidelines set out by the OIE for serological assays involves optimization of the protocol with the use of a limited number of samples and a set of standard references (OIE Terrestrial Manual 2008 chapter 1.1.4). The first stage is to ensure the methodology is repeatable and can produce reliable data with every run of the assay. Determining the analytical specificity and sensitivity of the assay platform in ideal conditions without the variability of test serum is the subsequent step. Analytical sensitivity is the smallest amount of antibody that can be detected in a test sample before being undistinguishable from background signal and is normally determined with a serial dilution of test samples. Analytical specificity is the ability of the test to differentiate between the proper components within the test serum. Testing this involves the use of a panel of different samples to show the assay is able to perform without unfavourable cross-reactivity (Wright 1998). It is also important to determine the range of signal that can be produced by the assay; from the highest, to the lowest point were signal is indistinguishable from background signal. This is carried out with a multi-step serial dilution using samples of known titers to observe how the signal decreases over a dilution series (Wright 1998).

After analytical performance is calculated and estimated thresholds are established, diagnostic performance is evaluated by testing large quantities of samples from reference and experimental animals. Diagnostic sensitivity is determined with a set of known infected samples that give positive results. Diagnostic specificity is the proportion of known uninfected animals, which give negative results. The diagnostic sensitivity and specificity are influenced by the analytical sensitivity and specificity and cannot exceed the limits that were established during analytical testing (Wright 1998).

This step is often the most complex and involves the testing of thousands of serum from a variety of sources to ensure that the assay is robust enough to produce reliable data from a variety of samples.

Once the assay has been approved and successfully tested by a number of collaborating labs, the total sum of data is interpreted and referenced to allow for international recognition of the validation of the assay. From here the assay will be approved for implementation in other labs and will be continually monitored to ensure that the assay performs up to the standards laid out in the initial reports.

2.0 Literature Review

Outbreaks of highly pathogenic avian influenza virus (HPAI) and virulent forms of avian Paramyxovirus-1 (APMV-1) infection continue to occur across the world and cause serious illness in domestic poultry. Both of these diseases are reportable to the OIE and current solutions to control the spread of disease rely on vaccinations and depopulation of poultry from infected areas (Molesti *et al.* 2013). Continuing development and validation of increasingly sensitive and specific serological assays for detecting antibodies to avian influenza virus (AIV) and APMV-1 in avian species represents some of the main objectives for experts (Molesti *et al.* 2013). Continued spread of HPAI subtypes H5 and H7, and the emergence of new dangerous strains that could potentially pose a threat to humans show that influenza presents an ongoing pandemic threat (Yang *et al.* 2012). The most common diseases in poultry from 2006-2009 according to the world livestock disease atlas were High Pathogenic Avian Influenza (HPAI), Avian infectious bronchitis, Low Pathogenic Avian Influenza (LPAI), and Newcastle disease. Improving methods for detection and management of these diseases is of upmost importance for the continuing success of the poultry industry both here in North America and throughout the world (Olson *et al.* 2014).

2.1 Avian Immune System

The Thymus and Bursa of Fabricius are the two most important immune system organs in broiler chickens and are the cellular factories for lymphocyte production and maturation (Bar-Shira *et al.* 2003; Fagerland and Arp 1993; van Ginkel *et al.* 2012). The Bursa of Fabricius is a specialized immune organ that is found only within birds and is a small round organ located in the hind gut, and functions as the site of B cell differentiation (Glick 1985). All avian species have nucleated red blood cells which allow the blood cells to replicate, and although they do still have bone marrow to produce erythrocytes, the lower density bone found in avian species relies on other organs to produce B and T cells. The avian

immune system serves fundamentally the same purpose as the immune system in other animals with only a few exceptions (Schat 2008). The chicken immune system develops before hatching but is not immediately fully functional. The first three weeks of life are the most important for total immune system maturation, but especially gut epithelial maturation (Rodriguez *et al.* 2012; Klasing *et al.* 2007). T cell selection occurs in the thymus but migration of lymphocytes to the thymus only begins at day 6 post hatch, and only IgM antibodies are found circulating in the body until this point (Kajiwara *et al.* 2003; Linna *et al.* 1969). The young broilers are given limited immune protection from the egg. The yolk within the egg contains a high concentration of antibodies from the maternal laying hen which may be high enough to provide a high background signal during testing on serological assays. As the broiler embryo ingests the nutrients within the yolk, the antibodies become increasingly concentrated until the day of hatch. At this point the chick consumes the last of the yolk containing the concentrated antibodies, and the hatched chick inherits a passive supply of antibodies to fend off pathogens in the first few days post hatch (Lee *et al.* 2009). The more advanced immune system organs: Cecal tonsil, Mecke's diverticulum, Harderian gland and diffuse lymphoid tissues within the gut and respiratory system are incomplete at hatch and require time for further development (Dibner *et al.* 1998). All of these systems develop slower without proper early broiler nutrition, and can mature more rapidly if probiotics are added early in the diet of broiler chicks. This process of immune system development is important to understand as it will provide the background knowledge to properly evaluate the results produced by a developing serological assay, and the results from time course samples that progress from 0 to 28dpi.

2.2 Avian Disease Surveillance

In Canada, regular surveillance for influenza in avian species began in 2005 with the introduction of wild bird surveys, and NDV surveillance is ongoing despite common vaccination practices. Keeping track of these reportable diseases can have a large impact on restrictions for import and export of

poultry (Hall 2004). Regular surveillance of wild birds is carried out by joint programs of government, industry, and producers within Canada (Pasick *et al.* 2012). The presence of these diseases is rarer in Canada and the surveillance methods are used more as a proof to trading partners that the poultry being shipped across borders are safe. Countries that are prone to outbreaks use surveillance to track disease spread, and monitor the evolution of the virus while also keeping track of new outbreaks (Comin 2012).

2.2.1 Surveys

One of the major surveys conducted in Canada is the Wild bird Influenza Survey which specifically looks for reportable and dangerous strains of influenza in thousands of wild birds (Pasick *et al.* 2010). Samples are collected from wild ducks, both alive and dead to determine current prevalent strains and are used as an early indicator for new potentially dangerous strains (Parmley *et al.* 2008). Ducks interact with domestic poultry water supplies, as well as lakes and areas across the country and are primary reservoirs for influenza A viruses. Staying informed about the current strains of influenza virus moving in ducks is a good indicator of which viruses may turn up in domestic poultry flocks. The migratory patterns of wild birds have been shown to greatly influence the movement of many diseases between countries and across borders, including both influenza and Newcastle disease (Jourdain *et al.* 2007). The yearly migrations of wild birds are greatly affected by temperature variations from season to season and also influences whether multiple species of bird migrations will occur at the same time, increasing the diversity of species in one place, and increasing the likelihood of virus mixing and transmission in the water sources along the paths of migration (Hill *et al.* 2012; Sivay *et al.* 2012).

Another survey program that is conducted in Canada is the "Canadian Notifiable Avian Influenza Surveillance System (CanNAISS)" (Christensen *et al.* 2011). This surveillance system looks more closely at domestic poultry flocks to detect and eliminate the presence of reportable strains of influenza before

they become widespread and out of control. This program includes poultry from all corners of the poultry industry including wild bird surveillance, passive surveillance in domestic poultry and targeted surveillance when reportable strains of influenza are found. Testing is also done in pre-slaughter poultry as well as hatcher supply flocks and even within poultry genetic exporters (Pasick *et al.* 2010a, Pasick *et al.* 2010b, Parmley *et al.* 2011).

2.3 Luminex Technology for Serological Assays

Multiplexed Particle-Based Flow Cytometry is a relatively new technology that started becoming more common around the year 2000 (Fulton *et al.* 1997; Vignali 2000). The technology was hosted by a few companies but since has been mostly supported by Luminex Corporation. The Luminex technology is based on a combination of flow cytometry, latex microspheres; two technologies that were previously available for different purposes and have since been modified to work together (Horan *et al.* 1979; Vignali 2000). The technology in its earliest form was initially termed "FlowMetrix™" and was mostly used for human cytokine analysis (Oliver *et al.* 1998), viral nucleic acid detection (Smith *et al.* 1998), and human immunoglobulin detection (Gordon and McDade 1997). The technology has always employed the use of small beads as the solid state of the assay, but as the technology has improved and become more popular over the last decade with the use of magnetic beads becoming widely used. Initially, the FlowMetrix™ system used three different lasers (530nm, 585nm, 650nm) instead of the modern two lasers for detection because the beads were different sizes as well as containing different quantities of fluorescent dye (Fulton *et al.* 1997). The platform is currently capable of high-throughput while still being versatile and flexible to accommodate different methods and antigens. Everything from nucleic acids, antigen-antibody binding, enzyme reactivity, and receptor-ligand binding to different kinds of protein interactions are now compatible with this technology in many formats but the real breakthrough was the ability of the assay to detect results from independent beads simultaneously (Biagini *et al.* 2003; Gageldonk *et al.* 2008; Chowdhury *et al.* 2008). The assay was also initially designed to be used with a

"no wash" protocol because the beads could be easily separated from the rest of the reagents in each well (Vignali 2000). Luminex assays have been developed in a wide range of research fields from health and life science research and diagnostics, to food safety and bio-defence. Many different types of biological samples are compatible as well including serum, plasma, tissue, cell lysate, saliva, sputum, and Broncho alveolar lavage (Zhang *et al.* 2014; Langenhorst *et al.* 2012;). The assays have also shown to be more sensitive than popular methods such as the ELISA (McHugh *et al.* 1997). It is possible that the luminex platform may grow to become the next standard for serologic surveillance and diagnostics, but in the meantime is still effectively used as a primary screening tool (Basile *et al.* 2013).

A major factor for comparison of ELISA and Luminex assay is the cheaper cost associated with performing the assay in terms of the amount of reagents used and time spent performing the test despite the increased initial investment of the luminex equipment (Knight *et al.* 2003; Anderson *et al.* 2011).

2.3.1 Magnetic Beads

The current Luminex technology uses 6.5µm diameter magnetic microspheres made out of polystyrene that are internally labelled with two different fluorophores which when excited by a 635nm laser emit light at two distinct wavelengths 658 and 712nm (Dunbar 2006). The beads are made up of a core consisting of polystyrene divinylbenzene, and a polymer layer of polystyrene methacrylic acid which is where the dye is infused, and also the magnetite for allowing reaction with magnets. This magnetic property is what allows for the possibility of a "wash-free" assay. The outer surface of this layer is what can be treated to accept covalent bonds to the desired proteins for the assay. The nature of the bonding allows for a higher available surface area for coating, and more room for interacting with analytes (Vignali 2000; Zhang *et al.* 2014). When the fluorphores are slightly altered to alter the emission ratios, up to 100 different signals can be expressed which can be differentiated by the Luminex

machine. This allows 100 unique bead regions to be generated and allows for 100 simultaneous reactions to occur within a single well of a test plate (Martins 2002, Zhang *et al.* 2014). The beads are pushed through a detection chamber in single file and the red classification laser (635nm) determines the bead region, while the green laser (532nm) determines the quantity of fluorescent dye that is associated with that specific bead. The target-specific molecules that are bound to the beads may be antigens, antibodies, oligonucleotides, enzyme substrates, or receptors (Dunbar 2006).

2.3.2 Luminex Assay Methods

The method of coupling protein to the surface of the beads greatly depends on the type of microbeads being used in the assay, as well as the type of protein or material being coupled to the beads. Bead coupling involves the use of activation buffer (100mM monobasic sodium phosphate pH6.2), as well as N-hydroxysulfosuccinate (Sulfo-NHS) and 1-ethyl-3-3-dimethylaminopropyl carbodiimide (EDC) to activate and prepare the surface of the magnetic beads for recombinant protein coating (Xia *et al.* 2010; Anderson *et al.* 2011). A very common quantity of beads of each type per well is between 2,000 and 5,000 according to manufacturer's instructions. These quantities of beads allow the assay to maintain accuracy and speed without becoming a less economical means of determining results (Bozza *et al.* 2007; Langenhorst *et al.* 2012; Lin *et al.* 2011).

2.3.3 Luminex Internal Controls

The ability to monitor the success and reliability of results in real-time is an additional benefit of utilizing the Luminex technology. Internal controls within a Luminex assay allow the researcher to monitor the assay's performance, or keep track of known cross-reactivities as the data is being analyzed (Basile *et al.* 2013; Lenhoff *et al.* 2008). Internal controls can be used to ensure the correct sample addition, sufficient quantities of detection antibody have been added, or the quality of the test serum added to the assay which may explain unexpected results during assay performance (Pinette *et al.*

2014). Other groups have included internal controls which allow them to identify interfering substances in their assay, monitor instrument performance, and correct reagent addition (Martins 2002).

2.3.4 Recently Developed Uses of Luminex Technology

Use of Luminex technology has become very common in the past decade as researches continue to find new ways to optimize the technology and develop assays to suit their specific needs. The method is growing in popularity due to its primary advantages of simultaneous detection, low labour intensity, and rapid generation of useable results. There are many assays which focus on the detection of antibodies for a variety of human and animal diseases, and some of them have similar objectives:

A multiplexed luminex immunoassay for simultaneous IgG detection of antibodies associated with viral hemorrhagic fever (Wu *et al.* 2013); multiplex microsphere Immunoassays for the detection of IgM and IgG to Arboviral Diseases (Basile *et al.* 2013); development of a bead-based immunoassay to routinely measure vimentin autoantibodies (Fhied *et al.* 2014); simultaneous detection of antibodies against Apx (Gimenez-Lirola *et al.* 2013); henipavirus microsphere immuno-assays for detection of antibodies against Hendra virus (McNabb *et al.* 2014); development of whole-virus multiplex serological assay for diagnosis of infections with kaposi's sarcoma-associated herpes (Ryan and Rose 2013); fluorescent microsphere immunoassay for antibody detection against porcine reproductive and respiratory syndrome virus (Langenhorst *et al.* 2012); simultaneous detection of antibodies to foot and mouth disease by a luminex assay (Clavijo *et al.* 2006).

There are also many new assays that focus more on the detection of cytokines and monitoring gene expression: Multiplex analysis of cytokines/chemokines as biomarkers that differentiate healthy contacts from tuberculosis patients in high endemic settings (Anbarasu *et al.* 2013); optimizing the quantification of cytokines present at low concentrations in small human mucosal tissue samples (Staples *et al.* 2013); disease cytokine profiles (Bozza *et al.* 2007). Another unique assay is able to detect

pospiviroid plant pathogens and has an internal assay method of quality control (Brunschot *et al.* 2014). The majority of these methods include very similar methods and assay conditions to those described in this thesis.

2.4 Viruses

2.4.1 Avian Influenza Virus

Avian Influenza Viruses (AIV) belong to the *Orthomyxoviridae* virus family and evidence of this virus and serious disease affecting birds dates back to 1878 and was known as "fowl plague" (Ritchey *et al.* 1976). The virus is highly contagious and prone to constant changes and re-assortments, increasing its overall unpredictability. Viral lineages containing various genotypes can be determined and are usually sorted into two major categories of either Eurasian or American (Fries *et al.* 2014; Sivay *et al.* 2012). Avian influenza viruses are classified first by type (A, B), and then by host, geographic location of first isolation, strain identification number, year of isolation, and the antigenic subtype. The low pathogenic forms of the virus are usually carried by wild water fowl, and can then mutate into highly pathogenic strains once they begin interacting with poultry and spreading through the domestic bird populations (Hall 2004; Iowa state university 2010). Natural reservoir hosts of the virus include ducks, geese, swans, gulls, terns, wading birds, etc. (Fries *et al.* 2014; De Marco *et al.* 2014). The disease in natural reservoir birds is usually asymptomatic and highly pathogenic viruses are not usually carried by wild water fowl (Iowa State University 2010). Highly pathogenic strains of Eurasian H5N1 have caused disease in animals besides domestic poultry such as other mammals and many avian species. Experimental infections and models suitable for this virus study have been established in foxes, ferrets, mice and rabbits (Liu *et al.* 2014; Reperant *et al.* 2008; Sutton *et al.* 2014; Wallerstrom *et al.* 2014).

2.4.1.1 Influenza Virus Genome and Major Components

The genome is made up of 8 negative sense single stranded RNA segments that code for 11 proteins: Nucleoprotein (NP), Hemagglutinin (HA), Neuraminidase (NA), M1, M2, NS1, NS2, PA, PB1, PB1-F2, and PB2 (Ritchey *et al.* 1976; Palese and Schulman 1976). Only type A influenza viruses are known to cause natural infection in wild birds and all 16 hemagglutinin and all nine neuraminidase subtypes have been found in avian species in almost all of the possible combinations (Alexander 2000; Capua and Alexander 2004). They are classified into serological subtypes based on the surface glycoproteins - hemagglutinin (H1 to H16) and neuraminidase (N1 to N9). Influenza viruses are divided into low pathogenic (LPAI) and highly pathogenic (HPAI) viruses based on their potential to cause disease in domestic poultry (Alexander 2000). Low pathogenic AI causes a localized mild respiratory or enteric disease in domestic poultry. These viruses could belong to any of the existing 16 HA subtypes and are commonly found in wild birds but may not be associated with any clinical disease (Abolnik 2014). Highly pathogenic AI belong to viruses of H5 and H7 subtypes and cause systemic disease in domestic poultry with up to 100% mortality (OIE 2008). Characteristics that would suggest that the strain of influenza is HPAI include: being lethal for 75% to 100% of 8, 4 to 6-week old chickens within 10 days following intravenous inoculation with 0.2ml of a 1:10 dilution of infectious allantoic fluid (Hall 2004). Any viruses that do not meet these criteria are considered LPAI but are still reported to the OIE if they are of H5 or H7 subtype. It is also possible for LPAI viruses to mutate into HPAI viruses after many subsequent transmissions between birds (Monne *et al.* 2014). This change can be induced by insertions or substitutions of basic amino acids into the cleavage site, but is still not completely understood as sometimes the virus will shift from LPAI to HPAI quickly after introduction into a population, but may also require many passages, or months of transmission to mutate (Comin 2012).

A study was done to determine if the difference in the clinical outcome between HPAI and LPAI was due to acute cellular changes and uncontrolled induction of cytokines in the lung (Rebel *et al.* 2011).

They looked at differences in clinical signs as well as post-mortem microscopic examination of tissues. There was evidence of peri-bronchitis and pneumonia as early as 4 hours after infection independent of the LPAI or HPAI virus used. No major differences in onset and severity of pneumonia or bronchitis between HPAI and LPAI was seen, although LPAI infections appeared to be more localized, while HPAI infections seemed to spread to the entire lung (Rebel *et al.* 2011). There was some evidence that suggested that the HPAI was more efficiently replicated than the LPAI during infections. However, overall the infections themselves are very similar, as there were no major differences in lesions or viral load. Within the first 24hours, none of the investigated parameters could be related to the clinical outcome of these two viruses. A cytokine storm was not observed in the lungs of HPAI infected birds at the mRNA level after 24hours (Rebel *et al.* 2011).

2.4.1.2 Hemagglutinin Protein

Hemagglutinin transmembrane proteins are homotrimers found embedded within the lipid envelope of the virus (Palese and Schulman 1976). These proteins function in the process of target cell attachment and entry into the cytoplasm when bound and cleaved by specific sialic acids and enzymes on the surface of host cells (Samji 2009). The hemagglutinin protein itself is a precursor and is cleaved into two parts, HA1 and HA2. HA1 contains the receptor binding domain and HA2 contains the fusion peptide, and both of these are linked by disulphide bonds. The hemagglutinin protein requires the host proteases that are specific to the HA1/HA2 cleavage site to cleave the protein after translation to enable the virus particles to be infectious (Comin 2012). The subtype of hemagglutinin and its associated structure dictate which cell types are able to cleave the protein and allow access into the cell. Highly pathogenic AIV have a hemagglutinin protein that has a polybasic cleavage site and can be cleaved by an array of different proteolytic enzymes that are found more commonly throughout the body (Capua and Alexander 2009). These types of infections in birds tend to attack the tissues of the lower respiratory tract which cause pneumonias and can lead to systemic infections. Low pathogenic AIV is usually

restricted to less serious upper respiratory tract infections because their hemagglutinin cleavage site is not as widely recognized by the body and requires a more localized proteolytic enzymes (Comin 2012).

2.4.1.3 Genetic Drift and Shift of Avian Influenza Virus

Reassortment and genetic changes and shifts can occur frequently with avian influenza viruses and present important challenges for trying to control the spread (Molesti *et al.* 2013). These are abrupt changes, or "antigenic shifts" that occur when multiple influenza viruses of slightly different types infect a cell simultaneously, allowing genetic segments of the virus to mix between virus strains and get packaged differently, sometimes providing the virus with different properties. The influenza viruses that infect avian, human, swine, and equine species are able to react together in this way (Harder *et al.* 2013). These mixes can result in the formation of new subtypes. If a cell becomes infected with a swine and avian influenza virus, it is possible for some of the segments to switch and now allow parts of the avian virus to infect swine. Antigenic drift also affects the viral genome but at a much slower rate. These changes are a result of random point mutations that slowly alter the virus over long periods of time and are much less dramatic than antigenic shifts (Vasin *et al.* 2014). Sometimes even more unlikely events occur where entire viruses just jump from one species to another (Harder *et al.* 2013). These are usually unstable transfers and may never transmit to another individual of the same species, but sometimes these spontaneous shifts can result in new circulating subtypes of virus (Iowa State 2010). These transmissions present a higher risk because the host population may not have any resistance to the new strain of virus and may have an increased chance of causing an epidemic or pandemic.

2.4.1.4 Transmission of Avian Influenza Virus

Transmission of avian influenza virus occurs when the bird sheds virus in the feces, saliva, and nasal secretions (Abolnik 2014; De Marco *et al.* 2014). The largest loads of virus are usually found in the feces and the most common method of transmission is through the fecal/oral route. Wild birds that use the same water supply, or gather in large groups may become infected by the virus in this way because

the virus is able to persist in a low temperature water supply (Iowa State 2010). Although the estimated amount of respiratory transmission of avian influenza virus among wild birds is low, there is evidence that large groups of domestic birds on farms can transmit influenza virus this way (Nickbakhsh *et al.* 2013; Dorjee *et al.* 2013). Shedding of the virus can begin as shortly as one or two days post-infection and may continue up to two weeks. Aerosol transmission becomes a much more prominent issue because of the close proximity of the birds (Linster *et al.* 2014; Nickbakhsh *et al.* 2011). In these environments, fecal/oral transmission and respiratory/aerosol transmission are equally likely. Other methods of transmission may be fomites on the farm, as well as broken eggs from infected hens, because HPAI viruses have been found in the yolk from infected birds (Henzler *et al.* 2003). The best method for eliminating the spread of virus is the destruction of birds and sanitation of all farming equipment, but this still does not guarantee that the virus has been eliminated completely.

Transmission of avian influenza to mammals most likely happens when coming in contact with the dead bird carcass or eating raw products of the bird, but there have also been cases of transmission when in close contact with sick birds (Harder *et al.* 2013). Indirect exposure routes such as swimming in infected water, or coming into contact with infected avian feces have also been suggested to allow transmission of the virus.

2.4.1.5 Avian Influenza Viability

Avian influenza viruses can survive in the environment for long periods of time, but are influenced by temperature, pH, salinity, and the presence of organic material (Iowa State 2010). Strains of LPAI have been found to be viable up to 100 days in distilled water at 28°C, and 200 days at 17°C (Davidson *et al.* 2010). Other studies suggest that HPAI H5 and H7 strains may survive for shorter periods in water than LPAI, however they were still viable for 100 days at 17°C, and 26 days at 28°C. These viruses have also been shown to persist for about a month in chicken feces and survived the longest at cooler temperatures (Keeler *et al.* 2014).

2.4.1.6 Incubation period and Clinical Signs of Avian Influenza in Poultry

The incubation period of avian influenza viruses in poultry range from one to seven days until the first onset of symptoms (Iqbal *et al.* 2013). LPAI infections may cause mild respiratory disease, or decreased egg production, or otherwise non-serious infections (Niqueux *et al.* 2014). HPAI viruses can cause much more severe disease and the clinical signs are often variable. Sudden death of many birds is common evidence among HPAI outbreaks, but other broad symptoms such as depression, ruffled feathers, sinusitis, lacrimation, cyanosis of the head, comb and wattle, and also green to white diarrhea may be present (Guionie *et al.* 2010; Iowa State 2010). Further signs include coughing, blood-tinged nasal discharge, deformed or shell-less eggs. Although there are many possible symptoms associated with this virus, none of them are unique characteristic symptoms, but some of the most common in domestic birds are sinusitis, diarrhea, and rapid increase in flock mortality (Mondal *et al.* 2013; Costa-Hurtado *et al.* 2014).

2.4.1.7 Avian Influenza Post-Mortem Lesions

The post-mortem lesions found on poultry are similar to other avian diseases but are not unique which makes immediate diagnosis difficult without further testing. The lesions are prominent in diseases caused by HPAI viruses and include: swollen sinuses and comb, edematous, hemorrhagic, congested, and cyanotic (Iqbal *et al.* 2013). There may be subcutaneous edema on the head and neck, edema and diffuse subcutaneous hemorrhages on the feet and shanks, possibly bloody fluid in the oral cavity, and swelling and/or hemorrhages of the conjunctivae (Charlton *et al.* 2009). Hemorrhagic tracheal lesions may be seen and the lungs may also have hemorrhages and congestion. There may also be fluid in the lungs. Hemorrhages may also be found on the mucosa of the glands of the proventriculus, beneath the lining of the gizzard, and in the intestinal mucosa. The kidneys may be plugged with urate deposits, and the ovaries may be degenerated with areas of necrosis. The peritoneal cavity often contains yolk from ruptured ova, which may cause severe airsacculitis and peritonitis

(Abolnik 2014; Harder *et al.* 2013; Iqbal *et al.* 2013; Ladman *et al.* 2008; Henzler *et al.* 2003; Cappucci *et al.* 1985; Shalaby *et al.* 1994; Niqueux *et al.* 2014). Airsacculitis is unique to birds because of their unique respiratory system being a uni-directional flow of air through a series of air sacks. The posterior air sacks are usually the first to become infected as inhaled material becomes immediately deposited in this region (Pan *et al.* 2012).

2.4.1.8 Treatment and Prevention of Avian Influenza in Poultry

Vaccination with live HPAI is not conducted in poultry because of the viral shedding that occurs during infection and the possibility of reassortment of the vaccine strain with a field strain, or random mutation that may lead to an outbreak (Kiseleva *et al.* 2013). Some countries (Egypt, China, Vietnam, Mexico) that have problems with subsequent outbreaks of AIV have been using inactivated or recombinant AIV vaccines to control subsequent HPAI outbreaks (Molesti *et al.* 2013). Vaccinations may also put selection pressure on circulating strains of influenza and cause the emergence of resistant strains of the virus (Lee *et al.* 2014).

Treatment of HPAI is not really an option as infections in poultry reach upwards of 90-100% morbidity and mortality, and survivors usually remain in poor condition for many weeks after the virus has passed. In the case of farm depopulation, the carcasses are either buried, or rendered, and the facility is completely disinfected (Abdelwhab *et al.* 2014; Rankin *et al.* 2013). The feed and manure is completely cleaned down to bare concrete, or if soil, the top few inches of soil are completely removed along with all manure. The removed soil is either buried below 5 feet deep, or composted for 90 days or longer covered with polyethylene sheets. Feathers may be burnt or composted, and all building facilities and equipment should be cleaned with pressure washing equipment equipped with disinfectant solution. Newer methods have also been developed which use carbon dioxide gas or water-based foam to quickly cull large populations of domestic birds (Rankin *et al.* 2013; Benson *et al.* 2012).

2.4.1.9 Laboratory Diagnosis of AI

Diagnosis of AIV in the lab can be determined by a variety of methods which include both molecular and serological methods. The choice of test is dependent on the level of specificity required and the laboratory facilities that are available. Samples may be taken from the bird by oral, tracheal, or cloacal swab, and often blood/serum samples are collected. Feces may also be collected, and organs (air sacs, trachea, intestine, spleen, brain, liver, and heart) may be tested from dead birds (Iowa State 2010).

Real Time – PCR (RT-PCR) is the method of choice for the detection of AI virus in clinical specimens, and a benefit of the PCR assay for detection is that highly pathogenic viruses may kill the host bird so quickly that there was no chance for antibodies to develop (Deregt *et al.* 2006). Serology is still considered valuable for overall surveillance of entire domestic flocks and can effectively show the absence of disease as well. A direct comparison was done between the results from a real-time PCR assay and a luminex assay and the results concluded that the two assays are both reliable methods for testing serum although the RT-PCR did produce slightly higher levels of sensitivity and specificity (Munro *et al.* 2013).

2.4.1.9.1 Agarose Gel Immunodiffusion (AGID) to detect AIV antibodies

Agarose gel immunodiffusion (AGID) is a gold standard test for AIV antibody detection and is another example of an indirect antibody detection technique (Pearson and Knowles 1984; Smith and Stewart 1978). Agarose gel is made and placed in a plastic petri dish and six 8mm wells are cut out, leaving a 4mm space between all wells. 100µl of the antigen is placed in the center well and 100µl of test antigen are placed in the surrounding wells. As the antigen seeps into the agarose, precipitin lines begin to form in the area where the serum and antigen interact and results can be read as positive or negative (Smith and Stewart 1978). The AGID test detects antibodies against the highly conserved

nucleoprotein (NP) and matrix (M1) proteins. However, the assay is less sensitive and is prone to producing higher rates of false negatives (Watson *et al.* 2009; Zhou *et al.* 1998, Snyder *et al.* 1985).

2.4.1.9.2 Virus Neutralization Assay for AIV Antibody Detection

Virus Neutralization assays exhibit high specificity and are very reliable serological methods that are commonly used for determining if test samples contain antibodies against influenza virus (Hassantoufighi *et al.* 2010; Gauger and Vincent 2014; Havlickova *et al.* 2012; Niqueux *et al.* 2010). The test has a higher specificity than other serological tests while maintaining sensitivity but the test takes a few days to complete. The test involves serial dilutions of test sera which has been heat inactivated into a 96-well plate and incubated with a pre-determined quantity of live virus. Following the incubation, cells which are susceptible to the virus are added to the mixture of virus and test sera. The plate with added cells is incubated for 2-3 days and read by examining each well for the presence of viral infection in the form of cytopathic effect (CPE). Those sera which contained neutralizing antibodies will have neutralized the virus and the cells will be uninfected. When no neutralizing antibodies are present in the test sera, the virus is not neutralized and is able to infect the cells which were added to the test sera solution. The titre of the serum sample is determined based on which serial dilution is no longer able to neutralize all of the virus that was added (Schmidt *et al.* 1976).

2.4.1.9.3 Enzyme Linked Immunosorbent Assay (ELISA) for AIV Antibody Detection

Enzyme-linked immunoassays are a well-known and effective method of serological detection of antibodies within serum in a wide array of situations. This method also uses an indirect detection method for measuring the strength of antibodies in the test sample. There are many variations of the ELISA test that are used for the detection of AIV antibodies and can be performed in a few ways: indirect, sandwich, and competitive (Chappell *et al.* 2014; Curran *et al.* 2014; Wu *et al.* 2014; Park *et al.* 2014; Jensen *et al.* 2013). The indirect method first has a 96-well plate coated with recombinant viral antigen. Next there is the addition of serum sample, followed by an enzyme-labeled antibody

(Horseradish peroxidase) to detect the immunoglobulin bound to the protein in the well. Lastly, a substrate such as 3,3',5,5'-Tetramethylbenzidine (TMB) is added and reacts with the HRP enzyme to generate a colorimetrically measurable product to quantify the amount of bound test serum to the well (Katz *et al.* 2011).

2.4.1.9.4 Hemagglutination Inhibition Assay for AIV antibody detection

Hemagglutination inhibition is still considered to be the "gold standard" for avian influenza antibody detection and is widely put into use (Molesti *et al.* 2013; Shi *et al.* 2014). The hemagglutination inhibition assay remains the most widely used assay to detect subtype-specific serum antibodies to influenza (Katz *et al.* 2011). The property of red blood cell agglutination was first discovered when infected allantoic fluid within the egg agglutinated red blood cells from ruptured embryo blood vessels (Hirst 1941). After that a procedure was refined to conduct the test in a reproducible way to detect AIV antibodies (Salk 1944). The test takes advantage of the hemagglutination property interaction between virus hemagglutinin proteins and the surface of chicken red blood cells. The serum is two-fold serially diluted and combined with equal volume of live or killed virus. The sample and virus are incubated before the addition of a standardized quantity of chicken red blood cells. Those samples that form a uniform covering on the bottom of the well are considered to be positive for agglutination, and likely does not contain antibodies against the particular virus used in the test because the virus was able to interact with the chicken cells. When the sample does contain adequate antibodies to block the agglutination of red blood cells, a "button" of cells drops to the bottom of the well as the quantity of serum antibodies specific for that viral subtype were able to prevent the interaction between the virus and the cells (Charlton *et al.* 2009).

Unlike the AGID test, the HI assay specifically detects hemagglutinin subtype specific antibodies using H1-H16 antigens, however it has several drawbacks: it is labour intensive, difficult to automate as results are read visually, it requires subtype specific antigens that are difficult to produce under ordinary

lab conditions, the antigens are hard to standardize as they need to be calibrated for each assay, and the assay depends on fresh red blood cells from chickens that could be problematic to get on time (Upadhyay *et al.* 2009; Zhou *et al.* 1998; de Witt *et al.* 2007; Comin *et al.* 2012). In addition, the reference antigen used in the assay needs to be updated on yearly bases with respect to the currently circulating viruses otherwise this may impair the sensitivity of the assay dramatically.

2.4.1.9.5 Fluorescent Microsphere Immunoassay (FMIA) for AIV Antibody Detection

The Luminex methodologies have already been described at length in the previous section 2.3, and this section will highlight luminex assays developed for the detection of AIV antibodies. Due to the simultaneous detection which occurs with this format, the associated assays are also able to detect mixed infections containing antibodies against AIV and NDV. There is one assay which detects antibodies against avian influenza virus by also using recombinant NP, H5, and H7 protein produced in Baculovirus (Watson *et al.* 2008). Other recent developments include: Detection of Influenza NP and H5 antibodies in Chicken sera (Lupiani *et al.* 2010), and microsphere immunoassay for detecting antibodies to avian influenza (Deregt *et al.* 2006). The methods employed in each of these assays are very similar, and are all successful at correctly identifying the presence of AIV antibodies within test serum.

2.4.2 Newcastle Disease Virus (Avian Paramyxovirus - 1)

Newcastle disease virus (NDV) also known as avian paramyxovirus serotype-1 (APMV-1) belongs to the family of *Paramyxoviridae*, genus *Avulavirus* along with eight other APMV serotypes (Capua and Alexander 2004; Alexander book). Of these, only APMV-2 (mild respiratory disease in Turkeys), 3 (Mild respiratory disease in turkeys but serious egg production problems), 6(Mild respiratory disease in ducks, and slightly elevated mortality in turkeys), and 7(Mild respiratory disease in turkeys) are known to cause diseases in poultry. The virus has a single stranded negative sense RNA genome containing 6 genes that code for 9 structural and non-structural proteins. The genes being: Nucleocapsid protein,

Phosphoprotein, Matrix protein, Fusion protein, Hemagglutinin-neuraminidase, and Large protein (Briand *et al.* 2013). APMV-1 can range in virulence from lentogenic strains that usually cause asymptomatic to mild clinical disease, and may sometimes even be used as vaccines (Alexander 1988; Alexander 1993); mesogenic – causing respiratory disease but not usually fatal; and velogenic – most pathogenic and often fatal (Aldous *et al.* 2001; Capua and Alexander 2004; Kapczynski *et al.* 2013). This disease is still a serious issue in many countries including Africa, Asia, and parts of Central and South America even though some areas utilize vaccination. Although not as dangerous as avian influenza to humans, the virus is still considered "A biological agent that can cause human disease, yet is unlikely to spread throughout the community" (Capua and Alexander 2004). In developed countries, outbreaks of this disease are extremely costly as control measures and constant vaccination cause strain on the industry. In developing countries, APMV-1 is more commonly endemic and enhances the challenge of establishing trade links and sustainable businesses.

2.4.2.1 NDV (APMV-1) Genome and Major Components

The variability of the onset of this virus makes it difficult to identify in regions where it has not caused infection in the past. The fusion protein is the most important agent for determining the level of virulence of that particular virus (Toyoda *et al.* 1987). The viral fusion protein has a similar function to the hemagglutinin protein within avian influenza viruses and is responsible for fusing the virus membrane and cell membrane so that the virus genome can enter the host cell (Capua and Alexander 2004). Once the virus has fused and entered the host cell, it establishes itself to replicate entirely within the cytoplasm (Alexander 1993). The fusion protein is manufactured as a precursor glycoprotein F0 that must be cleaved into F1 and F2 segments by host cell proteases in order for the virus to become infectious. The sequence of certain amino acids at the F0 cleavage sites determines which host proteases are able to cleave the protein, and then dictates which host tissues and organs the virus will be able to enter and replicate within. The lentogenic viruses are only able to be cleaved by trypsin-like

proteases found primarily in the respiratory system of chickens, while the velogenic viruses have F0 proteins that are cleavable by proteases in a variety of organs. Once the production of viral proteins is complete and the negative sense genome has been replicated, the genetic material is encapsulated into the viral particles grouped at the inner surface of the cell membrane and the new virus buds out of the host cell (Alexander 1993).

The nucleocapsid protein and fusion protein are the major immunogenic proteins within Newcastle disease virus and are highly conserved between avian paramyxovirus subtypes. The nucleocapsid protein has a predicted molecular weight of 53kDa and is essential for viral replication and plays a role in the varying degree of virulence of the different APMV-1 strains (Seal *et al.* 2002). The methods of determining the virulence of a particular APMV-1 strain suggest that the APMV-1 strains can be grouped based on how quickly they can produce mortality in chicken embryos after allantoic inoculation. The virus was considered velogenic if chickens died at <60 hours, mesogenic at 60-90 hours, and lentogenic at >90 hours (Hanson and Brandly 1958). Another method of determining the severity of the infection is the intracerebral pathogenicity index (ICPI) in one-day old chicks, or intravenous pathogenicity index (IVPI). 6-8 week old chicks are inoculated with infected allantoic fluid and if the birds develop clinical signs and die within a short period, the virus is classified as velogenic (Vickers and Hanson 1982). Avian paramyxoviruses are quite distinct from other paramyxoviruses as the entire family of viruses is quite diverse and are responsible for many diseases in animals and humans with varying severity (Seal *et al.* 2002).

2.4.2.2 NDV (APMV-1) Transmission

APMV-1 has shown evidence to pass between birds via aerosols, and in naturally occurring infections these aerosols may be generated by disturbing dust or feces in poultry houses (Alexander 1988; Briand *et al.* 2014; Cappelle *et al.* 2011; Dai *et al.* 2013). The aerosolized particles may be inhaled and come into contact with chicken mucous membranes and trigger the infection. From the first

inoculation or exposure, the speed of the appearance of clinical signs vary based on the virulence of the virus, but typically average to be about 5-6 days of incubation (Alexander 1988).

2.4.2.3 Clinical Signs of ND

Clinical signs depend on the severity of the virus causing the infection but usually begin with increased respiration, lethargy, and weakness, ending with prostration and death (McFerran 1988). The velogenic form of the disease causes hemorrhagic lesions of the digestive tract and green diarrhea is frequently seen in birds that do not die in the early stages of infection, as well as edema around the eyes and head (Alexander 1988). Sudden severe respiratory signs are common followed by neurologic signs. Morbidity may reach 100% but mortality is generally about 50% among adult birds, but closer to 90% in younger birds. When the birds near death, muscular tremors, torticollis, paralysis of legs and wings, and abnormal rigid posturing may be seen (Alexander 1988). Mesogenic strains of APMV-1 do not cause high mortality, with the exception of very young or immunocompromised birds, and usually cause respiratory disease and a temporary drop in egg production. The lentogenic form of the virus normally doesn't cause serious disease in adult chickens, but younger birds can be more affected, and the case can be worsened by co-infection with other pathogens while the young chicken is in a weakened immunological state (Alexander 1988).

2.4.2.4 ND Post-Mortem Lesions

Gross lesions within the gastrointestinal tract are normally found in the proventriculus, cecal tonsil, and small intestine (Hanson *et al.* 1973). The lesions are dark red patches of hemorrhagic tissue that appear as a result of necrosis of the tissue. There aren't always lesions found within the respiratory system, but when they are present, they are mainly hemorrhagic lesions within the trachea (Alexander and Allan 1974). Lesions and edema may also be found externally on the wattle and comb.

2.4.2.5 NDV (APMV-1) Vaccination and Immune Response

Vaccination against APMV-1 is quite common for most poultry farms, and a combination of live and inactivated vaccines are used by all major poultry producers to control NDV infection. Aerosol generators within poultry housing have been used and often lentogenic strains of APMV-1 are used to immunize the flocks as they cause an immune reaction but will not cause major illness in the birds (Meulemans 1988). The major immune response to APMV-1 in the early stages is cell-mediated immunity and is active even before serum antibodies can be detected (Ghumman and Bankowski 1976). Antibodies normally begin to be detectable after 6-10 days of the initial infection. From here the quantity of antibody peaks around 3-4 weeks and can remain detectable in serum for up to a year (Kim and Spradbrow 1978). Although all NDV belong to the same serotype (APMV-1), there is evidence that conventional vaccines (La'sota, B1, etc.) may be losing their effectiveness against genotype VII strains within APMV-1 serotype (Kapczynski *et al.* 2013).

2.4.2.6 Laboratory Diagnosis of ND

Diagnosis of NDV in the lab is carried out very similarly to that of influenza virus because both viruses are able to agglutinate chicken red blood cells. These two diseases have such similar clinical presentations that make it difficult to differentiate them, making effective methods of diagnosis even more important (Miller and Torchettie 2014). Samples are mainly collected in the form of blood / serum samples to be further analyzed with serological methods. One of the largest issues making molecular diagnosis more popular among suspected NDV cases is that serological assays are not able to distinguish between antibodies as a result of vaccination, and those from an active infection.

2.4.2.6.1 Agarose Gel Immunodiffusion (AGID) to detect NDV antibodies

Agarose gel immunodiffusion (AGID) is a method which may be used to detect Newcastle disease antibodies as well as influenza antibodies but the AGID method does not seem to be as widely used for detecting NDV antibodies (Roy and Venugopalan 1997).

2.4.2.6.2 Virus Neutralization Assay for AIV detection

Virus Neutralization assays have been shown to be effective for NDV diagnosis although the HI test is still far more widely used (Reynolds and Maraqa 1999).

2.4.2.6.3 Enzyme Linked Immunosorbent Assay (ELISA) for NDV Antibody detection

Enzyme-linked immunoassays are commonly used for detection of antibodies against NDV and have been developed by different laboratories as well as by commercial companies. (Rodriguez-Sanches *et al.* 2008; Moro de Sousa *et al.* 2000; Thayer *et al.* 1987).

2.4.2.6.4 Hemagglutination Inhibition Assay for NDV antibody detection

Hemagglutination inhibition is regarded as the gold standard and most reliable method for detecting antibodies against NDV. (Beard and Wilkes 1985; Choi *et al.* 2013).

2.4.2.6.5 Fluorescent Microsphere Immunoassay (FMIA) for NDV Antibody Detection

The Luminex assay developed in this project is the only known luminex assay which can detect antibodies against NDV (Pinette *et al.* 2014).

2.5 Types of Interference and Nonspecific Binding

There are a number of unpredictable effects which may interfere with the assay signal. Interference is a large issue that must always be addressed when using or developing an immunoassay. Substances which can interfere with the assay are present in samples and can usually interact with the assay in a variety of unfortunate ways. Interference can be in the form of cross reactivity, unspecific binding, matrix effects, and others (Boscato and Stuart 1988; Andersson *et al.* 1989; Balsari and Caruso 1997; Rauch *et al.* 2005).

Cross reactions occur when the test serum or components of the assay are able to bind to other structures other than those intended. In most cases, the incorrectly bound material has great similarity

to the actual target (Rauch *et al.* 2005). In the case of Luminex assays, cross reactivity may occur between the protein bound to each bead region and cause either bead to bead reactivity, or some beads may pick up signal from the same test samples, or have proteins bound to their surface that allow interaction with greater than the desired amount of subtypes (Boscato and Stuart 1988).

Non-specific binding is a type of interference that is difficult to distinguish from cross reactivity, in that they both result in the similar effects. However non-specific reactions are normally based on substances which are in excess of the target analyte (Rauch *et al.* 2005).

Matrix effects are the names for all of the interfering components that appear within the test sample and impact the outcome of the assay. Another method of interference here are endogenous components found within the serum that have a high affinity for assay components and produce a false signal, such as complement proteins.

There are methods used to reduce or eliminate some of the interfering components of test sera which may include a one hour 56°C heat inactivation to disrupt interfering components, as well as the use of neuraminidase digesting enzymes which can be added to test samples before HI assay to eliminate the neuraminidase protein within the sample (Kim *et al.* 2012; More and Copeman 1991).

2.6 Baculovirus Protein Production

Baculoviruses are double stranded circular, supercoiled DNA viruses in rod-shaped capsids, and mostly infect insects of the order Lepidoptera (Hitchman *et al.* 2009). This family of viruses can exhibit both lytic and lysogenic life cycles that may independently develop throughout viral replication. In the regular insect hosts, these viruses quickly spread throughout the insect's open circulatory system and infect multiple cell types. The difference between natural Baculovirus infections and those designed to produce recombinant proteins is that the polyhedron gene is replaced with the gene of interest. This causes the virus to mass produce the desired protein in the late phase of the infection rather than the

protein coat needed to continue on the life cycle of the virus (Hitchman *et al.* 2009; Hitchman *et al.* 2011). Baculoviruses also do not cause any infections in animals or plants, and even among insects are only specific to the insect family *Lepidoptera*.

There are three phases of the Baculovirus infection in insect cells, beginning with the early phase, where the virus prepares the host cell for its own viral DNA replication. This includes attachment, entry, uncoating, early gene expression and interrupting normal host cell functions. This stage occurs about 0.5 to 6 hours post infection. After this is the late phase, where viral genes that code for replication of viral DNA and assembly are expressed. This is between 6 to 12 hours after infection. 18 to 36 hours post infection is when the virus has reached peak extracellular release of non-occluded virus. In the very late phase, occlusion bodies are formed between 24 and 96 hours post infection and multiple virions are produced and surrounded by a crystalline polyhedral matrix (Volkman *et al.* 1977; Volkman *et al.* 1976; Invitrogen Baculovirus info). It is important to be able to differentiate between initial viral budding out of the cells about 10hours post infection, and the initiation of protein expression at approximately 24 hours post infection so that you can harvest the cells at the right time to optimize the amount of recombinant protein harvested.

2.6.1 Glycosylation

Baculovirus is a prime candidate for growing recombinant proteins because the cell machinery that produces proteins enables the correct post-translational modification and high yields (Belzhelarskaia 2011; Murphy and Piwnica-Worms 2001; Hitchman *et al.* 2011; O'Shaughnessy and Doyle 2011). Insects regularly synthesize oligomannosidic and paucimannosidic N-glycan structures which are similar to those found in other species. Insects have the machinery to produce N-glycan structures but there is actually quite limited data on such configurations in nature (Rendic *et al.* 2007).

The first studies done to evaluate the N-glycans in insect cells were done on mosquitos and were found to contain high mannose structures, but no complex oligosaccharides (Hsieh and Robbins 1984). Studies continued and found that honeybee enzymes were also able to produce N-glycans, which continued to lead to those resembled in lepidopteran species (Rendic *et al.* 2006). *Spodoptera frugiperda* (Sf9) is one of the most commonly used insect cell lines and although does not produce glycosylation exactly like human cells do, their ability still exceeds *E.coli*'s inability to produce glycosylated proteins at all (Rendic *et al.* 2007). The first human protein produced using a protein expression system in insect cells was N-acetylglucoasaminyltransferase (GnTI) (Wagner *et al.* 1996). This system is suitable for use with influenza A HA proteins because extensive study has shown that the insect cell baculovirus system produces essentially the same glycosylation of these proteins that occur in vertebrates (Wagner *et al.* 1996).

2.6.2 Baculovirus production of Influenza Hemagglutinin proteins

The Baculovirus commonly used for these techniques is *Autographa californica* and is normally grown in the insect cell *Spodoptera frugiperda* (Sf9). The cells are usually cultured ahead of time to acquire a steadily growing population before infection with virus. The virus then grows in the cells and produces protein for 3-5 days. This is the optimal period until the majority of cells are dead and it is important to harvest the protein before it starts to degrade in the media, or there are so many dead cells that they're not producing protein any longer (O'Shaughnesy and Doyle 2011). The recombinant protein that is incorporated into the Baculovirus is engineered to contain an N or C-terminal grouping of 6 or more His amino acids in a row. This is called a his-tag and allows for affinity purification with Ni-NTA resin or columns. Other proteins may also be tagged on the desired recombinant protein such as GFP protein.

HA protein is normally translated at membrane-bound polysomes, translocated by means of an amino-terminal signal sequence into the lumen of the endoplasmic reticulum and transported through the golgi to the cell surface, this is the time where the protein normally undergoes N-glycosylation, trimerization, and cleavage into the two major fragments HA1 and HA2 (Kuroda *et al.* 1986; Wagner *et al.* 1996). When the HA is produced in insect cells, the same post-translational modifications have been shown to occur, but the trimerisation and cleavage steps were significantly less efficient when inside insect cells, possibly because of the lack of other influenza proteins present during trimerization (Kuroda *et al.* 1991). Direct comparisons of the glycosylation that occurs within the insect cell systems and actual chicken embryo cells have been done and found that only high mannose type structures and short truncated trimannosyl structures were detected on HA from Sf-9 cells, while the trimannosyl structures replaced the complex oligosaccharides found on HA produced in chicken embryo cells (Wagner *et al.* 1996).

2.7 Receiver Operating Characteristic Analysis

Receiver operator characteristic analysis was a method of statistical decision making developed during world war two with radar, to determine if radar blips were enemy aircraft or background noise. The test has become useful for assessing the accuracy of predictions with data that is difficult to analyze with alternative means (Maxion and Roberts 2004). This determination is based on a threshold value which is directly related to the trade-off between the ratio of true positives (sensitivity) and true negatives (specificity) (Maxion and Roberts 2004). The ROC analysis can also be used to determine the overall accuracy of the data generated by calculating the area under the curve (AUC) (Hajian-Tilaki *et al.* 1997). This test is for use when data transformation is not sufficient to change the skew of the data to fit a normal distribution. The sensitivity and specificity varies as the chosen cut-off varies. This is an important way to measure the performance of diagnostic tests and allows the visualization of overall assay accuracy when balancing sensitivity vs. specificity (Fan *et al.* 2006; Fawcett 2005). Using data

which fits a normal distribution is usually beneficial because it allows for the use of highly accurate and accepted methods of data analysis such as averages, standard error, and standard deviation. If all outliers have been taken into account, and the data has been attempted to be transformed via log or root transformations and still does not fit the normal distribution than it cannot be accurately analyzed using parametric statistical analysis. The ROC analysis generates a table displaying all of the possible threshold values with their associated assay sensitivities and specificities. In this sense, the threshold values are customizable to find a balance between sensitivity and specificity, or to favour one or the other. Many studies that involve Luminex technology have used ROC analysis to determine cut-off values and thresholds (Xia *et al.* 2010; Bozza *et al.* 2007; Langenhorst *et al.* 2012; Anderson *et al.* 2011; Lin *et al.* 2011).

3.0 Manuscript 1

Development of a Duplex Fluorescent Microsphere Immunoassay (FMIA) for the Detection of Antibody Responses to Influenza A and Newcastle Disease Viruses

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3.1 Abstract

Highly pathogenic avian influenza virus (HPAI) and virulent forms of avian paramyxovirus-1 (APMV-1) cause serious illnesses in domestic poultry, both of which are reportable to the World Organization of Animal Health (OIE 2008). The clinical presentation of avian influenza (AI) and APMV-1 infections are difficult to differentiate, emphasizing the importance of rapid and sensitive serologic assays that are able to distinguish them. Currently, a variety of serological assays are used for the serologic diagnosis of both diseases, but these assays are not used in multiplex formats. In this study, development of a duplex fluorescent microsphere immunoassay (FMIA) based on xMAP Luminex technology is described. The assay employs MagPlex magnetic microspheres that are covalently coated with recombinant avian influenza virus nucleoprotein and APMV-1 nucleocapsid antigens produced in a baculovirus insect cell expression system. The assay is able to detect AIV antibodies against all existing hemagglutinin (H1-H16) subtypes and simultaneously detect antibodies against APMV-1. In the process of this assay development different bead coupling conditions were compared. The assay has the capability of detecting serum antibodies from chickens and turkeys and optimization was accomplished by using 2,462 chicken and 446 turkey field and experimental sera and had a comparable detection capability with currently used assays in the laboratory. Assay threshold values were calculated with Receiver Operating Characteristic Analysis (ROC) in non-parametric analysis due to a highly skewed data distribution; this analysis resulted in AIV nucleoprotein relative diagnostic sensitivity and specificity of 99.7%, and 97.3% respectively. The APMV-1 nucleocapsid relative diagnostic sensitivity and specificity were 95.4%, and 98.5% respectively.

Keywords: Luminex, Baculovirus Insect Cell Expression System, Avian influenza, Newcastle disease, Antibodies, Fluorescent Immunoassay, Serologic diagnosis, Chickens, Turkey

3.2 Introduction

Recent outbreaks of avian influenza (AI) and avian paramyxovirus-1 (APMV-1) infections in domestic poultry in Canada, Mexico, China, India, Vietnam, Colombia, Russia, Italy, Japan, Korea, parts of the Middle East, and parts of Africa, have led to the destruction of a large number of flocks with a total economic impact in the millions of dollars (Mutinelli *et al.* 2003; Pasick *et al.* 2012; Pawar *et al.* 2012; Wang *et al.* 2008; Watson *et al.* 2009). From 2006 to 2009, the OIE with its 176 member countries, reported that APMV-1 caused outbreaks in 56 countries while HPAI caused outbreaks in 28 countries (World Livestock Disease Atlas, 2011). The highly contagious nature and similar clinical characteristics of both APMV-1 and AI infections emphasize the importance of being able to perform rapid and accurate serologic diagnosis that can determine which disease is causing the outbreak (Alexander and Senne, 2008; Leijon *et al.* 2011).

Avian influenza viruses belong to the influenza A genus of the Orthomyxoviridae family and have a genome that is made up of 8 single-stranded RNA segments of negative sense that code for 11 proteins: Nucleoprotein (NP), Hemagglutinin (HA), Neuraminidase (NA), M1, M2, NS1, NS2, PA, PB1, PB1-F2, and PB2. They are classified into serological subtypes based on their viral surface glycoproteins - hemagglutinin (H1 to H16) and neuraminidase (N1 to N9). Based on their potential to cause disease in chickens, AIV are divided into low pathogenic (LPAI) and highly pathogenic (HPAI) viruses. Low pathogenic AIV causes a localized mild respiratory or enteric disease, while few viruses belonging to H5 and H7 subtypes are considered highly pathogenic AIV and cause systemic disease of domestic poultry with up to 100% mortality. Low pathogenic AI and HPAI H5 and H7 are both reportable to the OIE.

Newcastle disease virus (NDV) also known as APMV-1 belongs to the family of *Paramyxoviridae*, genus *Avulavirus*. The virus has a single stranded RNA genome of negative sense containing 6 genes that code for 9 structural and non-structural proteins. The OIE defines velogenic NDV as a virus with an intracerebral pathogenicity index of >0.7 for day-old chicks, or with a polybasic cleavage site in the F protein (OIE 2008). Newcastle disease can also be caused by lentogenic strains that cause sub-clinical to mild clinical disease; mesogenic – causing non-fatal respiratory disease; and velogenic – most pathogenic and often fatal (Aldous *et al.* 2001; Kapczynski *et al.* 2013; Seal *et al.* 2002).

Currently, a variety of serological assays are being used for the detection of AIV and APMV-1 antibodies in domestic poultry to monitor the antibody response due to infection, or for the purpose of monitoring vaccine efficacy. Although different commercially available ELISA kits are used for detecting APMV-1 antibodies in domestic poultry, the HI assay continues to be the “gold standard” for detecting APMV-1 antibodies. For the detection of AIV antibodies, the agar gel immunodiffusion (AGID) and hemagglutination inhibition (HI) test remain as the "gold standard" methods approved by the OIE. The AGID test detects antibodies against the highly conserved nucleoprotein (NP) and matrix (M1) proteins in addition to antibodies to HA and NA proteins. However, the assay is less sensitive and is prone to producing higher rates of false negatives (Snyder *et al.* 1985; Watson *et al.* 2009; Zhou *et al.* 1998). Unlike the AGID test, the HI assay specifically detects hemagglutinin subtype specific antibodies using H1-H16 antigens, however it has several drawbacks: it is labour intensive, difficult to automate as results are read visually, it requires subtype specific antigens that are difficult to produce under ordinary lab conditions, the antigens are hard to standardize as they need to be calibrated for each assay,

and the assay depends on fresh red blood cells (RBC) from chickens that can be difficult to acquire (Comin *et al.* 2013; Upadhyay *et al.* 2009; Zhou *et al.* 1998). In addition, the reference antigen used in the HI assay needs to be updated on a yearly basis with respect to the currently circulating viruses, otherwise this may impair the sensitivity of the assay dramatically.

Currently, different laboratories use the HI assay in combination with an ELISA to check the immune status of vaccinated flocks (Desvaux *et al.* 2012; Toffan *et al.* 2010). Despite their robustness and higher sensitivity, ELISAs don't have multiplexing capabilities, and for each individual analyte to be detected a separate test must be carried out making the use of ELISAs time consuming and laborious. In the current study, the development of a duplex florescent microsphere immunoassay (FMIA) based on the xMAP technology for simultaneous detection and differentiation of antibody response to AIV and APMV-1 infections is described. The duplex FMIA is based on AIV nucleoprotein (AIV-NP) and APMV-1 nucleocapsid protein (APMV-1-NC) that are the most abundant proteins in AIV and APMV-1 respectively. Both AIV-NP and APMV-1-NC proteins are highly conserved and immunogenic, which therefore makes them suitable candidates for the detection of virus-specific antibodies and for serologic diagnosis of each individual disease.

3.3 Materials and Methods

3.3.1 Cloning of APMV-1 Nucleocapsid and AIV Nucleoprotein Genes

Avian Influenza nucleoprotein from A/Duck/AB/AFLB-C16/2008 (H7N7) and APMV-1-NC from APMV-1 vaccine (La'Sota) were selected for the development of the assay. The AIV-NP gene was amplified using universal AIV-NP primers as described previously (Hoffmann *et al.* 2001) and cloned into PCR^{4}TOPO cloning vector (Invitrogen, Ontario, CA). The coding region of AIV-NP was re-amplified using forward primers: ACG CGC CCG CTA TGG CGT CTC AAG GCG CCA A and reverse primers: CGG TCT GAG TTA ATT GTC ATA CTC CTC TGC ATT. The full nucleocapsid gene from APMV-1, La'Sota strain was amplified using primers: ATG TCT TCC GTA TTT GAT GA (forward) and TCA ATA CCC CCA GTC GGT GT (reverse). The amplified AIV-NP and APMV-1-NC PCR products were sub-cloned in-frame into the *Not 1* and *Xba 1* restriction enzyme sites of pAB-bee-FH transfer vector which included an 8X HIS tag for later protein purification (AB Vector, California). Correct frame and sequence of the gene in the transfer vector (pAB-bee-FH – APMV-1-NC or AIV-NP) were confirmed by sequencing.

3.3.2 Transfection and Generation of Recombinant Baculovirus

The APMV-1-NC or AIV-NP genes that were cloned in the transfer vector were co-transfected into *Spodofera frugiperda* (Sf-9) cells with ProFold-ER1 linearized baculovirus DNA (AB Vector, California, USA). All procedures for the production of recombinant proteins, including plaque purification, production of high titer stocks and optimization of protein expression were performed according to the manufacturer's protocols (AB Vector, California, USA). The APMV-1-NC or AIV-NP recombinant proteins from the cell pellets and media

supernatants were purified by batch procedure using Ni-NTA agarose (Qiagen, Maryland, USA) according to manufacturer's instructions.

3.3.3 Coupling of Proteins to MagPlex Luminex Magnetic Beads

Coupling of proteins to MagPlex Luminex magnetic beads was carried out according to the manufacturer's instructions (Luminex Corporation, Texas, USA). Prior to coupling, the recombinant proteins were desalted using micro-biospin 6 columns (Bio-Rad, California, USA) according to the manufacturer's instructions to exchange the buffer from imidazole to PBS, and quantified using Pierce BCA protein Quantification Kit (Thermo Scientific, Maryland, USA). Recombinant APMV-1-NC protein were coupled to MagPlex region #012 microspheres and AIV-NP were coupled to region #015 (Luminex Corporation, Texas, USA). Twelve micrograms (ug) of each recombinant protein were coupled to a fixed amount of beads (1.25 X 10 10^6) per 1X reaction.

3.3.4 Duplex FMIA

Coupled APMV-1-NC and AIV-NP beads at concentration of 2000 beads/well were combined and blocked for 2 hours in 5% (v/v) normal donkey serum (Jackson ImmunoResearch Laboratories Inc., Pennsylvania, USA) in PBS. Twenty five microliters of bead suspension and 25 µl of test serum diluted 1:500 in 1% (v/v) normal donkey serum in PBS were added to each well. The plate was left at room temperature in the dark on a shaking incubator for 1 hour. A wash program consisting of 3 washes with 200µl of PBS-tween 20 in magnetic plate washer was completed (Bio-Tek, 405 Select TS), and the plates were incubated with 50 µl of 2 µg/ml biotinylated donkey anti-chicken immunoglobulin (Jackson ImmunoResearch Laboratories, Inc.,

Pennsylvania, USA) for 30 min at room temperature. Plates were washed again before the final addition of 75 µl of 10 mg/ml streptavidin-phycoerythrin conjugate (Jackson ImmunoResearch Laboratories Inc., Pennsylvania, USA) and incubation at room temperature for 15 minutes. Following the final wash, each well of beads were re-suspended in 125 µl of wash buffer and analyzed with a BioPlex 100 instrument running Bioplex Manager 6.1 software (BioRad, California, USA). The results were expressed as arbitrary units of median fluorescence intensity (MFI).

3.3.4.1 Luminex Reference Curve

A 12-step reference curve was developed for use with the assay as a method of variability monitoring between consecutive runs by comparing the changes in fluorescent signal of the reference curve, and using this data to calculate a correction factor which was applied to all data sets. The references were prepared by mixing AIV and APMV-1 hyper-immune sera of known titers and diluted 2-fold in negative chicken serum to produce a 12-step curve. These reference samples were tested for the presence of AIV and APMV-1 antibodies using the IDEXX ELISA kits and hemagglutination inhibition assays. The hemagglutination inhibition titers of both the APMV-1 and AI sera that were used for making the standards were 512 and 128 consecutively.

3.3.4.2 Luminex Internal Controls

Region 18 and 19 MagPlex beads were used for internal controls for the assay. Highly purified chicken IgY (Jackson Immuno Research Inc., Pennsylvania, USA) and donkey anti chicken IgY (Jackson Immuno Research Inc., Pennsylvania, USA) 0.1µg/ml concentrations each were coupled to these respective bead regions as described above, and included in the assay

for the purpose of verifying the addition of secondary detection antibody and test serum respectively.

3.3.5 Hemagglutination Inhibition Assay

Hemagglutination inhibition (HI) assay was performed according to the methods described in the OIE manual (OIE 2008). For testing the serial bleeds from experimentally inoculated chickens, 4 HA units of homologous virus was combined with 2-fold serial dilutions of each serum sample. Chicken red blood cells (0.5% v/v suspension) were then added and HI endpoints read. Serum HI titre > 1:4 was considered positive.

3.3.6 IDEXX ELISA

Commercial IDEXX AIV and APMV-1 ELISA kits were used to determine presence of AIV or APMV-1 antibodies in poultry sera. Depending on the source of the serum, the indirect APMV-1 antibody test kits for turkey and chicken sera were used (IDEXX Laboratories, Maine, USA). The competitive MultiS-Screen ELISA kit was used for AIV antibody detection in turkey and chicken sera. Both assays were performed according to the manufacturer's protocols. Results were quantified by reading plates at 650 nm with Spectra Max plus microplate reader (BioTek Instruments Inc., Vermont, USA) using Softmax software (BioTek Instruments, Vermont, USA).

3.3.7 Competitive Enzyme-linked Immunosorbent Assay (cELISA)

Competitive ELISA (cELISA) was performed as described by Ming *et al.*, 2008. Microtiter plates coated with 1 µg of recombinant baculovirus AIV-NP protein in carbonate buffer (pH 9.6) at 100 µl/well and plates were incubated 4°C overnight. After washing, equal volumes (50 µl)

of diluted test sera (1:5) and hybridoma culture supernatants containing AIV-NP monoclonal antibody (1:200) were added to the plates and incubated at 37ºC for 1 hour with agitation. Then, HRP conjugated anti-mouse IgG (Jackson Immuno Research Inc., Pennsylvania, USA) was added and incubated for 1 hour at 37ºC with subsequent washing. Lastly, the enzyme substrate, 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma-Aldrich, Missouri, USA), was added and color development was stopped after 15 minutes with 50 µl/well of 2.0 M sulphuric acid. The OD was determined at 450 nm on an automated plate reader (Spectro Max plus, Molecular Devices, California, USA).

3.3.7 Serum samples

2,462 serum samples that were collected from SPF chickens, broilers chickens, layers and breeder chickens and an additional 446 turkey serum samples that were collected from turkey poults and breeders were used in the validation of the assay. All sera were pre-screened by a combination of assays including HI, in-house developed cELISA, and IDEXX ELISA to determine the presence or absence of detectable antibodies.

3.3.7.1 Reference AIV and APMV-1 antisera

Reference antisera against all existing 16 hemagglutinin subtypes of AI viruses and avian paramyxovirus (APMV) types 1-9 (Table 1) were produced by inoculating six-week-old white leghorn specific pathogen free (SPF) chickens that were obtained from the animal care unit of the Ottawa Laboratory Fallowfield (OLF), Canadian Food Inspection Agency (CFIA). The OLF SPF stock was developed from Charles River/SPAFAS birds (Storrs, Connecticut, USA). Negative SPF chicken sera used in this study were also collected from the same flock.

3.3.7.2 Field serum samples

Field serum samples used in this study were collected from broilers, turkey and chicken breeder flocks; some of which were vaccinated for APMV-1 using a combination of live and inactivated APMV-1 vaccines. Some serum samples were collected from turkeys that were vaccinated with inactivated H3N4 AIV vaccine (Berhane *et al.* 2012). Chicken field serum samples used in this study were donated to us by the Animal Health Laboratory (AHL), Guelph, Ontario or from archived serum database of NCFAD.

3.3.7.3 Production of experimental serum samples

During the production of experimental serum samples, all animal care and handling procedures were done according to the Canadian Council on Animal Care guidelines and as outlined in protocols approved by the institute.

3.3.7.3.1 Chicken Serum

For the production of anti-APMV-1 serum samples, one group of chickens (n=20) were inoculated intranasally and ocularly with 1 ml of inoculum containing 10^4 egg infective dose 50 [EID_{50}] of La'Sota APMV-1 vaccine strain. For the production of AIV serum samples, a second group of chickens (n=20) were also inoculated intranasally and ocularly with 1 ml of inoculum containing 10^4 plaque forming units (PFU) of low pathogenic influenza virus A/Anhuii/1/2013 (H7N9). Serum samples were collected at 0, 4, 7, 14, 21 and 28 days after infection (dpi). The H7N9 group of chickens was kept in enhanced level 3 animal cubicles

3.3.7.3.2 Turkey serum

For experimental turkey serum production, 8 to 10 week old influenza free turkey poults were bought from a commercial supplier in Manitoba. The turkeys were bled and pre-tested for the presence of influenza A group specific antibodies (NP) using cELISA and confirmed negative. The first group of turkeys (n=10) were inoculated intranasally and ocularly with A/turkey/ON/1963 (H6N8), and the second group (n=10) with A/turkey/MN/12877/1285/81 (H9N2). The turkeys were inoculated with 10^6 egg infectious does$_{50}$ (EID_{50})/ml of virus inoculated intranasally and ocularly. Serum samples were collected at 0, 7, 14, 21 and 28 days after infection (dpi).

3.3.8 Data Analysis

All statistics were done using the Microsoft Excel add on "XLstat – LIFE". Basic frequency versus signal histograms were generated to visualize the distribution of the data. Receiver Operating Characteristic Analysis (ROC) was used to determine accurate threshold values for the assay.

3.4 Results

3.4.1 Recombinant Protein Production and Purification

The AIV-NP and APMV-1-NC cloned into pAB-bee-FH transfer vectors were co-transfected with linearized ProFold™-ER1 baculovirus genomic vector into SF9 cells. The presence *of Aequorea victoria* green-fluorescent protein (GFP) in ProFold™-ER1 allowed for the convenient monitoring of recombinant baculovirus infection. Expression of both recombinant proteins (AIV-NP and APMV-1-NC) in the cell culture supernatants and cell pellets were monitored by running the denatured protein product in 10% Bis-Tris precast gels (Invitrogen) followed by Coomassie blue staining and Western blot analysis. The APMV-1-NC or AIV-NP recombinant proteins from the cell pellets and media supernatants were purified using Ni-NTA agarose and the purity was confirmed by SDS-PAGE followed by Coomassie Blue staining (Fig. 1A) and the authenticity of the purified recombinant proteins was confirmed by Western blot analysis (Fig. 1B).

3.4.2 Bead Coupling Dose Response and Protocol Optimization

The quantity of protein used in each microsphere coupling reaction was based on recommendations within the Luminex literature to range from 5µg to 12µg of purified protein per 1X coupling reaction (1.25 X 10^6 beads per 100 µl reactions). Five confirmed negative serum and 6 confirmed positive sera were used to test the different quantities of protein bound to the beads. Doses of 6 µg, 12 µg, 23 µg, and 45 µg of each recombinant protein were tested in each bead coupling reaction. For both AIV-NP and APMV-1-NC proteins, coupling with 12 µg of protein per reaction gave the maximum signal-to-noise ratio (Fig. 2). The lowest background to noise ratio was achieved when the beads were blocked for 2 hrs in 5% (v/v) normal donkey

serum in PBS and the test sera diluted at 1:500 in PBS solution containing 1% (v/v) normal donkey serum.

3.4.3 Assay development

The reference curve developed for monitoring assay variability between consecutive runs was also used to demonstrate that the assay was not affected when detecting antibody with both beads simultaneously. Monoplex and duplex FMIA results for both AIV-NP and APMV-1-NC were analyzed using the non-parametric Wilcoxon, Mann-Whitney comparison test with 5% significance level (XLStat 2013). Neither result suggested to reject the null hypothesis (H_0: The difference between the samples is equal to 0), so the monoplex and duplex FMIA results were not found to be significantly different (AIV-NP p-value 0.865; APMV-1-NC p-value 0.706). The AIV-NP coated beads were able to detect antibodies developed against all existing hemagglutinin subtypes (H1 to H16) while showing no cross reactivity with antisera developed against APMV-1, 2, 3, 4, 6, 7, 8, and 9 serotypes (Fig. 3). The APMV-1-NC beads did not cross react with any of the H1 to H16 subtypes of influenza, and were able to detect antibodies to APMV-1 very strongly. The APMV-1-NC cross-reacted weakly with anti-sera developed to APMV-7 and APMV-9 (Fig 3).

The antibody coupled microspheres (chicken IgY and donkey anti-chicken IgY) were used as internal assay controls to confirm the addition of critical reagents. The presence of donkey anti-chicken IgY coupled microspheres ensured that test serum has been added. The presence of chicken IgY coupled microspheres, ensured that the secondary detection antibody has been added. The presence of these internal controls helped elucidate false negatives caused by user

error. MFI values greater than 8000 for each internal control microsphere indicate successful reagent addition.

3.4.4 Duplex FMIA Analytical Sensitivity

In order to determine the analytical sensitivity of the assay, AIV and APMV-1 reference antisera that were developed in chickens were diluted 2-fold in negative SPF chicken serum (Sigma Aldrich, USA) in a 12-step dilution series. The APMV-1 reference antiserum was developed in chickens that were inoculated with La'Sota NDV vaccine strain. The hemagglutination inhibition titer of this antiserum to homologous antigen was 512. The AIV reference antiserum was produced in chickens that were inoculated with A/Chicken/BC/514/2004 (H7N3) and HI titer of this antiserum to homologous antigen was 256. These 12 serial dilutions of AIV and APMV-1 antisera were tested using the AIV-NP and APMV-1-NC duplex FMIA. The AIV-NP beads were able to detect antibodies in serum at dilutions as low as 1/128, and APMV-1-NC beads could detect antibodies at dilutions as low as 1/1024 before falling below the assay background (Fig. 4).

3.4.5 Statistical Normalization of Results

The standard curve results were compiled for every run of the FMIA, providing a summary of how the assay performed over time. Statistical ANOVA testing was carried out across the individual reference controls and revealed a minor difference between runs. This prompted the normalization of the overall data to correct for assay variation. Averages of the MFI values of each standard were calculated across all runs and the calculated averages of the standards were used to generate an overall correction factor to determine the correct assay values. When values of the standards were higher or lower than the average, the entire standard curve usually reflected this shift either wholly above, or wholly below the average.

For example, if standard 1 read a greater value than normal, the rest of the reference samples in that run also read greater values than normal. This allowed us to create a separate correction factor for each run of the assay and apply it uniformly to either raise or lower the observed values to normalize the values of the standard curve. The corrected assay values were then used for statistical analysis to determine the assay cut-off.

3.4.6 Receiver Operating Characteristic Analysis for Threshold Determination

Receiver operating characteristic (ROC) analysis was used for assay threshold determination because of the inability to transform the data to fit a normal distribution even after attempted $\log_{10}$ and fourth root transformations (Fig. 5). The ROC analysis was used to calculate the threshold value determined by the ratio of true positives to false negatives. The median fluorescence intensity (MFI) at which the ratio of false positives and false negatives are lowest determines the optimal threshold. The IDEXX ELISA results were used as a guide for "true positive" and "true negative" status of the test serum undergoing analysis as the assay has been commercially validated. The diagnostic specificity of the APMV-1 IDEXX ELISA was 100% (n=40 SPF sera), and the assay showed sensitivity of 91.5% in relation to HI results (n=47) (IDEXX APMV-1 ELISA Validation Report 2010). The AIV MultiS-Screen ELISA has a diagnostic specificity of 99.7% (n=5,007), and diagnostic sensitivity that matched the HI (IDEXX AIV ELISA Validation Report). In the events where the IDEXX ELISA and the HI results were in disagreement, "true positivity or negativity" was assigned according to the HI as it is the current gold standard and also the assay that the IDEXX was validated against.

The AIV antibody response detection threshold of the duplex FMIA was calculated to be 1835 median fluorescent units with an associated diagnostic sensitivity of 99.7% and diagnostic

specificity of 97.3% relative to the IDEXX ELISA. This threshold value yielded 332 relative true positives, 56 false positives, 1 false negative, and 1,987 true negatives.

The avian paramyxovirus antibody response detection threshold of the duplex FMIA was calculated to be 2284 median fluorescent units (MFU) with an associated diagnostic sensitivity of 95.4% and diagnostic specificity of 98.5% relative to the IDEXX ELISA. This threshold yielded 313 true positives, 18 false positives, 15 false negatives, and 1,149 true negatives.

All values were statistically significant within a 95% confidence interval (XLstat 2013).

3.4.7 Monitoring the kinetics of antibody response using the duplex FMIA.

3.4.7.1 Chickens

The duplex FMIA was used to monitor the kinetics of antibody responses in 2 groups of chickens that were experimentally infected with influenza and APMV-1 LaSota strain. Serum samples that were collected at 0, 4, 7, 14, 21 and 28 days after infection (dpi) were tested using the duplex FMIA. In addition, the serum samples were also tested for the presence of APMV-1 antibodies using the indirect IDEXX APMV-1 ELISA kit and HI assay. In addition to the FMIA, for detecting AIV antibodies the IDEXX AIV multiscreen ELISA kit was also used. All pre-bleed samples from chickens of both groups tested negative for AIV and APMV-1 antibodies using all test methods.

Chickens that were infected with APMV-1 started to develop antibody responses at 7 dpi and reached peak MFU by 14 dpi. The results were comparable to APMV-1 IDEXX ELISA results, but the duplex FMIA was more sensitive than HI assay. Results of this study are summarized in Fig. 6A.

Serum samples collected from the 2nd group of chickens that were infected with A/Anhuii/1/2013 (H7N9) were tested on the duplex FMIA. Infected chickens started to develop antibody responses starting at 4 dpi based on the IDEXX ELISA and duplex FMIA results. By 7 dpi, all samples tested positive on the IDEXX ELISA and duplex FMIA. However, there were only 3 samples that were positive on the HI assay. At 14dpi, all samples tested positive on the IDEXX ELISA and on the duplex FMIA, however only 50% of samples had anti H7 hemagglutinin antibodies. By 21dpi, all assays were detecting a positive immune response from all test serum. Results of this study are summarized in Fig. 6B.

3.4.7.2 Turkeys

The duplex FMIA assay was also used to detect the kinetics of antibody responses in turkey poults that were infected with LPAI viruses. For this purpose, turkeys in the 1st group were inoculated with A/turkey/ON/1963 (H6N8), and the 2nd group with A/turkey/MN/12877/1285/81 (H9N2). Pre-bleed serum samples collected from these turkeys tested negative for APMV-1 and AIV antibodies using the FMIA or IDEXX ELISA tests for both antibodies. Based on the IDEXX ELISA and FMIA results, almost 100% of the turkeys in both groups elicited humoral immune response against AIV starting at 7 dpi and reached peak responses by 14 dpi. According to the HI assay results, anti-hemagglutinin antibodies were detected in the 90% of turkeys in the H9N2 group by 14 dpi and all turkeys seroconverted by 21 dpi and remained at higher level until the end of the study at 28 dpi. Sixty percent of turkeys that were inoculated with the H6N8 virus developed anti hemagglutinin antibodies at 14 and 21 dpi. Results of this study are summarized in Fig 7.

3.5 Discussion

The most commonly used serological methods for the detection of antibody response to APMV-1 and AIV infections in domestic poultry are ELISA and HI assay; in addition the AGID test is used for detecting AIV antibodies. Although AGID and HI are gold standards, both assays are labour intensive and are less suited for high-throughput screening. The current project reports the development of multiplex immunoassay with the ability to simultaneously detect antibody responses to AIV and APMV-1 infections using the Luminex xMAP technology.

The application of Luminex technology for the detection of antibody responses to multiple pathogens in a single assay format has been demonstrated previously (Andersen *et al.*, 2011; Bergmann *et al.* 2003; Chen *et al.* 2009; Clavijo *et al.*, 2006; Dias *et al.*, 2005; Lupiani *et al.*, 2010; Martins, 2002;). Most of these developed assays used either recombinantly expressed proteins, or purified, or semi-purified antigens coupled to the beads. In the current study, expression of the AIV-NP and APMV-1-NC proteins were performed by using the baculovirus expression system. Producing the recombinant antigens in the baculovirus expression system has significant advantages including accurate glycosylation, speed and volume of protein expression, as well as proper folding (Invitrogen, Ontario, CA). Expressing the recombinant proteins fused to a His-tag had additional advantages in attaining a very clean protein product that helped minimize the occurrence of possible cross-reactions, lowered unwanted background signal in the assay, and also increased confidence of the results.

Many veterinary diagnostic laboratories currently use the IDEXX ELISA or other similar ELISAs for serologic screening of both AIV and APMV-1 disease infections in domestic poultry.

The performance of the duplex FMIA was compared to the widely used IDEXX ELISA kits for both infections. In addition, for detecting AIV antibodies an in-house developed cELISA was used which employed identical capture antigen that was used in the FMIA (data not shown). Based on the comparative data presented in this study, the duplex FMIA assay performance was almost identical to the IDEXX ELISA kits for both diseases. Although the IDEXX ELISA kits or other commercially available ELISA kits allow for the processing of a very large number of samples in a relatively short period of time, they lack the multiplexing capabilities, and individual tests have to be carried out for each analyte to be detected. Therefore, ELISA testing becomes laborious and time consuming. The duplex FMIA reported here has a number of advantages as it is able to detect antibody responses to AIV and APMV-1 infection using a single serum dilution and also could easily be automated and used for processing of large amounts of serum submissions. The assay's potential to detect multiple targets in the same platform could offer substantial benefits for routine serological testing through reduced staff and reagent costs, and simplified laboratory operation.

As described previously by Martins (2002), the duplex FMIA assay also incorporates an additional two microspheres as internal controls that allow for the establishment of quality control parameters to ensure that each individual reaction has received the correct reagents. This allows the monitoring of whether the correct concentration of each individual reagent such as test sera, conjugate, and substrate has been added. The secondary antibody addition control ranged in from 9,000-12,000 MFI on average. MFI values below 8,000 were considered untrustworthy and repeated. The control for proper serum addition ranged from 6,000-10,000 MFI on average. MFI values below 6,000 were considered unreliable and were retested.

Internal controls were tested on plates where serum or secondary antibody were intentionally left out or added incorrectly, and the controls successfully identified all problematic wells. Internal controls could also be utilized to increase precision and accuracy by monitoring instrument fluctuations, allowing intra- and inter-assay normalization (Martins, 2002).

The performance of the developed duplex FMIA was also compared with IDEXX ELISA kits in studying the kinetics of immune responses in chickens and turkeys that were infected with different subtypes of LPAI viruses. Based on these studies, in most infected animals, AIV antibodies could be detected as early as 4-7 dpi; reaching their peak by 14 dpi and remaining at a high level until 28 dpi. The results of duplex FMIA were almost identical to the IDEXX ELISA kit values for most of the groups infected with different subtypes of AIV. In addition, the performance of the assay was almost identical to the commercial ELISA platform when studying the kinetics of antibody responses in chickens immunized with the LaSota APMV-1 vaccine. Antibodies to ND-NC could be detected as early as 4 – 7 dpi and remained at peak levels until 28 dpi. This study demonstrates the usefulness of the assay in monitoring antibody response in chickens or turkeys that were exposed to APMV-1 and AIV. The assay was also useful in monitoring antibodies in field serum samples collected from turkey breeders immunized with inactivated AIV and APMV-1.

The stability of AIV-NP and APMV-1-NC coupled beads stored at 4℃ was tested for a period of 4 months. The reference curve was used as a benchmark to track the sensitivity of the assay for this period of time, and four month old protein coupled beads have yet to show a drop in fluorescence values (data not shown).

In the duplex FMIA assay, there was limited cross reactivity between the APMV-1-NC antigen with APMV-7 and APMV-9 antisera. There is a possibility that this could be due to the presence of some conserved immunogenic antigenic sites on the nucleocapsid protein between these viruses and APMV-1 serotypes. Traditionally, APMVs are grouped into different serotypes (APMV-1 to AMPV-9) based on the HI test (Alexander, 1988), however, antibodies to prototype viruses representative of the nine currently defined APMV serotypes are already known to cross react with other APMV serotypes and with mammalian paramyxoviruses (Alexander *et al.*, 1978, 1981; Kessler *et al.*, 1979; Lipkind *et al.*, 1982; Shortridge et al, 1980; Tumova *et al.*, 1979). It is possible the APMV-1-NC may be too conserved among APMV serotypes to provide enough resolution for the exact classification.

A number of Luminex based assays have been developed for serologic diagnosis of avian diseases including AIV (Chowdhury *et al.* 2008; Drummond *et al.* 2008; de Giavedoni, 2005; van Gageldonk *et al.* 2008; Jager *et al.* 2005;). To our knowledge this is the first study where a duplex FMIA was developed for serologic diagnosis and differentiation of two important poultry diseases. The time saving value of the FMIA even makes it a feasible option for high-throughput surveillance testing (Seideman *et al.* 2002).

Receiver Operating Characteristic analysis (ROC) is a statistical non-parametric function that was used to determine the assay threshold values by analyzing the trade-off between true positive results and false positive results (Fawcett 2005). Overall performance of the assay is consistent, and is based on a large data set. It is important to include a set of reference standards to allow for independent threshold determination without the need to normalize results. However, if the assay were to only be used with chicken and turkey serum from the

same geographical region, once a suitable threshold value has been determined, there is no need to undergo the complex statistical analysis again. In its current state, the assay could be effectively used for surveillance of AIV and APMV-1 in poultry flocks. New work is under way to incorporate two additional bead sets which will allow for the specific differentiation of AIV infections of H5 or H7 subtypes. These are the notifiable forms of AIV and also have the capacity to become highly pathogenic strains.

3.6 Manuscript 1 Figures

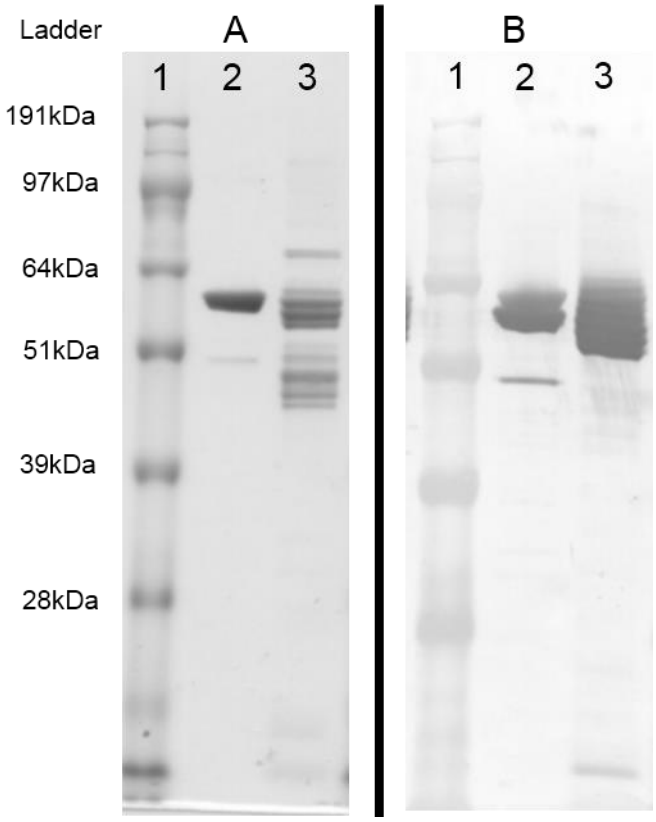


Figure 1. Analysis of recombinant avian influenza nucleoprotein (2A and 2B) and avian paramyxovirus-1 nucleocapsid protein (3A and 3B) that were expressed using the baculovirus expression system were successfully purified and underwent sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie Blue staining (Figure 1A) and western immunoblot (Figure 1B) using anti his antibody. Equivalent volumes of each protein (5µl) were used for both Coomassie and western immunoblot analysis.

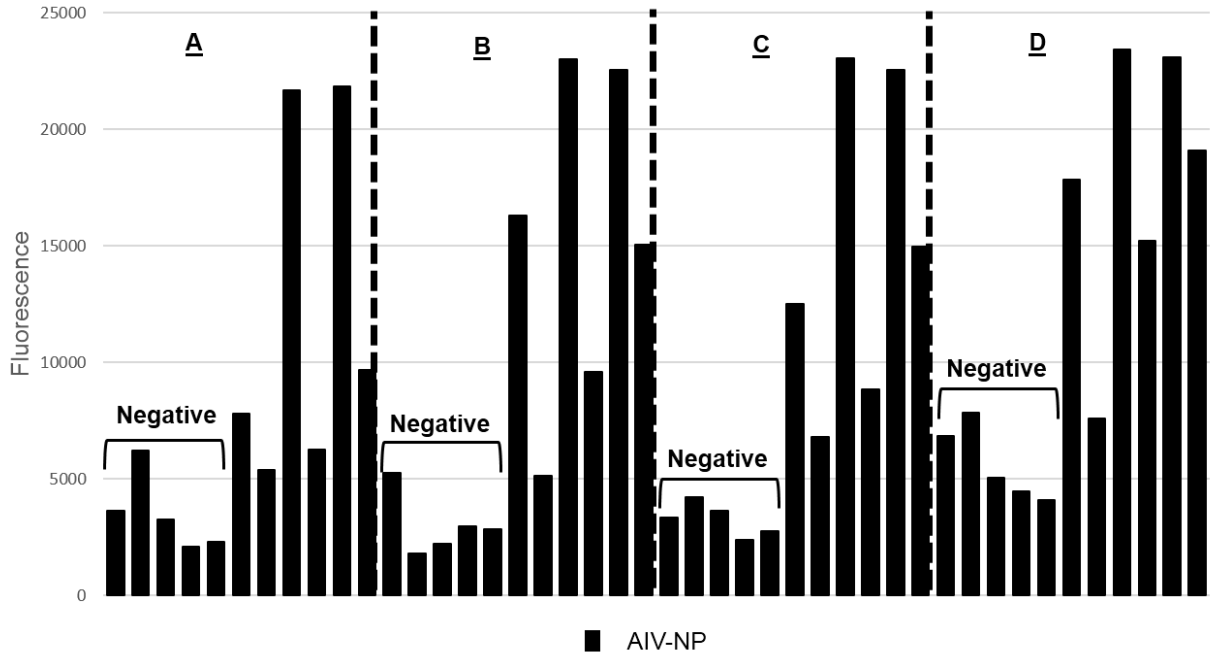


Figure 2. The optimization of optimal amount of recombinant protein required for coupling to the MagPlex luminex magnetic beads (Luminex Corporation, Austin, Texas, USA) was assessed by testing 4 different concentrations of recombinant avian influenza nucleoprotein at 5.6µg (A), 11.2µg (B), 22.4 µg (C) and 44.8µg (D) with 5 known negative and 6 known positive chicken sera. Beads that were coupled with 11.2µg of recombinant avian influenza nucleoprotein showed the most favorable Sample/Negative ratio and also displayed the lowest overall background signal.

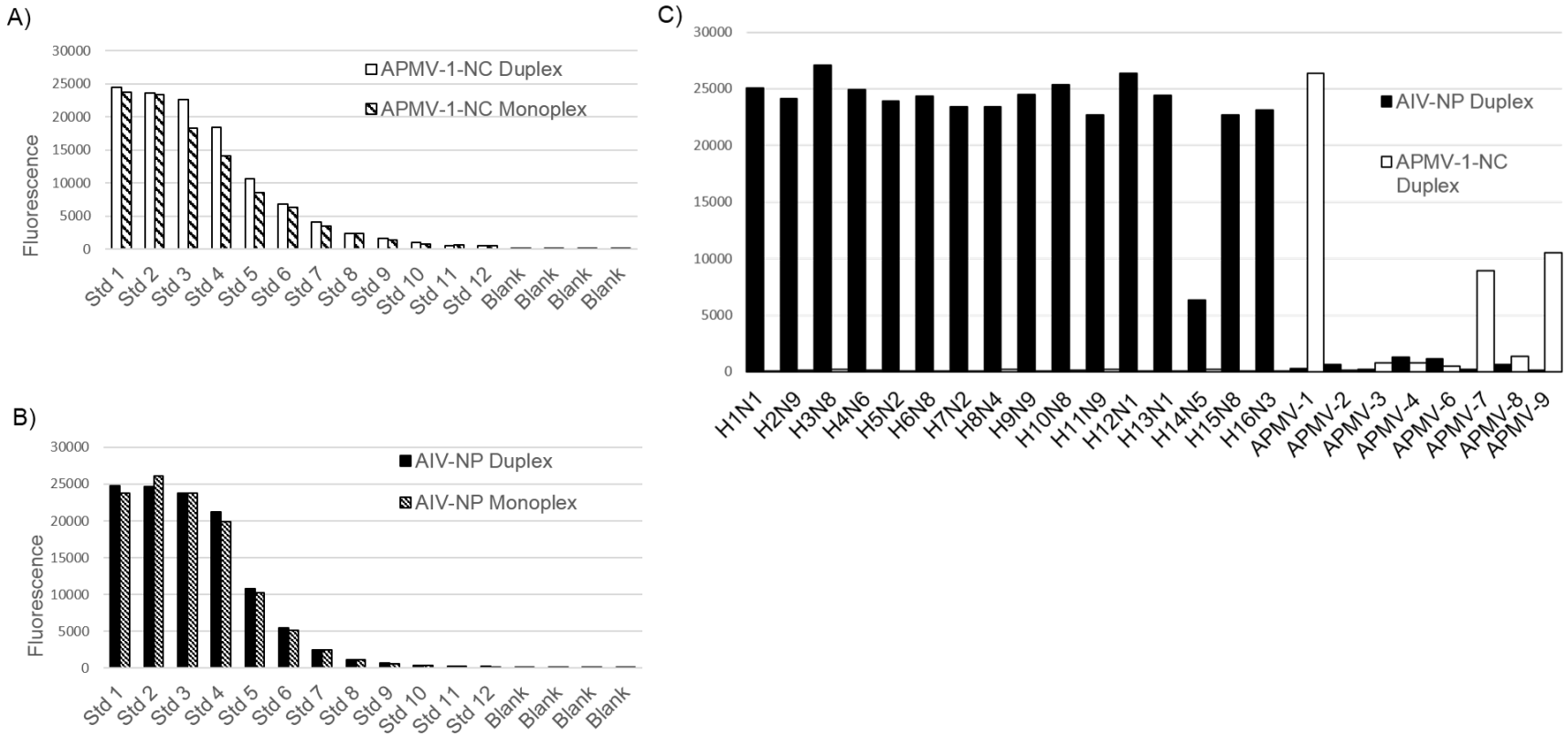
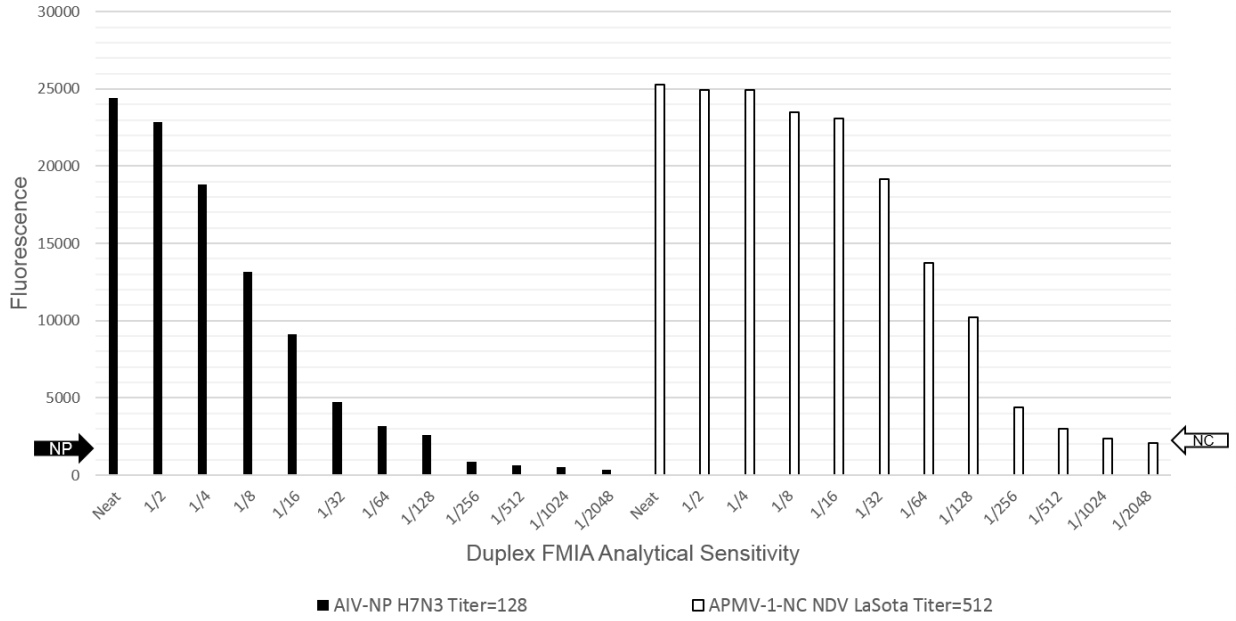


Figure 3A & 3B. Comparison of results of the FMIA using recombinant avian influenza nucleoprotein and avian paramyxovirus type 1 nucleocapsid coated beads when tested in monoplex and duplex formats with the Luminex reference sera. Fig. 3A describes avian paramyxovirus type 1 FMIA in monoplex and duplex formats and Fig. 3B – avian influenza monoplex and duplex FMIA. There was no significant difference in fluorescent mean values (MFI) when the assays were run in duplex or monoplex formats.

Figure 3C. The duplex fluorescent microsphere immunoassay for serologic diagnosis of avian influenza and avian paramyxovirus type 1 infection was tested using a panel of reference antisera against all the existing 16 hemagglutinin subtypes (H1 to H16) and avian paramyxoviruses type 1-9 (excluding type-5). The luminex beads coated with avian influenza nucleoprotein were able to detect antibodies against all 16 hemagglutinin subtypes and beads coated with avian paramyxovirus-1 nucleocapsid protein were able to detect reference antisera against type 1 with minimal cross reactivity to type 7 and 9.



	H7N3 A/CK/BC/514/2004 Titer=128		LaSota APMV-1 Titer=512	
Dilution	AIV IDEXX <0.5 positive	AIV-NP Luminex >1835 positive	NDV IDEXX >0.2 positive	APMV-1-NC Luminex >2284 positive
Neat	0.102	24404.9	7.669	25296.3
1/2	0.117	22877.4	6.596	24961.8
1/4	0.107	18819.4	4.299	24942.3
1/8	0.124	13177.4	3.530	23502.3
1/16	0.236	9135.9	2.440	23112.3
1/32	0.290	4753.9	1.568	19183.8
1/64	0.430	3159.4	0.882	13708.3
1/128	0.552	2571.9	0.629	10225.3
1/256	0.693	890.4	0.338	4382.3
1/512	0.776	616.4	0.569	2989.3
1/1024	0.870	501.9	0.152	2367.3
1/2048	0.832	355.9	0.058	2106.3

Figure 4. A 12 step 2-fold serial dilutions of reference AI and APMV-1 sera were conducted to determine the analytical sensitivity of the duplex FMIA. The AIV-NP beads were able to detect dilution of antisera at1/128, and APMV-1-NC beads were able to detect down to 1/1024.

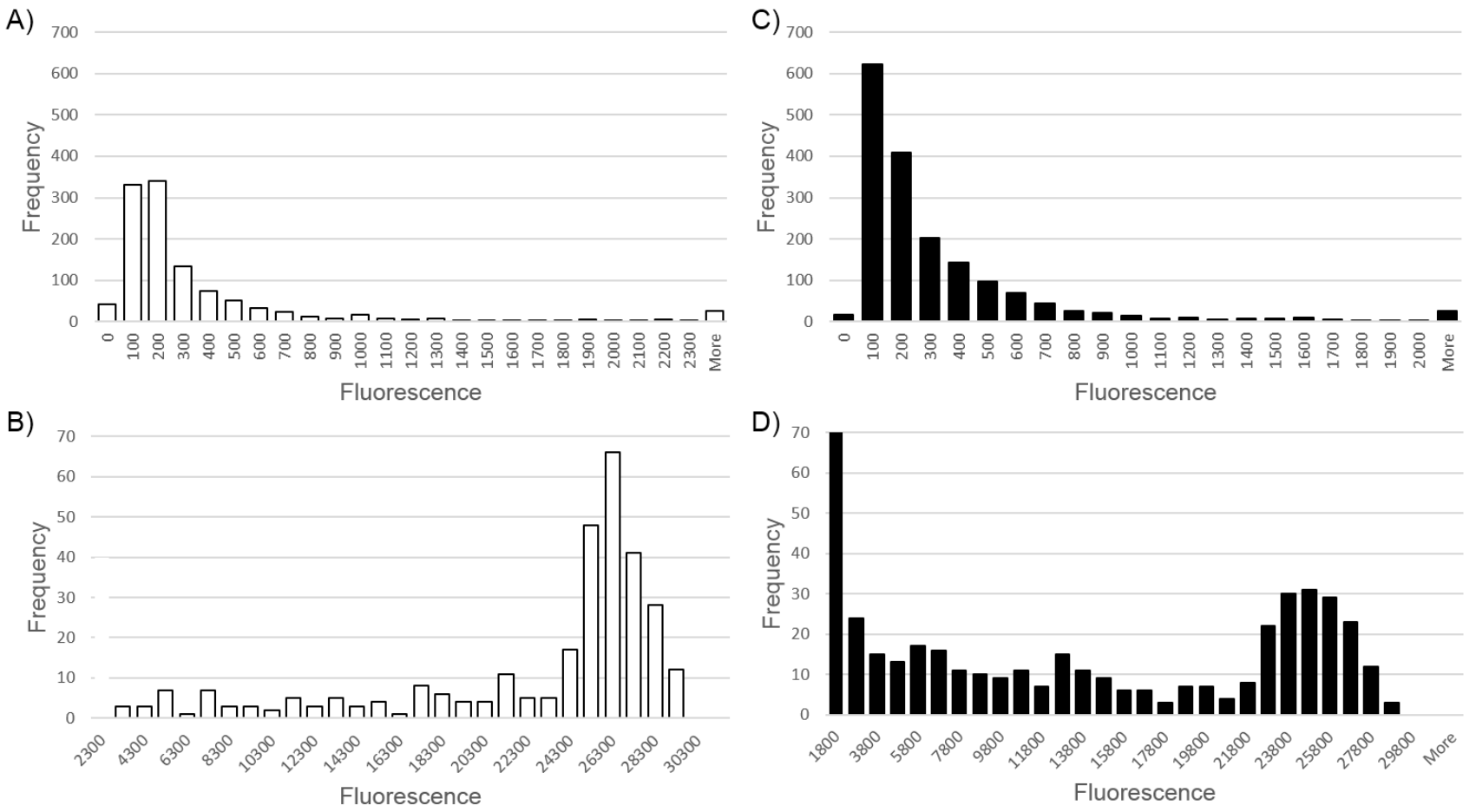
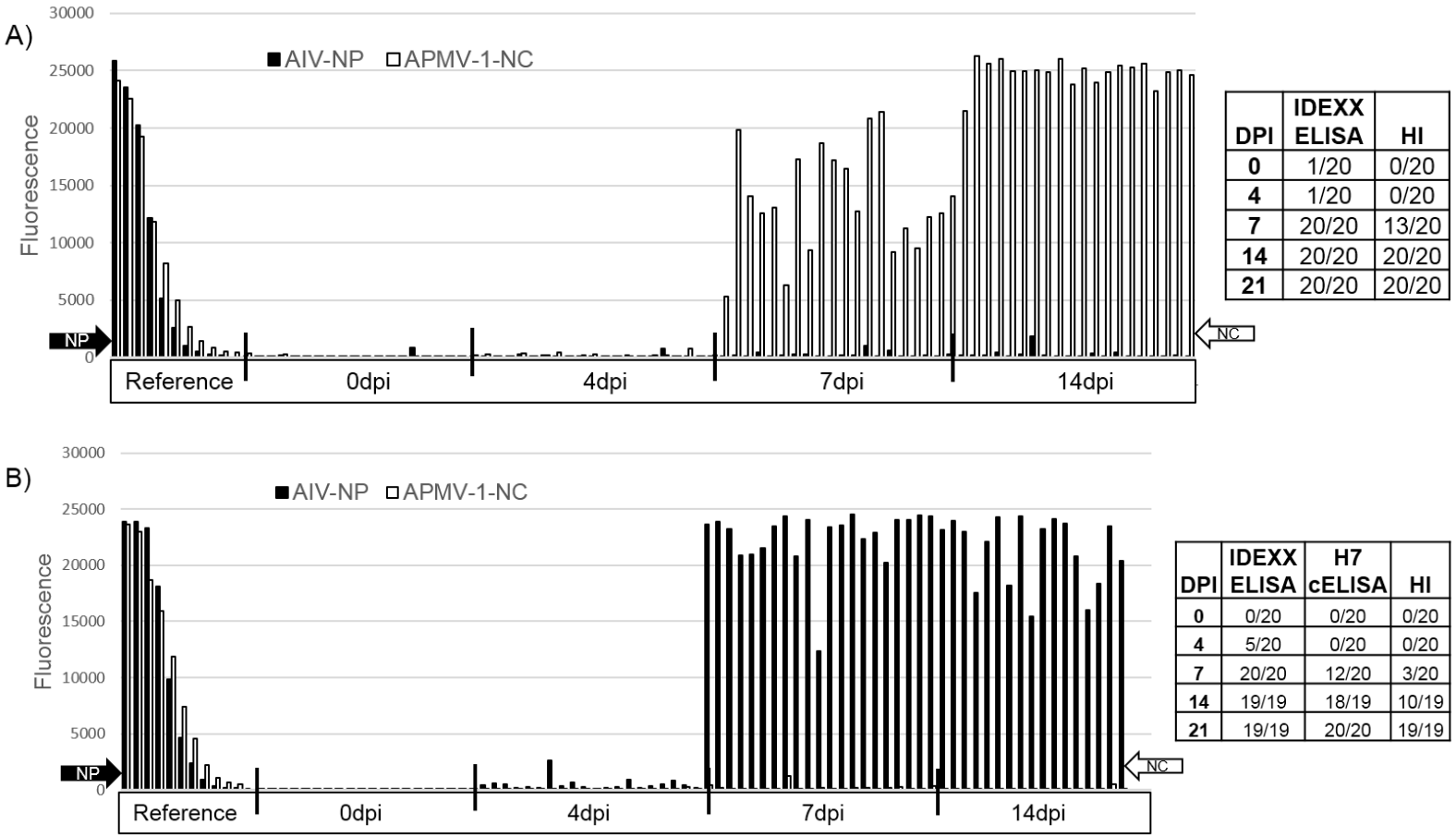


Figure 5. Figures describe the combined frequency distribution of all chicken and turkey serum data for threshold determination, separately analyzing positive and negative samples. A) Avian paramyxovirus 1 negative serum samples; B) Avian paramyxovirus type 1 positive serum samples; C) Avian influenza negative serum samples; D) Avian influenza positive serum samples.

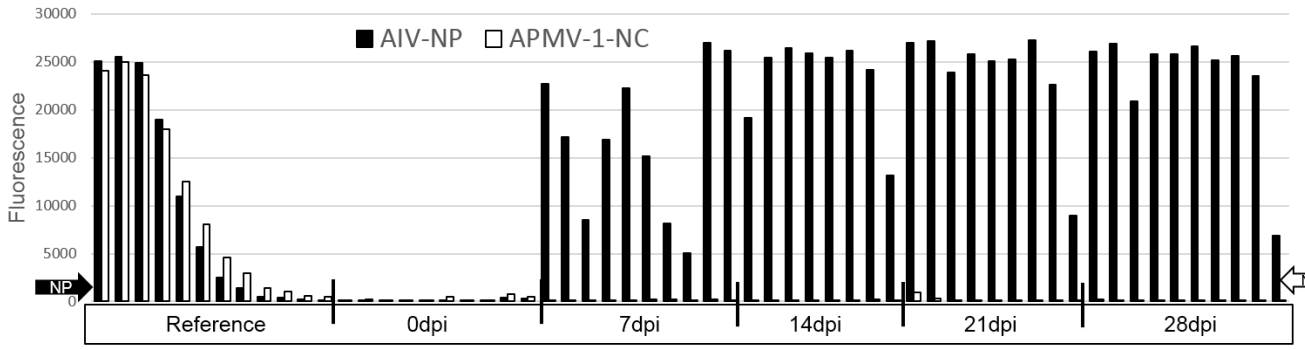


DPI	IDEXX ELISA	HI
0	1/20	0/20
4	1/20	0/20
7	20/20	13/20
14	20/20	20/20
21	20/20	20/20

DPI	IDEXX ELISA	H7 cELISA	HI
0	0/20	0/20	0/20
4	5/20	0/20	0/20
7	20/20	12/20	3/20
14	19/19	18/19	10/19
21	19/19	20/20	19/19

Figure 6. 6A). Kinetics of antibody response in chickens that were immunized with NDV La'Sota vaccine strain using the duplex FMIA, NDV IDEXX ELISA kit (IDEXX Laboratories, Maine, USA) and hemagglutination inhibition assay using homologous La' Sota antigen. According the FMIA data, 100% of chickens were able to sero-convert by 7dpi according to the duplex assay and IDEXX ELISA. 6B) Kinetics of antibody response in chickens that were infected with LPAI A/Anhuii/1/2013 (H7N9) using the duplex FMIA, avian influenza multiscreen ELISA kit (IDEXX Laboratories, Maine, USA) and hemagglutination inhibition assay with homologous antigen. 100% of chickens were able to sero-convert by 7dpi according to the duplex assay and the IDEXX ELISA.

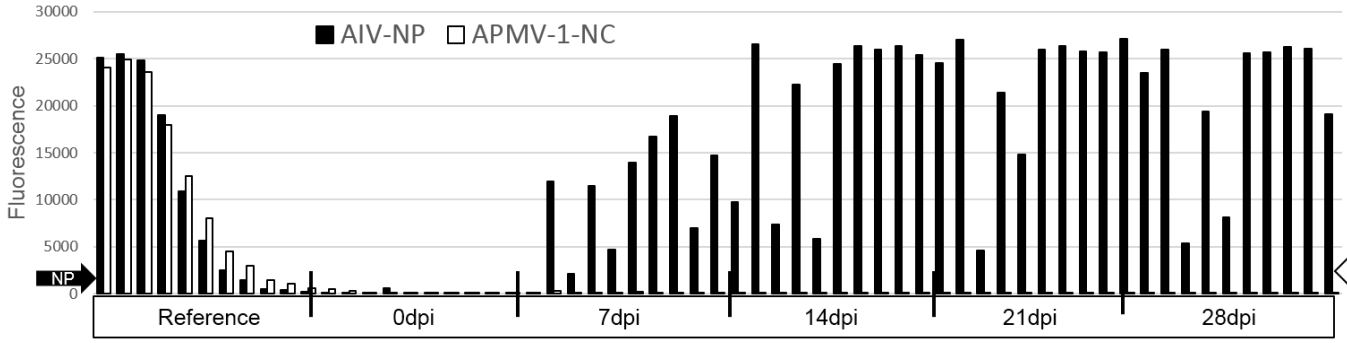
A)



H9N2

DPI	IDEXX ELISA	HI
0	0/10	0/10
7	9/10	0/10
14	10/10	9/10
21	10/10	10/10
28	10/10	10/10

B)



H6N8

DPI	IDEXX ELISA	HI
0	0/10	0/10
7	10/10	0/10
14	10/10	6/10
21	9/10	6/10
28	10/10	3/10

Figure 7. Kinetics of antibody response in turkeys that were infected with either LPAI A/turkey/MN/12877/1285/81 (H9N2) (7A) or A/turkey/ON/1963 (H6N8) (7B) using the duplex FMIA, avian influenza multiscreen ELISA kit (IDEXX Laboratories, Maine, USA) and hemagglutination inhibition assay using corresponding homologous antigens. Almost 100% of turkeys were able to sero-convert by 7dpi according to the duplex assay and the IDEXX ELISA.

3.6 Acknowledgments

The project was funded by CFIA (WIN-A-1304), Manitoba Egg producers and MAFRI (Manitoba Agriculture – Food and Rural Initiative). The authors would like to acknowledge Dr. Kathleen Hooper McGreevy and Animal Health Laboratory (AHL), Guelph, ON for providing the field sera used in the studies. Dr. Kurtis Swelka and his animal care staff for helping out with animal experiments. Dr. Andre Broes (Bio-Vet) for his input in assay development. Dr. Gary Crow for his statistical analysis advice. CSF/Avian staff and Kate Hole for their help in some aspects of the assay validation.

3.7 Manuscript 1 Bibliography

Aldous, E.W., Alexander, D.J., 2001. Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). Avian Pathol. 30, 117-128.

Alexander, D.J., 1988. Newcastle disease virus - An Avian Paramyxovirus. In D.J. Alexander (ed.). Newcastle Disease. Kluwer Academic Publishers: Boston, MA 11-22.

Alexander, D.J., Chettle, N.J., 1978. Relationship of parakeet/Netherlands/449/75 virus to other avian paramyxoviruses. Res Vet Sci. 25(1):105-106.

Alexander, D.J., Collins, M.S., 1981. The structural polypeptides of avian paramyxoviruses. Arch. Virol. 67(4):309-323.

Alexander, D.J., Senne, D.A., 2003. Newcastle disease, other avian paramyxoviruses and pneumovirus infections: Newcastle disease, other avian paramyxoviruses. In: Saif, Y.M. (ed) Diseases of Poultry, 7th edn. Iowa State University Press, Ames, 63-92.

Anderson, S., Wakeley, P., Wibberley, G., Webster, K., Sawyer, J., 2011. Development and evaluation of a Luminex multiplex serology assay to detect antibodies to bovine herpes virus 1, parainfluenza 3 virus, bovine viral diarrhoea virus, and bovine respiratory syncytial virus, with comparison to existing ELISA detection methods. J. Immunol. Methods 366, 79-88.

Bergmann, I.E., Neitzert, E., Malirat, V., Ortiz, S., Colling, A., Sanchez, C., Correa Melo, E., 2003. Rapid serological profiling by enzyme-linked immunosorbent assay and its use as an

epidemiological indicator of foot-and-mouth disease viral activity. Arch. Virol. 148, 891-901.

Berhane, Y., Kehler, H., Handel, K., Hisanaga, T., Xu, W., Ojkic, D., Pasick, J., 2012. Molecular and antigenic characterization of reassortant H3N2 viruses from turkeys with a unique constellation of pandemic H1N1 internal genes. PLoS One. 7(3): e32858.

Chen, T., Lee, F., Lin, Y., Pan, C., Shih, C., Lee, M., Tsai, H. 2009. Development of a Luminex assay for the detection of swine antibodies to non-structural proteins of foot-and-mouth disease virus. J. Immunol. Methods .

Chowdhury, F., Williams, A., Johnson, P., 2009. Validation and comparison of two multiplex technologies, Luminex® and Mesoscale Discovery, for human cytokine profiling. J. Immunol. Methods 340, 55-64.

Clavijo, A., Hole, K., Li, M., Collingnon, B. 2006. Simultaneous detection of antibodies to foot-and-mouth disease non-structural proteins 3ABC, 3D, 3A and 3B by a multiplexed Luminex assay to differentiate infected from vaccinated cattle. Vaccine 24, 1693-1704.

Comin, A., Toft, N., Stegeman, A., Klinkenberg, D., Marangon, S., 2013. Serological diagnosis of avian influenza in poultry: is the haemagglutination inhibition test really the 'gold standard'? Influenza Other Respi Viruses 7, 257-264.

Desvaux, S., Garcia, J.M., Nguyen, T.D., Reid, S.A., Bui, N.A., Roger, F., Fenwick, S., Peiris, J.S., Ellis, T., 2012. Evaluation of serological tests for H5N1 avian influenza on field samples

from domestic poultry populations in Vietnam: consequences for surveillance. Vet. Microbiol. 156, 277-284.

Dias, D., Van Doren, J., Schlottmann, S., Kelly, S., Puchalski, D., Ruiz, W., Boerckel, P., Kessler, J., Antonello, J.M., Green, T., Brown, M., Smith, J., Chirmule, N., Barr, E., Jansen, K.U., Esser, M.T., 2005. Optimization and validation of a multiplexed Luminex assay to quantify antibodies to neutralizing epitopes on human papillomaviruses 6, 11, 16, and 18. Clin. Diagn. Lab. Immunol. 12, 959-969.

Drummond, J.E., Shaw, E.E., Antonello, J.M., Green, T., Page, G.J., Motley, C.O., Wilson, K.A., Finnefrock, A.C., Liang, X., Casimiro, D.R., 2008. Design and optimization of a multiplex anti-influenza peptide immunoassay. J. Immunol. Methods 334, 11-20.

Fawcett, T., 2006. An Introduction to ROC Analysis. Pattern Recognition Letters , 861-874.

van Gageldonk, P.G.M., van Schaijk, F.G., van der Klis, F.R., Berbers, G.A.M., 2008. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus. J. Immunol. Methods 335, 79-89.

Giavedoni, L.D., 2005. Simultaneous detection of multiple cytokines and chemokines from nonhuman primates using Luminex technology. J. Immunol. Methods. 301, 89-101.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275-2289.

de Jager, W., Prakken, B.J., Bijlsma, J.W.J., Kuis, W., Rijkers, G.T., 2005. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. J. Immunol. Methods 300, 124-135.

Kapczynski, D.R., Afonso, C.L., Miller, P.J., 2013. Immune responses of poultry to Newcastle disease virus. Dev. Comp. Immunol. 41,3, 447-53.

Kessler, N., Aymard, M., Calvet, A., 1979. Study of a new strain of paramyxoviruses isolated from wild ducks: antigenic and biological properties. J. Gen. Virol. 43, 273-282.

Leijon, M., Ullman, K., Thyselius, S., Zohari, S., Pedersen, J.C., Hanna, A., Mahmood, S., Banks, J., Slomka, M.J., Belak, S., 2011. Rapid PCR-based molecular pathotyping of H5 and H7 avian influenza viruses. J. Clin. Microbiol. 49, 3860-3873.

Lipkind, M., Weisman, Y., Shihmanter, E., Shoham, D., 1982. Isolation of yucaipa-like avian paramyxovirus from a wild mallard duck (Anas platyrhinchos) wintering in Israel. The Veterinary Record. 110,1,15-16.

Lupiani, B., Mozisek, B., Mason, P.W., Lamichhane, C., Reddy, S.M., 2010. Simultaneous detection of avian influenza virus NP and H5 antibodies in chicken sera using a fluorescence microsphere immunoassay. Avian Dis. 54, 668-672.

Martins, T.B., 2002. Development of internal controls for the Luminex instrument as part of a multiplex seven-analyte viral respiratory antibody profile. Clin. Diagn. Lab. Immunol. 9, 41-45.

Mutinelli, F., Capua, I., Terregino, C., Cattoli, G., 2003. Clinical, gross, and microscopic findings in different avian species naturally infected during the H7N1 low- and high-pathogenicity avian influenza epidemics in Italy during 1999 and 2000. Avian Dis. 47, 844-848.

Pasick, J., Pedersen, J., Hernandez, M.S., 2012. Avian influenza in North America, 2009-2011. Avian Dis. 56, 845-848.

Pawar, S.D., Kale, S.D., Rawankar, A.S., Koratkar, S.S., Raut, C.G., Pande, S.A., Mullick, J., Mishra, A.C., 2012. Avian influenza surveillance reveals presence of low pathogenic avian influenza viruses in poultry during 2009-2011 in the West Bengal State, India. Virol. J. 9, 151-422X-9-151.

Seal, B.S., Crawford, J.M., Sellers, H.S., Locke, D.P., King, D.J., 2002. Nucleotide sequence analysis of the Newcastle disease virus nucleocapsid protein gene and phylogenetic relationships among the Paramyxoviridae. Virus Res. 83, 119-129.

Seideman, J., Peritt, D., 2002. A novel monoclonal antibody screening method using the Luminex-100™ microsphere system. J. Immunol. Methods 267, 165-171.

Shortridge, K.F., Alexander, D.J., Collins, M.S., 1980. Isolation and properties of viruses from poultry in Hong Kong which represent a new (sixth) distinct group of avian paramyxoviruses. Research in Veterinary Science, 255-262.

Snyder, D.B., Marquardt, W.W., Yancey, F.S., Savage, P.K., 1985. An enzyme-linked immunosorbent assay for the detection of antibody against avian influenza virus. Avian Dis. 29, 136-144.

Toffan, A., Olivier, A., Mancin, M., Tuttoilmondo, V., Facco, D., Capua, I., Terregino, C., 2010. Evaluation of different serological tests for the detection of antibodies against highly pathogenic avian influenza in experimentally infected ostriches (Struthio camelus). Avian Pathol. 39, 11-15.

Tumova, B., Robinson, J.H., Easterday, B.C., 1979. A hitherto unreported paramyxovirus of turkeys. Research in Veterinary Science, 135-140.

Upadhyay, C., Ammayappan, A., Vakharia, V.N., 2009. Detection of NP, N3 and N7 antibodies to avian influenza virus by indirect ELISA using yeast-expressed antigens. Virol. J. 6, 158-422X-6-158.

Wang, L., Pan, C., Severinghaus, L.L., Liu, L., Chen, C., Pu, C., En, Huang, D., Lir, J., Chin, S., Chen, M., Lee, S., Want, C., 2008. Simultaneous detection and differentiation of Newcastle disease and avian influenza viruses using oligonucleotide microarrays. Veterinary microbiology 127, 217-226.

Watson, D.S., Reddy, S.M., Brahmakshatriya, V., Lupiani, B., 2009. A multiplexed immunoassay for detection of antibodies against avian influenza virus. J. Immunol. Methods 340, 123-131.

World Organization for Animal Health (OIE), 2008. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth Edition ed. OIE, Paris, France.

Yang, M., Berhane, Y., Salo, T., Li, M., Hole, K., Clavijo, A. 2008. Development and application of monoclonal antibodies against avian influenza virus nucleoprotein. J.Virol.Methods. 147, 2, 265-274.

Zhou, E.M., Chan, M., Heckert, R.A., Riva, J., Cantin, M.F., 1998. Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein. Avian Dis. 42, 517-522.

4.0 Manuscript 2

Multiplex Fluorescent Microsphere Immunoassay (FMIA) for Simultaneous Detection of Antibody Responses to Avian Influenza Nucleoprotein, H5, and H7 and Newcastle Disease Virus Nucleocapsid Protein

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4.1 Abstract

Highly pathogenic avian influenza virus (HPAI) and virulent forms of avian paramyxovirus-1 (APMV-1) cause serious illnesses in domestic poultry, both of which are reportable to the World Organization of Animal Health (OIE). The clinical presentation of avian influenza (AI) and APMV-1 infections are difficult to differentiate, emphasizing the importance of rapid and sensitive serologic assays that are able to distinguish them. Currently, a variety of serological assays are used for the serologic diagnosis of both diseases, but these assays are not used in multiplex formats. In this study, development of a quadriplex fluorescent microsphere immunoassay (FMIA) based on xMAP Luminex technology is described. The assay employs MagPlex magnetic microspheres that are covalently coated with recombinant avian influenza virus nucleoprotein, APMV-1 nucleocapsid, influenza H5 and H7 hemagglutinin proteins, produced in a baculovirus insect cell expression system. The assay is able to detect AIV antibodies against all existing hemagglutinin (H1-H16) subtypes, while subtyping the virus to H5 or H7 subtypes, and simultaneously detecting antibodies against APMV-1. In the process of this assay development the previously published duplex assay was expanded to include the new H5 and H7 beads. The assay has the capability of detecting serum antibodies from chickens and turkeys and optimization was accomplished by using 2,694 chicken and turkey samples accumulated from field and experimental sources and had a comparable detection capability with currently used assays in the laboratory. Assay threshold values were calculated with Receiver Operating Characteristic Analysis (ROC) and favoured values with high ROC-accuracy levels to maximize the assay specificity. The AIV-NP bead threshold value has a fluorescent value of 1253, and associated sensitivity, specificity, and ROC accuracy of 98.4%, 98.1%, and

98.2% respectively. The APMV-1-NC bead threshold value has a fluorescent value of 3595, and associated sensitivity, specificity, and ROC accuracy of 90.1%, 99.2%, and 97.6% respectively. The AIV-H5 bead threshold value has a fluorescent value of 1585, and associated sensitivity, specificity, and ROC accuracy of 92.7%, 99.4%, and 99.1% respectively. The AIV-H7 bead threshold value has a fluorescent value of 2585, and associated sensitivity, specificity, and ROC accuracy of 71.7%, 98.9%, and 97.2% respectively.

4.2 Introduction

The importance of having reliable assays for serologic monitoring and surveillance of domestic poultry disease cannot be understated. Despite available vaccines and prevention strategies currently in place, highly pathogenic avian influenza virus (HPAI) and Newcastle Disease (ND) continue to be two of the most economically damaging poultry diseases worldwide (Bahl *et al.* 1977; Berhane *et al.* 2014; Kapczynski *et al.* 2013).

Diseases caused by avian influenza viruses (AIV) belonging to H5 and H7 subtypes are reportable to the OIE and have caused outbreaks in Asia, North and Central America, the Middle East, and Australia already this year (OIE Disease report 2014). Wild migratory waterfowl act as the primary reservoir for AIV and shed large amounts of the virus into the environment. Virus spread to domestic poultry usually occurs as a result of contact with wild birds, fomites, or feeding and drinking from AIV contaminated water supplies (Claes *et al.* 2012). Monitoring of AIV is also important to human health due to the emergence of zoonotic viruses such as the Eurasian HPAI H5N1 virus and quite recently the H7N9 infections in China (Yu *et al.* 2014; Stallknecht 2007; OIE 2008; Pasick *et al.* 2005; Zhu *et al.* 2013). Low pathogenic strains (LPAI) are normally carried by wild birds and passed into domestic poultry. High pathogenic strains (HPAI) of AIV usually arise as the result of mutations after adapting in domestic poultry flocks, wild birds are not known to harbor HPAI viruses (Duan *et al.* 2007; Campitelli *et al.* 2004). HPAI is caused by quite a few viruses belonging to the H5 and H7 subtypes and these viruses contain a polybasic cleavage site in the hemagglutinin (HA) protein that allows them to replicate in a variety of tissues causing systemic infections (Capua and Alexander 2007; Lu *et al.* 2014). The external domains of the HA proteins are the major targets for host neutralizing antibodies along with the nucleoprotein, and are the foundation of how viruses are subtyped. The HA proteins are significantly different from one another and antibodies targeting one subtype will not react with other subtypes. The HA protein forms

the 4th segment of the viral genome, and aside from the neuraminidase, all other gene segments are responsible for producing the internal virus proteins (Lu *et al.* 2014).

Outbreaks of ND are caused by virulent viruses belonging to Avian Paramyxovirus serotype 1 (APMV-1) and despite extensive use of vaccines to control the disease, outbreaks are still occurring (Kapczynski *et al.* 2013). ND vaccines have been in use since before 1975, and seem to confer protection against homologous and heterologous ND viruses (Kaliannan *et al.* 1975). The most recent outbreaks in poultry have been attributed to contact with wild bird species (ducks, geese) as domestic birds like chickens and turkeys are not traditionally seen as the reservoir hosts for this virus (Spalatin and Hanson 1975). Newcastle disease can be caused by lentogenic strains -that usually cause asymptotic to mild clinical disease; mesogenic – causing respiratory disease but not usually fatal; and velogenic – most pathogenic and often fatal (Seal S. 2002; Aldous *et al.* 2001; Kapczynski *et al.* 2013). APMV-1 viruses are classified into two classes; class one viruses belong to a single genotype and class two contains 15 genotypes (genotype I to XV) (Diel *et al.* 2012). Despite this, the nucleocapsid protein is one of the most conserved genes among APMV-1 strains and is also immunogenic (Kapczynski *et al.* 2013).

Worldwide surveillance of wild birds and domestic poultry flocks are carried out on a large scale using a variety of molecular and serological methods (Molesti *et al.* 2013). Very popular methods of bird surveillance include Real-Time PCR (RT-PCR), but depend on whether the avian host is actually excreting the virus, and the time window when shedding occurs is very limited (Brown *et al.* 2010). After the cessation of shedding, the only way of determining that the birds have been exposed is using serological methods. Agar gel immunodiffusion (AGID) and Hemagglutination Inhibition Assays (HI) are currently viewed as the gold standard detection methods for serological surveillance of AIV infections, with Enzyme-Linked Immunoassays (ELISA) also being a common method (Claes *et al.* 2012; Swayne and Spackman 2013). HI and virus neutralization tests (VNT) have been primary choices for detecting NDV for some time (Spalatin and Hanson 1975). An ideal system would be a single consistent test that is

easily compared to other methods and capable of testing all sera samples to differentiate between AIV and NDV infections while simultaneously pathotyping antibody response to AIV infection to H5 or H7 subtype. One of the major problems with traditional serological testing is that it lacks the high sensitivity currently exhibited by the molecular RT-PCR testing. Although this is generally true for HI and some ELISAs, the new Quadriplex Fluorescent Microsphere-Immunoassay (Q-FMIA) in this development is proven to have high sensitivity which possibly surpasses that of the HI test, while maintaining the high specificity the HI is known for, resulting in a very effective method of surveillance (Cattoli and Capua 2007; Stallknecht *et al.* 2007).

The Q-FMIA is able to function with both chicken and turkey sera effectively due to antigenic relationships between the turkey and chicken IgY (Bencina *et al.* 2014; Choi *et al.* 2010; Hadge and Ambrosius 1986), and is effective with field sera as well as experimentally infected samples. Proper methods of assay validation are important and the power of the results are directly linked to the number of serum samples tested, as well as the overall variety of the samples (OIE 2008). Greater numbers of samples increase the power of the test because it portrays a more accurate representation of the population (Kistler *et al.* 2012). This study used 2,700 total samples from a wide variety of chickens and turkeys ranging from Specific Pathogen Free (SPF) birds, to field sample submission samples.

There has been testing of alternative surveillance methods in an attempt to replace the existing gold standards, but so far nothing has out-performed HI or ELISA tests (Nielsen *et al.* 2007). Despite these current methods being widely used and trusted, there are often discrepancies between the results making it difficult to compare results and come to a conclusion about the positive/negative nature of antibodies in the sample (Perez-Ramirez *et al.* 2010). A good surveillance test uses less serum and produces results faster than other methods, while limiting cross-reactivity and being able to detect new antibodies as soon as they become a high enough concentration within the serum. It is also important

to be able to detect all of the current subtypes and strains of the virus. The main issue with developing and optimizing a serological assay is determining a threshold value that limits false negatives and false positives to maintain a high sensitivity and specificity with a wide variety of samples. Other problems arise due to species differences, and non-specific inhibitors which may be more prevalent in field sera than experimental sera (Perez-Ramirez *et al.* 2010).

The current study describes the development of a Q-FMIA based on the XMAP technology for simultaneous detection and differentiation of antibody response to AIV and NDV infections while sub-typing the AIV infection to H5 or H7, and optimizing the operation of two internal controls to maintain assay performance. The Quadriplex assay is based on the AIV nucleoprotein (AIV-NP), H5 hemagglutinin protein (H5-AIV), H7 hemagglutinin protein (H7-AIV), and NDV nucleocapsid protein (NDV-NC).

4.3 Materials and Methods

4.3.1 Cloning of AIV Nucleoprotein, H5, H7 Hemagglutinin Proteins, and APMV-1 Nucleocapsid Protein

The avian influenza nucleoprotein, and APMV-1 nucleocapsid cloning process was carried out in the same way as previously described (Pinette *et al.* 2014), while continuing to use the influenza A/Duck/AB/AFLB-C16/2008 (H7N7) for AIV-NP, and APMV-1 vaccine (La'Sota) for APMV-1-NC recombinant proteins.

The H5 hemagglutinin protein from A/Turkey/ON was amplified using forward primer: CACGCGGCCGCAATCAGCGTTGTCAAAGGTG and reverse primer: CGGGATCCTTAACACTCTCCAATGGTAAGGGG. The amplified H5 product was digested by Not I and Bam HI (Promega, Wisconsin, USA) and then cloned into PCR pB3H cloning vector (Qiagen, Maryland, USA). The product was then sub-cloned into pAB B3H transfer vector which included a 3X his tag, and the correct frame and sequence were confirmed by sequencing.

The H7 hemagglutinin protein sequence from was highlighted and chosen to be used as recombinant protein and was ordered from Genescript (Genescript, New Jersey, USA). The protein sequence was delivered inside the pAB-bee-FH transfer vector to be easily transfected into insect cells using baculovirus.

4.3.2 Transfection and Generation of Recombinant Baculovirus

All four of the recombinant proteins were co-transfected into *Spodofera frugiperda* (Sf-9) cells, with ProFold-ER1 baculovirus DNA (AB Vector, California, USA). The ProFold DNA contained *Aequorea Victoria* green fluorescent protein (GFP) which allowed monitoring the progress of the Baculovirus infection. The protein was plaque purified, optimized, and produced in large batches in Hi5 cells according to manufacturer's protocols (AB Vector, California, USA). The cell pellet and media fractions

of recombinant proteins were purified from the Baculovirus protein expression system using Ni-NTA agarose (Qiagen, Maryland, USA) according to manufacturer's instructions. The AIV-NP, H5, and H7 proteins from the media fractions, and APMV-1-NC from the cell pellet fractions were chosen as best suited for use in the assay bead coupling.

4.3.3 Coupling Proteins to MagPlex Luminex Magnetic Beads

Prior to coupling, recombinant proteins are desalted according to manufacturer's instructions using micro-biospin 6 columns (BioRad, California, USA) and the buffer is exchanged to Phosphate Buffered Saline (PBS) prevent the Imidazole salts from interfering with the coupling reaction, and then quantified using Pierce BCA protein Quantification Kit (Thermo Scientific, Maryland, USA). The proteins used in the study are coupled to the MagPlex Luminex beads according to the manufacturer's instructions (Luminex Corporation, Texas, USA). Bead coupling involved activation of the beads with activation buffer (100mM monobasic sodium phosphate pH 6.2), as well as N-hydrosulfosuccinate (Sulfo-NHS), and 1-ethyl-3-3-dimethylaminopropyl carbodiimide (EDC) which prepares the surface of the magnetic beads for covalent protein coating (Luminex Corporation, Texas, USA).

Recombinant AIV-NP protein was coupled to MagPlex region #15, H5 protein was coupled to MagPlex region #13, H7 protein was coupled to MagPlex region #14, APMV-1-NC protein was coupled to region #12. Twelve micrograms of each recombinant protein were coupled to a fixed amount of beads (1.25×10^6) per 1X reaction. Different doses of each protein were tested previously to produce coupled beads with the maximum signal to noise ratio (Pinette *et al.* 2014).

Commercially purified proteins were used as internal controls for the assay including: chicken IgY protein (Jackson Immuno Research Inc., Pennsylvania, USA) coupled to MagPlex region #18, and donkey anti chicken IgY protein (Jackson Immuno Research Inc., Pennsylvania, USA) coupled to MagPlex region #19. Concentrations of 0.1ug/ml of the commercially produced proteins were used in each 1X

coupling reaction and used for verifying the addition of secondary detection antibody and test serum respectively.

4.3.4 Quadriplex FMIA (Q-FMIA)

The protein-coupled beads (AIV-NP, AIV-H5, AIV-H7, APMV-1-NC, CK IgY, DKαCK IgY) were combined at concentration of 2000 beads/well and blocked for 2 hours in 5% (v/v) normal donkey serum (Jackson ImmunoResearch Laboratories Inc., Pennsylvania, USA) in PBS. Twenty five microliters of bead suspension and 25 µl of test serum diluted 1:500 in 1% (v/v) normal donkey serum in PBS were added to each well. The plate was left at room temperature in the dark on a shaking incubator for 1 hour. A wash program consisting of 3 washes with 200µl of PBS-tween 20 in magnetic plate washer was completed (Bio-Tek, 405 Select TS), and the plates were incubated with 50 µl of 2 µg/ml biotinylated donkey anti-chicken immunoglobulin (Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA) for 30 min at room temperature. Plates were washed again before the final addition of 75 µl of 10 mg/ml streptavidin-phycoerythrin conjugate (Jackson ImmunoResearch Laboratories Inc., Pennsylvania, USA) and incubation at room temperature for 15 minutes. Following the final wash, each well of beads were re-suspended in 125 µl of wash buffer and analyzed with a BioPlex 100 instrument running Bioplex Manager 6.1 software (BioRad, California, USA). The results were expressed as arbitrary units of median fluorescence intensity (MFI).

4.3.4.1 Luminex Reference Curve

The reference curve previously described for use with the duplex assay (Pinette *et al.* 2014) was modified to include H5 and H7 hyper-immune sera of known titers to enable monitoring all of the beads within the assay. The curve was developed to monitor variability between runs of the assay and also a basis for calculating the S/P value, and alternatively generating a correction factor to apply to all data before determining the assay positive/negative threshold. The new serum included in the 12-step serial

dilution curve: 100ul A/CK/Vietnam AS 35dpi CK276 06-01-11 (H5N1), 100ul A/TY/MN/29200/83 39 dpi 03-12-18 (H7N9), 100ul NDV LaSota 1CE 99-12-01 20dpi 08-09-05, 150ul H5 CK DNA vaccine trial #99 21dpi 10-04-30, 50ul Neg CK Serum – Sigma heat inactivated 10-10-06 were all diluted 2-fold in negative chicken serum. The samples were previously tested for the presence of antibodies using IDEXX ELISA kits as well as hemagglutination inhibition assays.

4.3.5 Hemagglutination Inhibition Assay

The hemagglutination inhibition (HI) assay was performed according to the methods described in the OIE manual (OIE 2008). For testing the serial bleeds from experimentally inoculated chickens, 4 HA units of homologous virus was combined with 2-fold serial dilutions of each serum sample. Chicken red blood cells (0.5% v/v suspension) were then added and HI endpoints read. Serum HI titre > 1:4 was considered positive (Pinette *et al.* 2014).

4.3.6 IDEXX ELISA

Commercial IDEXX AIV and APMV-1 ELISA kits were used to determine the presence of AIV or APMV-1 antibodies in poultry sera (IDEXX Laboratories, Maine, USA). The competitive MultiS-Screen ELISA kit was used for AIV antibody detection in turkey and chicken sera. Both of these assays were performed according to the manufacturer's protocols. The plates were read at 650nm with Spectra Max plus microplate reader (BioTek Instruments Inc., Vermont, USA) using Softmax software (BioTek Instruments, Vermont, USA).

4.3.7 Competitive H5 Enzyme-linked Immunosorbent Assay (H5-cELISA)

The H5 cELISA involved 96-well micro-titer plates which were coated with 10 µg/ml of recombinant Baculovirus H5 protein (diluted 1:1000 in carbonate buffer (pH 9.6)) at 100ul/well and plates were incubated at 4°C overnight. Once washed, serum was diluted 1:2.5 in PBS-T and 50µl added to all wells. Immediately following, H5 monoclonal antibody 1:200 in PBS-T and 50µl was added to all

wells and plates were incubated at 37°C for 1 hour with shaking. Q1 was Positive control H5N1 anti-serum A/CK/Vietnam/14/05 diluted 1:2.5 in PBS-T. Q2 was 1:30 of Q1 in negative CK serum, then 1:2.5 in PBS-T. Q3 was 1:2.5 negative CK serum (Sigma, C4505) in PBS-T; 50µl was added of standard samples to the control wells. Plates washed after incubation, then the addition of 100ul/well of 1:2000 HRP conjugated goat anti-mouse IgG (Jackson Immuno Research Inc., Pennsylvania, USA) in PBS-T was added and incubated for 1 hour at 37°C. After the final wash, 100ul/well of the enzyme substrate 3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMB, Sigma-Aldrich, Missouri, USA) was added to each well and developed on shaker for 15 minutes at room temperature. Once developed, the reaction was stopped with 50ul/well of 2.0M sulphuric acid. The optical density was determined at 450nm with a Spectro Max plus plate reader (Molecular Devices, California, USA).

4.3.8 Competitive H7 Enzyme-linked Immunosorbent Assay (H7-cELISA)

The AIV-NP Competitive ELISA (cELISA) was performed as described by Ming *et al.* 2008, while the H7 cELISA was performed in the following way. The H7 cELISA involved 96-well micro-titer plates which were coated with 0.65mg/ml of recombinant Baculovirus H7 protein diluted 1:1500 in carbonate buffer (pH 9.6) at 100ul/well and plates were incubated at 4°C overnight. Once washed, serum was diluted 1:10 in 3% Fetal Bovine Serum (FBS) in PBS-T and 50µl added to all wells. Immediately following, H7 monoclonal antibody 1:1500 in 3% FBS in PBS-T and 50µl was added to all wells and plates were incubated at 37°C for 1 hour with shaking. Q1 was 1:10 Positive control H7N1 A/TY/ON/10-2/2000 28/29 pooled 03/08/21 in 3% FBS + PBS-T. Q2 was 1:40 Q1 into Q3 in 3% FBS + PBS-T. Q3 was 1:10 negative CK serum in 3% FBS + PBS-T; 50µl was added of standard samples to the control wells. Next, 100ul/well of 1:2000 HRP conjugated goat anti-mouse IgG (Jackson Immuno Research Inc., Pennsylvania, USA) in PBS-T was added and incubated for 1 hour at 37°C. After the final wash, 95ul/well of the enzyme substrate 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma-Aldrich, Missouri, USA) was added to

each well and developed on shaker for 15 minutes at room temperature. Once developed, the reaction was stopped with 50ul/well of 2.0M sulphuric acid. The optical density was determined at 450nm with a Spectro Max plus plate reader (Molecular Devices, California, USA).

4.3.9 Serum Samples

A common set of 2,700 unique serum samples made up of positives and negatives were used for the testing of all protein-coated beads including: Specific Pathogen Free Chickens (SPF) from Ottawa Laboratory Fallowfield (OLF), experimentally infected and vaccinated chickens and turkeys, as well as positive and negative chicken and turkey field serum. All sera were pre-screened with HI, ELISA, and/or AGID, for detectable levels of antibody.

4.3.9.1 Reference AIV, APMV-1, AIV-H5, AIV-H7 Antisera

A panel of reference sera for all 16 hemagglutinin subtypes of AIV, and all nine subtypes of APMV were assembled to assess cross-reactivity of the beads (Table 2). These sera were produced by inoculating six-week old white leghorn SPF chickens acquired from the animal care unit of the OLF, Canadian Food Inspection Agency (CFIA). The OLF SPF stock was developed from Charles River/SPAFAS birds (Storrs, Connecticut, USA). The panel also contained several sera samples from the Guelph Animal Health Laboratory (AHL) with infectious bursal disease antibodies, as well as duck and goose sera.

4.3.9.2 Field Serum Samples

Field serum used in the study was obtained from a variety of sources including: broilers, turkey and chicken breeder flocks; some of which were vaccinated for APMV-1 using a combination of live and inactivated APMV-1 vaccines. Many field sera were donated by the Animal Health Laboratory (AHL), Guelph, Ontario, and many were also from the archived serum database of NCFAD. Other turkey sera were from birds vaccinated with inactivated H3N4 AIV vaccine (Berhane *et al.* 2012), and chickens vaccinated for APMV-1 using combination of live and inactivated APMV-1 vaccines.

4.3.9.3 Experimental Serum Samples

- The study also included experimental chickens inoculated with recombinant H5N3, vaccinated with H7N3 then exposed to H5N1.

- Chicken and Turkey samples were also taken from the CRTI study with various H5 and H7 strains of AIV (Table 4). Experimental sera were also taken from an H5 DNA vaccine study.

During production of experimental sera, all animal care and handling procedures were done according to the Canadian Council on Animal Care guidelines as outlined in protocols approved by National Center for Foreign Animal Disease (NCFAD).

4.3.10 Data Analysis

Statistical analysis was done using the Microsoft Excel add-on "XLstat – LIFE" (Addinsoft 2014). The distribution of the data was visualized with frequency versus signal histograms, and the Receiver Operating Characteristic Analysis (ROC) was used to determine accurate threshold values for the assay.

4.4 Results

4.4.1 Recombinant Protein Production and Purification

In our previous work, we demonstrated the expression and purification of recombinant AIV-NP and APMV-1-NC using the Baculovirus insect expression system (Pinette *et al.* 2014). Similarly, the H5 and H7 genes were cloned into pAB-bee-FH transfer vectors and co-transfected with linearized ProFold™-ER1 baculovirus genomic vector into SF9 cells. The progression of the infection and evidence of recombinant protein production was monitored by the increasing amount of Green Fluorescent Protein (GFP) intensity in SF-9 cells infected with recombinant Baculoviruses encoding the H5 and H7 proteins. The cells were harvested when greater than 70% of cells expressed the GFP. The relative

quantities of protein expression in the media and cell pellets were visualized on a precast 10% Bis-Tris protein denaturing gel (Invitrogen, Ontario, Canada) followed by Coomassie blue staining and Western blotting. Depending on these results, media, cell pellet, or both will be purified using HIS-tag agarose resin purification of the recombinant protein products. After which, assessment of the purity, quantity, and quality of the purified protein products were assessed by SDS-PAGE followed by Coomassie Blue staining and His-tag Western blot analysis (Figure.8).

4.4.2 Assay Threshold and Overall Accuracy Determination

The assay thresholds differentiating negative sera from positive were calculated using Receiver Operating Characteristic Analysis (ROC) which is routinely used for luminex assay threshold determination because non-normally distributed data sets are common. A set of chicken and turkey field sera consisting of 1,709 unique samples were used for threshold determination according to the OIE assay validation method (OIE 2008). ROC analysis can be used to determine a value of overall accuracy which is a combination of the sensitivity and specificity associated with that threshold value. In simple terms, the accuracy is the probability that the associated threshold value will determine that a randomly chosen positive sample will test higher than a randomly chosen negative sample; and is a number representing how effectively the test has separated positives from negatives (Fawcett 2006). The final threshold values chosen for the assay highlight ROC accuracy, which produces a higher level of specificity to decrease the number of false negative test results.

The AIV-NP bead threshold calculation included all 1,709 field sera (1,307 chicken; 402 turkey), and was calculated to be 1,253 Mean Fluorescent Intensity (MFI) with an analytical sensitivity of 98.4% and analytical specificity of 98.1%. The ROC accuracy associated with this threshold value was 98.2%, and there were 252 true positives, 1,426 true negatives, 27 false positives, and 4 false negatives (Table 2).

The APMV-1-NC bead threshold calculation included 1,690 field sera (1,280 chicken; 410 turkey), and was calculated to be 3,595 MFI with an analytical sensitivity of 90.1% and analytical specificity of 99.2%. The ROC accuracy associated with this threshold value was 97.6%, and there were 273 true positives, 1,360 true negatives, 11 false positives, and 30 false negatives (Table 2).

The AIV-H5 bead threshold calculation included 1,340 field sera (1,148 chicken; 192 turkey), and was calculated to be 1,585 MFI with an analytical sensitivity of 92.7% and analytical specificity of 99.4%. The ROC accuracy associated with this threshold value was 99.1% and there were 51 true positives, 1,277 true negatives, 8 false positives, and 4 false negatives (Table 2).

The AIV-H7 bead threshold calculation included 1,628 field sera (1,288 chicken; 340 turkey), and was calculated to be 2585 MFI with an analytical sensitivity of 71.7% and analytical specificity of 98.9%. The ROC accuracy associated with this threshold value was 97.2% and there were 76 true positives, 1,506 true negatives, 16 false positives, and 30 false negatives (Table 2).

4.4.3 Internal Control Threshold Determination

To confirm the proper addition of secondary antibody into the Q-FMIA assay, Magplex beads coupled with Chicken IgY were used. All data combined from this internal control were found to follow a normal distribution with a mean of 19,691 MFI, with the majority of the 2,706 data points lying within two standard deviations of the mean, and all significant data to be found within 3 standard deviations of the mean. Values from 22,595 to 16,787 MFI outline the acceptable range of the control (Figure 9.).

The second internal control was intended to confirm the proper addition of test serum into the assay, and was Donkey anti-Chicken IgY bound to Magplex beads. After analysis, this control showed an ability to differentiate chicken from turkey sera, and further differentiate between sera from different sources/locations/experiments. As a result, the acceptable range depends on individual groups of sera,

and will be discussed in the discussion, although any sample over 2,000 MFI was considered to have been properly added to the assay (Figure 10 A & B).

4.4.4 Analytical Specificity

Analytical specificity was analyzed using a panel of reference antisera that were developed by infecting SPF leghorn chickens with AIV of subtypes H1 to H16, and all APMV viruses from serotype 1 to 9 (Table 3), to test whether cross-reactivity was occurring between specific beads (Figure 11.). The AIV-NP reacted strongly with all 16 of the hemagglutinin subtypes and did not cross react with any of the NDV-APMV sera. The APMV-1-NC bead reacted strongly with the APMV-1 La'Sota sera and moderately cross-reacted with APMV-7 and APMV-9 due to high sequence similarity between strains. The AIV-H5 bead reacted strongly with the H5N1 sera and did not show any significant cross-reaction with any other sera. The AIV-H7 bead showed a strong reaction with the H7N3 antisera and did not show any significant cross-reaction with any other sera.

4.4.5 Operating Range of the Q-FMIA

The optimal operating range of the Q-FMIA was determined separately for each bead by diluting hyper-immune sera of known titers two-fold into negative chicken sera. The 12-step dilution series of each sera reached a final dilution of 1/2048 which was not below the detectable range of the assay, and not yet indistinguishable from background signal, but represented a range of values which could be commonly seen as results on the assay, and the threshold values for each bead are displayed for reference (Figure 12). The sera used to determine the optimal range consisted of H7N3 A/CK/BC/514/2004 28dpi HA-256, H5N2 A/DK/BC/26-6 23dpi HA-256, and La'Sota NDV vaccine strain HA-256.

Determining the range of the assay also included analysis of whether the signals generated scaled proportionately with the amount of antibody within the sample. If so, the assay could be used in

the future for quantitation of antibody within test samples. The 12-step dilution curves were log transformed to approximate a linear relationship over the entire range of data. A high R^2 value can prove that values generated by the assay are proportional along the entire range of possible values. The AIV-NP bead tested with H7N3 sera had R^2 value = 0.976, and with H5N2 had R^2 value = 0.979. The APMV-1-NC bead tested with La'Sota sera had an R^2 value = 0.8746. The AIV-H5 bead tested with H5N2 sera had an R^2 value = 0.9885, and the AIV-H7 bead tested with H7N3 sera had an R^2 value = 0.9719 (Figure 13).

4.4.6 Reference Curve

The 12-step reference curve used to monitor assay variability and repeatability was not affected when used in multiplex as previously determined with the duplex AIV-NP and APMV-1-NC assay (Pinette *et al.* 2014). This set of references was repeatedly used on every run of the assay for later analysis of assay repeatability. The Quadriplex reference curves for each analyte were separately analyzed to show the average value and variance of the curves over all 36 runs of the assay (Fig 14 a,b,c,d). The reference curves showed expected levels of variability as the test was performed multiple times by a single technician over the course of six months. Variability was a minimum over the projected threshold range between reference 7 to 10 for all curves, meaning thresholds in this range were less affected by assay variability and remain valid across multiple runs. If antibody quantitation was taking place, normalization would be required to ensure all concentrations were accurate, however since quantitative analysis is not the goal of the assay, as long as limited variability is seen in the region of the threshold values, data normalization is not required.

4.4.7 Diagnostic Assay Performance and Kinetics with Experimental Sera

Assay diagnostic performance was tested with 985 sera (714 chickens; 271 turkeys) from a variety of experimentally infected birds. The sera were sourced from different experimental studies that

were conducted at NCFAD and included: Different NDV vaccination and challenge trials, efficacy of H5 DNA vaccine testing, and sera collected from different pathogenesis studies involving avian influenza viruses of H5 and H7 strains (Table 4). Using these experimental serum samples, the kinetics of antibody response and the estimated presence or absence of antigen in the samples of the assay was determined as a percentage representing correctly identified positives and negatives based on previous HI, AGID, IDEXX, and cELISA testing of the experimental sera (Table 5).

The performance of the AIV-NP bead relative to the IDEXX ELISA, from 868 total experimental sera made up of 636 chicken sera and 232 turkey sera, the sensitivity was 95.2% and specificity 95.0% with an overall ROC accuracy of 95.0%. Relative to the AGID, from 322 total experimental sera consisting of 100 chicken sera and 222 turkey sera, the sensitivity was 99.5% and specificity 91.9% with an overall ROC accuracy of 97.8%.

The performance of the APMV-1-NC bead relative to the IDEXX ELISA, from 827 total experimental sera made up of 596 chicken sera and 231 turkey sera, the sensitivity was 71.7% and specificity 98.8% with an overall ROC accuracy of 97.1%.

The performance of the AIV-H5 bead relative to the H5 HI, from 627 total experimental sera made up of 397 chicken sera and 230 turkey sera, the sensitivity was 70.5% and specificity 94.3% with an overall ROC accuracy of 89.0%. Relative to the H5 cELISA, from 377 total experimental sera consisting of 377 chicken sera and 0 turkey sera, the sensitivity was 69.7% and specificity 99.5% with an overall ROC accuracy of 87.3%.

The performance of the AIV-H7 bead relative to the H7 HI, from 322 total experimental sera made up of 89 chicken sera and 233 turkey sera, the sensitivity was 96.0% and specificity 93.5% with an overall ROC accuracy of 94.1%. Relative to the H7 cELISA, from 383 total experimental sera consisting of

383 chicken sera and 0 turkey sera, the sensitivity was 100.0% and specificity 91.6% with an overall ROC accuracy of 91.6%.

4.4.8 Experimental Sera from 0 to 28dpi

Many sets of sera were used with the Q-FMIA. To portray the gradual detection of the developing immune response in poultry, the various experimental time course data was sorted according to “days post infection/inoculation”. The data was graphed alongside the results from the gold standard methods to show the progression of the antibody response over time from the point of initial infection (0 dpi) to the final bleeds (28 dpi) (Figures 15 – 18).

4.5 Discussion

The Q-FMIA has been designed and optimized as a surveillance test to detect antibody responses in chicken and turkey sera against AIV nucleoprotein, H5 and H7 subtypes of hemagglutinin protein, as well as the nucleocapsid protein within NDV-APMV-1. The assay's use is targeted towards samples from experimental infections, vaccine studies, SPF chickens, and in avian populations that may be moving through industry or trade. These diverse sources of serum enable the assay to be robust and produce accurate results regardless of the samples being tested. The ability to test a broad range of samples has an impact on the decision of the ideal threshold values for the assay. While maintaining the highest ROC accuracy possible, the threshold values reflected a decrease in sensitivity in order to maintain high specificity with a wide range of sera from different sources. Results close to the threshold values, or suspicious via the internal controls could likely be confirmed with other serological methods such as HI, AGID, or ELISA. The process of assay validation is highly dependent on whether the test sera used is representative of the population, and for this study both field and experimental sera was used for analysis (Claes *et al.* 2012 ; Jacobson 1998). This assay also used a very high number of sera for use during validation which increases the power of the results.

The optimal operating range of the FMIA was established using samples from SPF birds, field sera, and hyper-immune sera to ensure that the assay would be functional with a single threshold value to evaluate all samples including: strong positives from hyper-immune sera, weak positives from developing immune responses, negative field samples with high background signal, and experimental samples with limited/absent background signal. Assay selectivity is the ability of an assay to differentiate between antibodies produced by different means (vaccination vs real infection). The Q-FMIA did not show significant levels of selectivity during testing and could not differentiate between antibodies produced during a DNA vaccination and those produced by a live virus infection, nor could it determine if the antibodies were a result of an ongoing infection, or from a previous exposure (Makkay

et al. 1999). These results were not unexpected, as most currently used serology methods cannot determine if antibodies are elicited by vaccination or natural infection. However, the assay was successful at detecting all of these antibodies from various sources without any issues.

After the optimal operating range was identified, a reference curve of diluted sera which mirrored this optimal range was developed to use with every run of the assay, tracking repeatability over time. Background signal level in the blank wells of the assay were considered acceptable if their values remained lower than the lowest value of the reference curve. Once optimized, the assay rarely experienced spikes in background signal, and was always the result of serum samples of poor quality. The curve data mainly served as a method to monitor the assay run at a glance, as any deviations from normal values would become immediately apparent.

The secondary antibody used in the assay was initially meant to be specific to chicken sera, but the assay performed with equal efficiency while using turkey sera which has been shown to be possible due to very close antigenic relationships between these IgY immunoglobulins (Hadge and Ambrosius 1986). One study suggests that there is 73% sequence homology between chicken and turkey IgY immunoglobulins, and only 54% homology between chicken and duck IgY, and only 53% between turkey and duck IgY, and that there is cross-reactivity between Immunoglobulins of chicken and turkeys (Bencina *et al.* 2014; Choi *et al.* 2010). We found these two species to be the limit of this Donkey anti Chicken IgY secondary detection antibody as the assay was ineffective with goose or duck sera. This limitation can likely be overcome in the future through changing the assay format to a competitive assay, where the species of the test sera would no longer be a factor affecting performance and accommodate a larger range of species. However, tests of this nature still require that species are analyzed independently, because they may not all react similarly on the same assay, also some assays may work better on certain species than others (Perez-Ramirez *et al.* 2010).

Analytical FMIA specificity was an important characteristic highlighted during assay optimization to ensure that beads had limited reactivity with other hemagglutinin subtypes, or members of the APMV family. The cross-reactivity between the APMV-1-NC bead and the APMV-7 and APMV-9 sera was an issue previously described (Pinette *et al.* 2014), due to the high level of similarity between the antigenic sites of the NC proteins. Assay inclusivity is defined as the ability of the test to detect antibodies of different strains of the same virus, while remaining consistent with hemagglutinin proteins combined with various types of neuraminidase proteins. The assay was able to detect all strains that were tested, which enhances its strength as a surveillance assay. Future work will include an expanded panel of antisera from other common avian diseases to confirm that no cross-reactivity occurs. The most common avian diseases are infectious bursal disease, infectious bronchitis virus, and reovirus (Silim and Venne 1989).

The Q-FMIA is able to detect very low quantities of antibody within low volumes of test serum at high dilutions (1 in 500), making it useful for detection during the early stages of antibody response. However, some field sera with previous vaccinations, remnants of maternal antibodies, and other antibodies generated over the course of living in a natural environment resulted in unexpected reactivity relative to the results of this serum tested with the IDEXX ELISA. These false positives in field sera with high background signal are the primary reason for the decreased sensitivity of the APMV-1-NC, and AIV-H5 beads (Table 5). The threshold values calculated by highlighting ROC-accuracy decreased the number of false positives by raising the threshold value. The ROC analysis plots provide a way to quantify this accuracy as the area under the curve, which is the probability that the assay will correctly identify positive from negative samples at the specified threshold value (Fawcett 2006). This increased threshold value did not have that large of an impact on sensitivity, but seemed to significantly increase the specificity. This measure of accuracy could also be calculated for different assay methods (ELISA, HI, etc.) and provide a unified value of accuracy as the basis of assay comparison (Greiner and Gardner

2000; Zweig and Campbell 1993). This is beneficial especially in the case of comparing different assays that use different volumes of sample or reagents in order to be effective (Perez-Ramirez *et al.* 2010).

The majority of experimentally infected samples consisted of repeated time-point samples from 0, 7, 14, 21, and 28dpi and were used to evaluate the assay kinetics of antibody responses and the ability to detect the developing antibody response (Figures 15-18). The results were used to calculate the percentage of correctly identified positive and negative samples relative to the gold standard methods. The level of antibody in the sample at early time points, influences the amount of antibody present in that sample at later time points, making the data points correlated. Methods of statistical analysis assume that all data points are independent of one another. Therefore, taking repeated samples from the same sets of birds can lead to calculated results that are biased because the calculations are not designed to take correlation between samples into effect. This can lead to inaccurate results that lead to either under-, or over- estimating the performance of the assay that would not occur if each sample had been taken from a unique bird. The data is still useful, but cannot be considered truly accurate values of diagnostic sensitivity and specificity. Combined with the fact that some of the gold standard data may be out dated and that the serum has been stored in the freezers for a very long time, provides reasonable evidence that the true performance of the assay may be better than described in these data.

There is also evidence that experimental conditions and methods of viral inoculation may produce antibody responses of a strength that are not necessarily representative of those which would occur naturally (Jacobson, 1998). Variables such as the quantity of virus in the inoculation, the route of administration, the condition of the virus, and the living environment of the birds are usually not representative to those found in the environment but are also nearly impossible to control. Although the current calculated results are suitable for the aim of this research, additional analysis with natural

cases of infected field birds are usually recommended, and should be tested over time to allow for refinement of assay threshold values to maintain the most accurate values possible (OIE 2008).

The 7 dpi experimental samples represent an initial period where the birds were responding to the vaccination or challenge and initiating sero-conversion. This was the most challenging period for comparison between gold standard methods because the reference ELISA test was recording positive results while the HI was still negative. Other studies have been done which show similar differences in developing infections while comparing different serological assay methods. In these studies, ELISAs are the primary tests being used and also show increased reactivity to early infections than the HI does. (Perez-Ramirez *et al.* 2010). The Q-FMIA data was separately compared with each gold standard method to see how the relative performance changed based on the assay used for confirmation.

Detailed comparisons of the luminex and the HI assays revealed that there are benefits and cautions/limitations associated with each assay. The HI is labour intensive, limited to monoplex, uses high reagent volumes, and requires the use of live virus and fresh blood cells for best results. However, the assay also has very high specificity and an incredibly low incidence of false positives. The luminex assay was shown to react with antibodies sooner than the HI in the 7dpi animals, suggesting that the luminex is more reactive to developing antibodies, however this higher reactivity leaves the luminex at a slightly increased risk of producing false positive signals in negative serum from field samples with serum matrix effects. The luminex is more user-friendly, can operate in multiplex, does not require the use of live virus, is not labour intensive and can test far more samples with less reagent in a shorter amount of time. However, both assays are still susceptible to matrix effects, which may be present in test serum (Perez-Ramirez *et al.* 2010).

The FMIA is very similar to the ELISA and the results regarding sensitivity and specificity were largely comparable in most cases because of the similar mechanism of action of the two assays. The

FMIA is still more beneficial than the ELISA because of the ability to multiplex, while still using less reagents and performing at a reduced cost per sample. However, there has also been work to show that sera tested with a variety of commercialized kits do not always perform exactly as intended and that assay validation is a very complex problem to solve (Claes *et al.* 2012).

The longevity of the coupled beads and their suitability for continued testing was also a factor in the analysis, and whether increased background levels were observed, or reduced signals when beads were stored for longer periods of time. Therefore, the beads were tested previously for these properties in the duplex assay with time extending over 4 months in length and no adverse effects such as reduced signal or increased background of coupled beads were seen (Pinette *et al.* 2014). In this study, no beads were left in storage greater than two months before being used up, so the bead storage ability was not a factor in any case. Beads stored long term were tested again with the quadriplex assay with the reference curve and no significant increases in background signal, or drops in detection signal were seen.

Upon completion of the preliminary steps of assay validation, concluded that the assay will be fit for the intended purpose of serological surveillance of domestic flocks of chickens and turkeys.

4.6 Manuscript 2 Figures

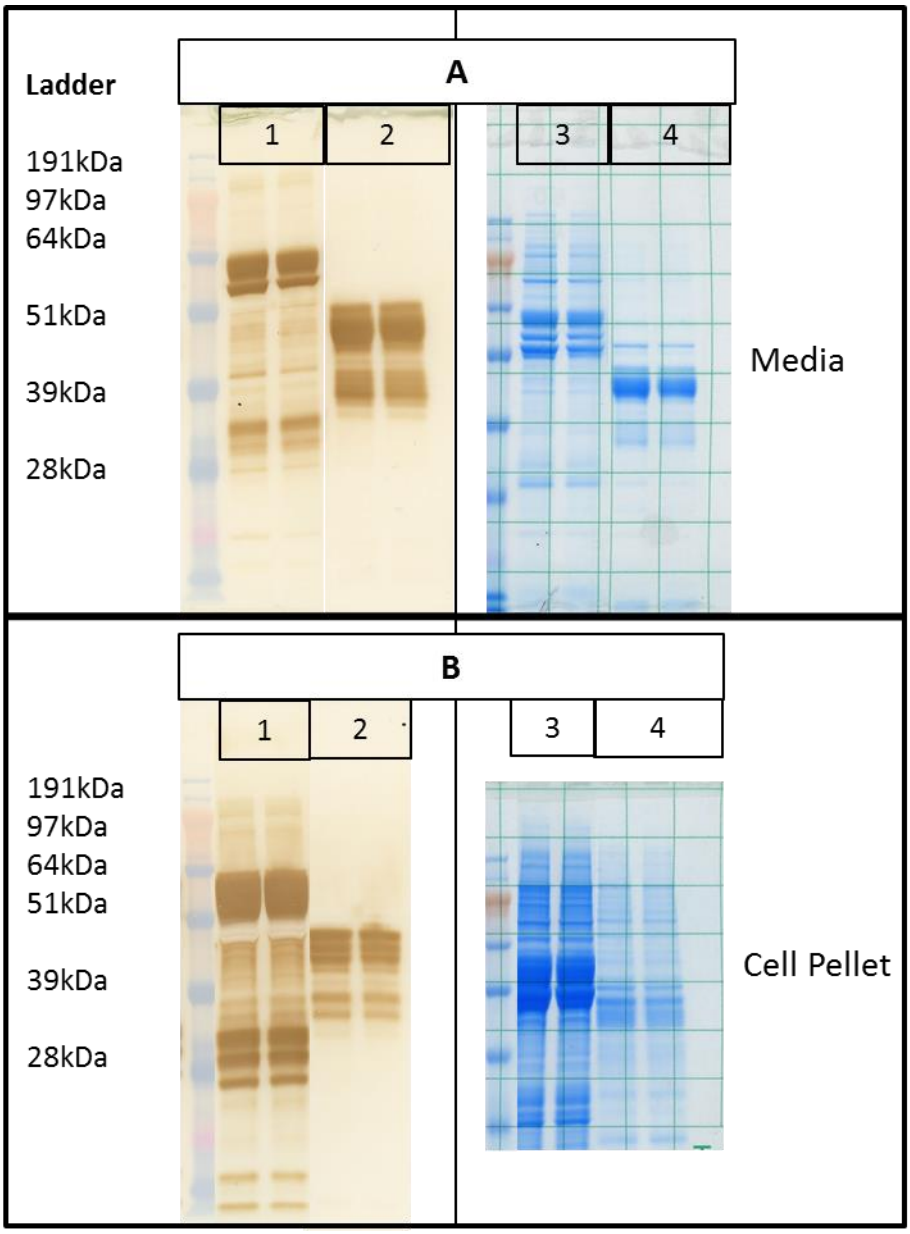


Figure 8. Analysis of recombinant avian influenza H5 hemagglutinin protein (1A, 3A and 1B, 3B) and H7 hemagglutinin protein (2A, 4A and 2B, 4B) that were expressed using the Baculovirus expression system. Both successfully purified and run on SDS-polyacrylamide gel electrophoresis (1A, 2A, 1B, 2B), and western immunoblot using anti-his antibody (3A, 4A, 3B, 4B). Equivalent volumes of each protein were used for both Coomassie and western immunoblot analysis and the media and cell pellet were purified separately.

Table 1. Q-FMIA Threshold Determination Maximizing ROC Accuracy

	Quadriplex AIV-NP	Quadriplex APMV-1-NC	Quadriplex AIV-H5	Quadriplex AIV-H7
# sera used for threshold determination	1709	1690	1340	1628
Chickens	1307	1280	1148	1288
Turkeys	402	410	192	340
# Positives on Gold Standards	256	308	55	106
# Negatives on Gold Standards	1453	1382	1285	1522
ROC threshold	**1253**	**3595**	**1585**	**2585**
Sensitivity	98.4%	90.1%	92.7%	71.7%
Specificity	98.1%	99.2%	99.4%	98.9%
ROC accuracy	**98.2%**	**97.6%**	**99.1%**	**97.2%**
True Positives	252	273	51	76
True Negatives	1426	1371	1277	1506
False Positives	27	11	8	16
False Negatives	4	30	4	30

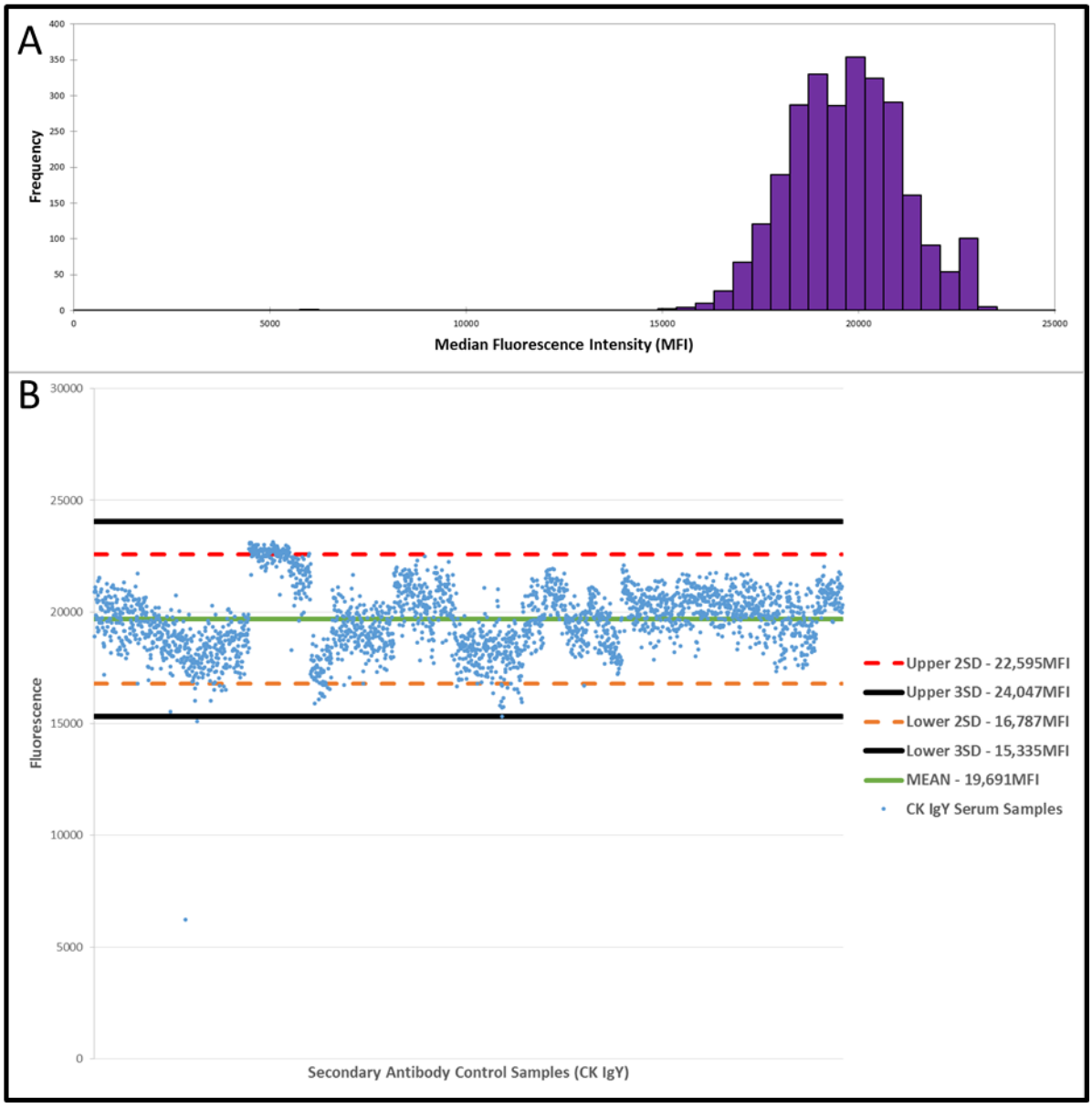


Figure 9. Secondary Antibody Addition Control (CK IgY) (A) showing the frequency distribution of all 2,706 Sera used in the Q-FMIA representing a normal distribution; and (B) determining the acceptable range of fluorescent signal between two standard deviations of the mean (22,595 to 16,787 MFI)

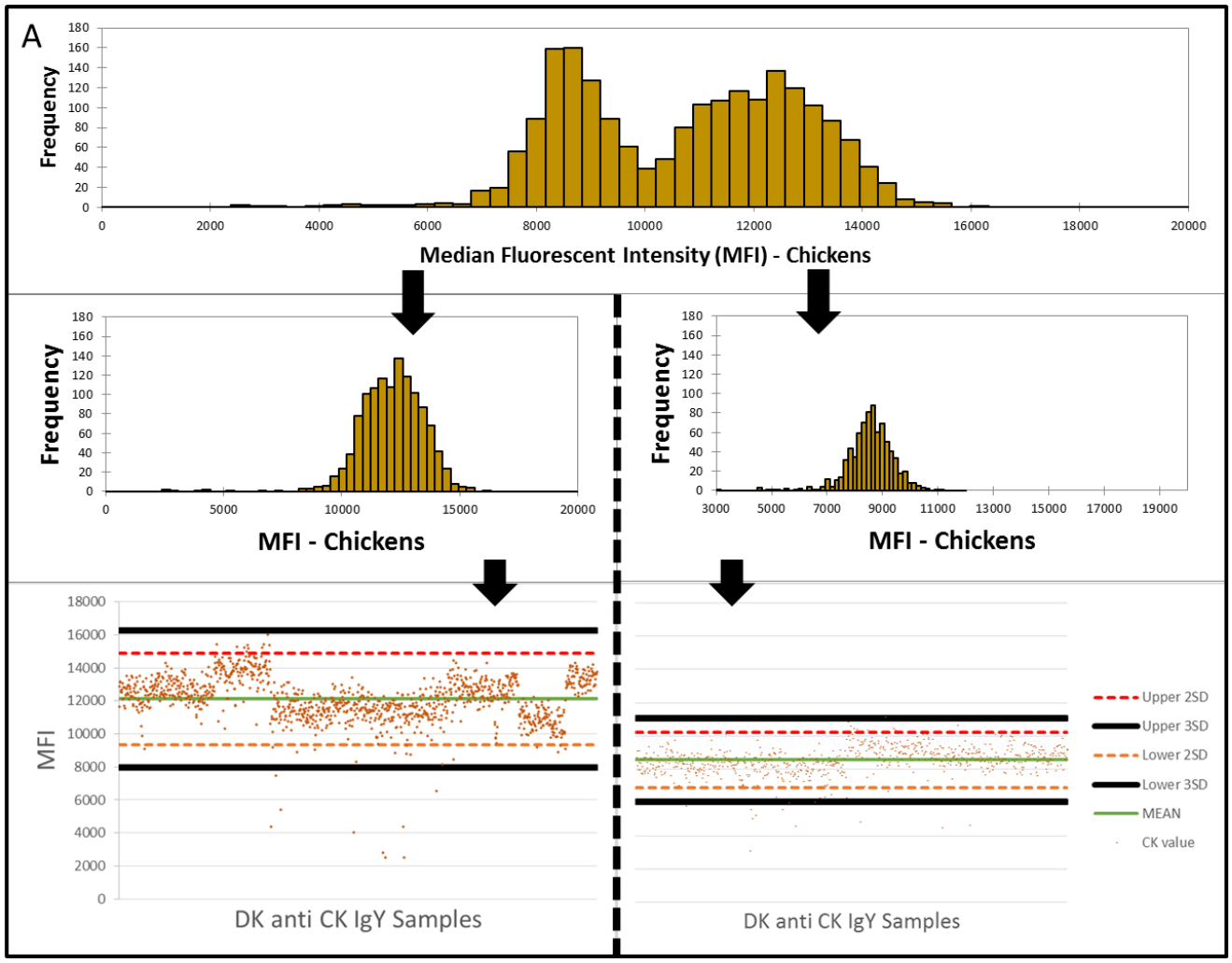


Figure 10A. Serum Addition Control (DK anti CK IgY) Chicken samples showing the frequency distribution of samples tested by the serum control and how chicken sample sets were separated into distinct groups by the internal control. Each group consisting of samples from the same location/breed/age, having a unique acceptable range and normally distributed set of data.

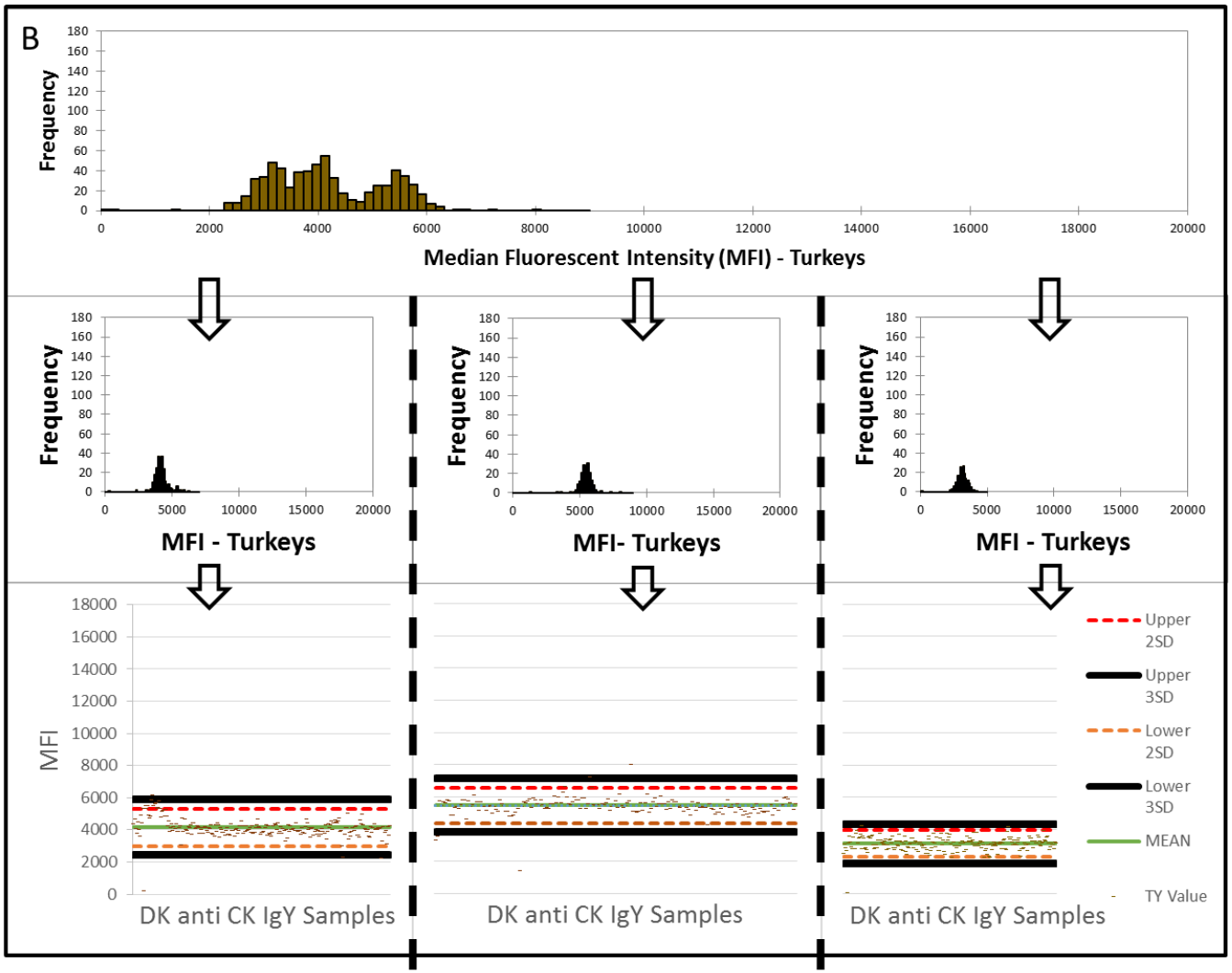


Figure 10B. Serum Addition Control (DK anti CK IgY) Turkey samples showing the frequency distribution of samples tested by the serum control and how turkey sample sets were separated into distinct groups by the internal control. Each group consisting of samples from the same location/breed/age, having a unique acceptable range and normally distributed set of data.

Table 2. Specificity Sera

Virus Type	Virus Name
H1N1	A/Mallard/ON/6/2005
H2N3	A/Mallard/1777/77
H3N2	A/Duck/ON/05/00
H4N8	A/Parrot/UK/70/1995
H5N1	A/Chicken/Scot/59/1994
H6N1	A/Chicken/ON/98HR00004
H7N3	A/Turkey/Ore/1971
H8N4	A/Turkey/ON/6118/62
H9N9	A/Pheasant/WA/37349/85
H10N7	A/Chicken/Ger/N/1949
H11N9	A/Duck/Memphis/546/74
H12N1	A/Duck/AB/60/76-BEL/1990
H13N1	A/Gull/MD/704/77
H14N5	A/Mallard/Gurjev/263/82
H15N8	A/Duck/Austria/341/83
H16N3	A/BHG/Sweden/5/99
APMV-1	La'Sota NDV vaccine strain
APMV-2	Chicken/California/Yucaipa/56
APMV-3	Parakeet/Netherlands/449/75
APMV-4	Duck/Hong Kong/D3/75
APMV-6	Duck/Hong Kong/199/77
APMV-7	Dove/Tennessee/4/75
APMV-8	Goose/Delaware/1053/78
APMV-9	Domestic Duck/New York/22/78
SPF Chicken	-
SPF Chicken	-
SPF Chicken	-
IBV, NDV Chicken	-
Goose Serum	-
Duck Serum	-

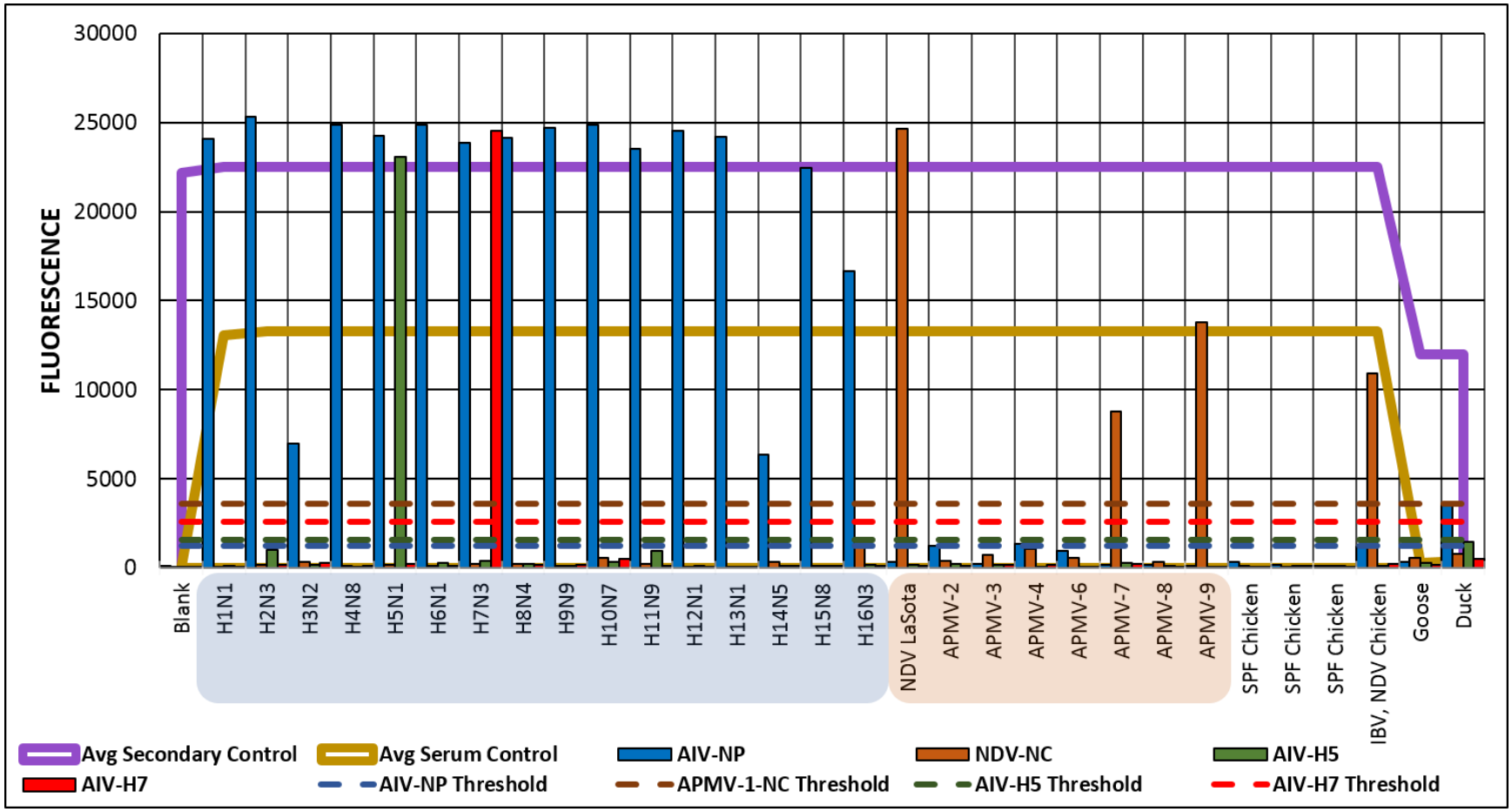


Figure 11. The Q-FMIA was tested using a panel of reference antisera against all of the existing 16 hemagglutinin subtypes (H1 to H16) and avian paramyxoviruses serotype 1-9 (excluding type-5), as well as with IBV and Goose and Duck Sera. The AIV-NP beads were able to detect antibodies against all 16 hemagglutinin subtypes and beads coated with APMV-1-NC were able to detect reference antisera against type 1 with minimal cross reactivity to type 7 and 9. AIV-H5 beads were able to detect H5 antisera and the AIV-H7 beads were able to detect H7 antisera and did not cross react with any of the other antisera. The Q-FMIA is not compatible with the Goose or Duck sera as the bead values are incorrect and the values are not consistent on the internal controls.

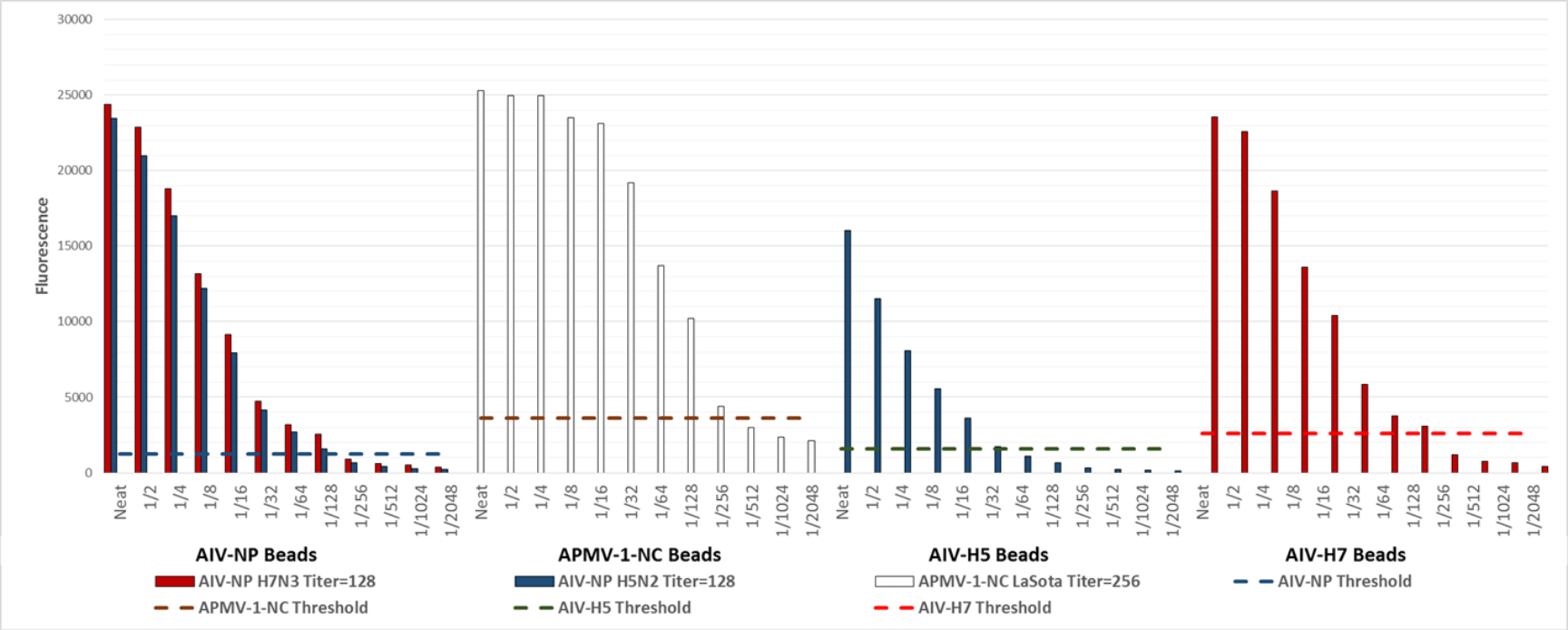


Figure 12. The optimal operating range of Q-FMIA was determined by performing two-fold dilutions with known titer sera into negative chicken sera to evaluate the range of values to be expected when using field or experimental sera.

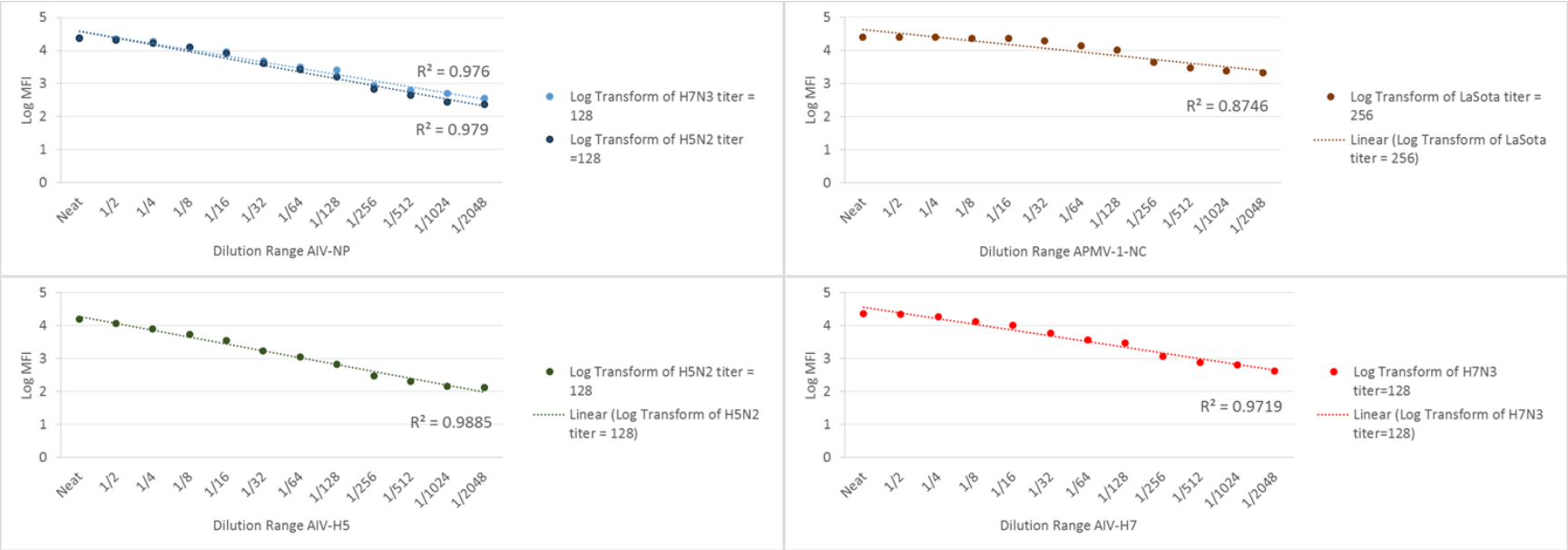


Figure 13. The dilution curves were log-transformed and linearized to evaluate the proportionality of the signal generated by the assay over the entire operating range. All beads were shown to have signal that increased linearly with the amount of antibody within the sample.

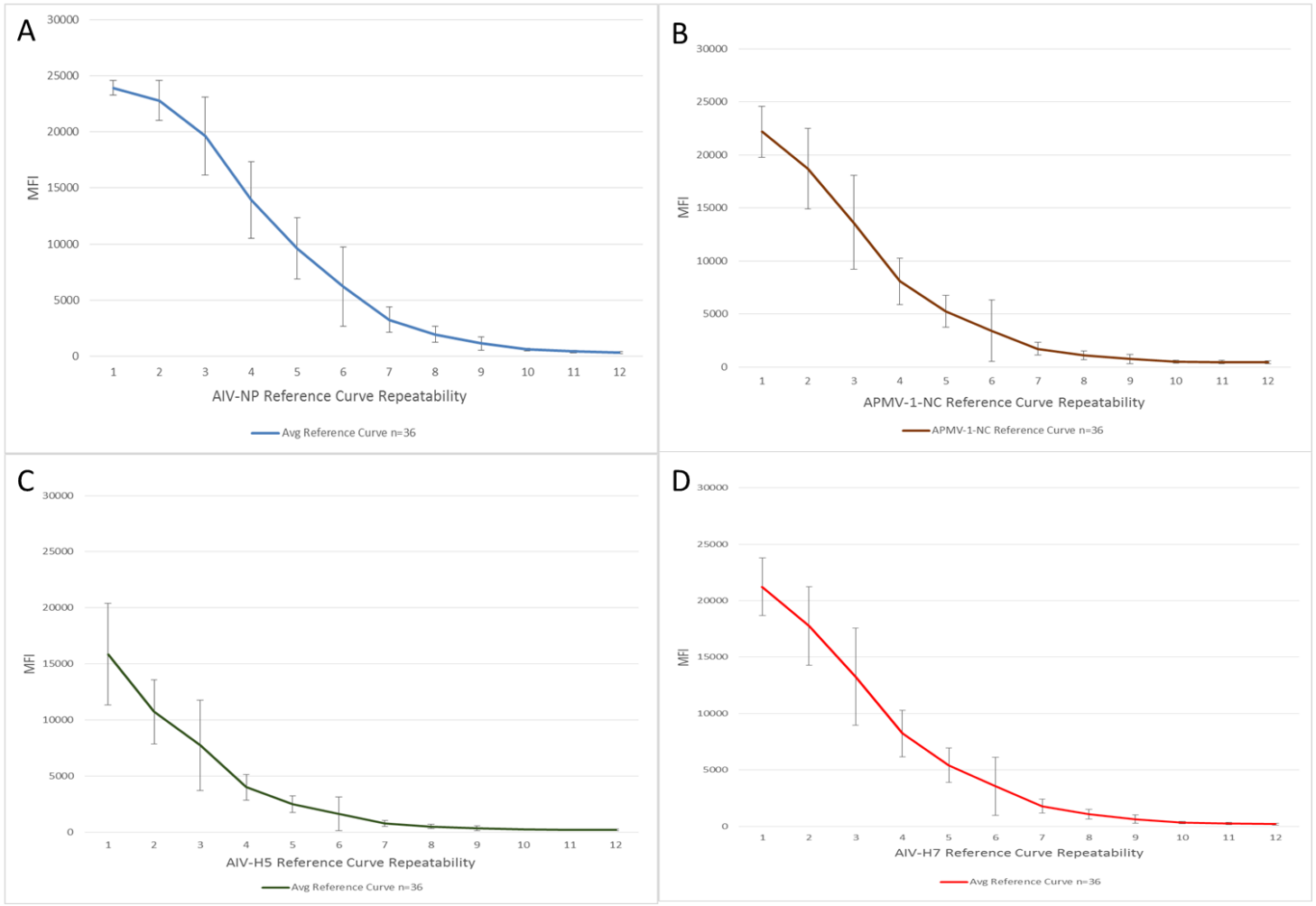


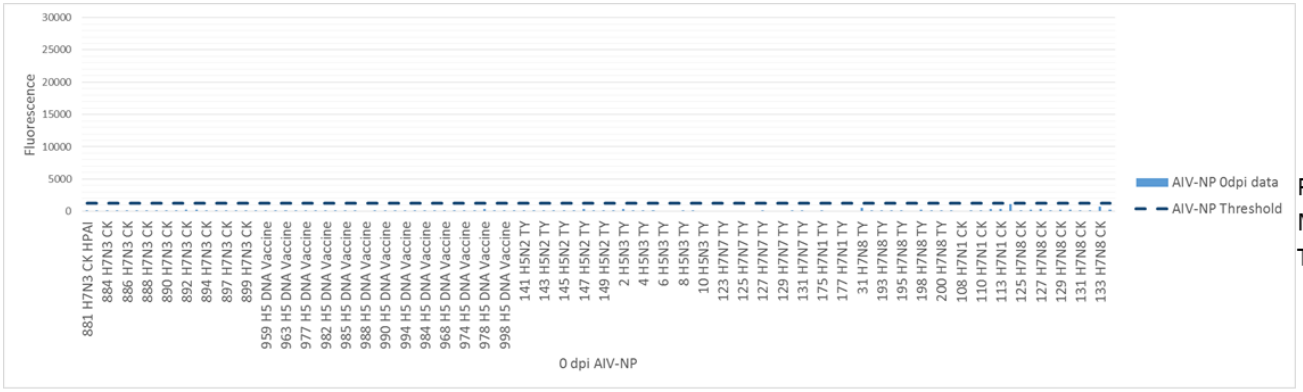
Figure 14. The reference curve data accumulated from all runs of the assay combined to show variation of the reference curve between runs of the assay. References 7, 8, and 9 in the threshold range show the least variation overall. Assay results can be accurately compared between runs of the assay without a normalization factor being applied. (A) AIV-NP (B) APMV-1-NC (C) AIV-H5 (D) AIV-H7.

Table 3. Experimental Sera Used for Diagnostic Performance of Q-FMIA

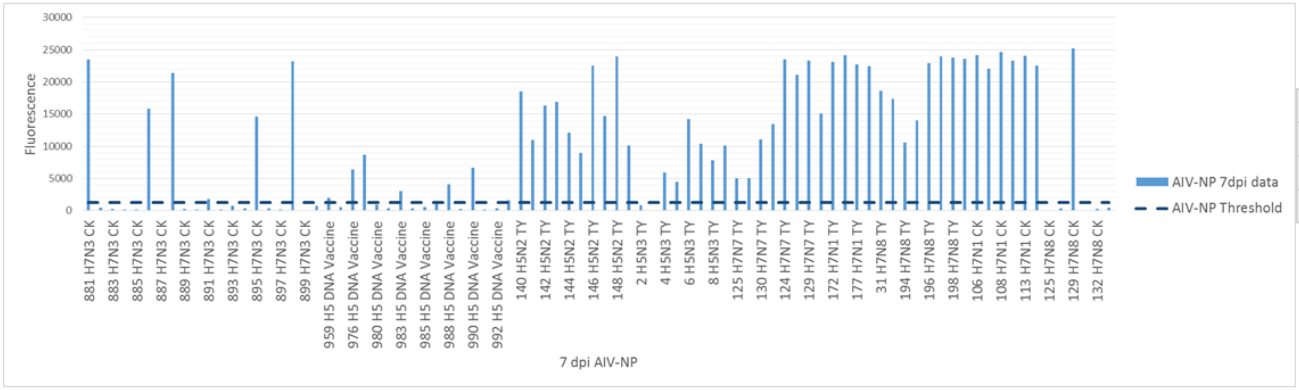
Inoculation	Description
H5 DNA Vaccine	6-8 week old commercial layer CK all NDV vaccinated
NDV Colombia Vaccine	6-8 week old leghorn CK, single vaccine inoculation
#1 H5N9	A/TY/ON/7732/1966 HPAI Sex: Mixed, Age: 4 weeks, Breed: Turkey / mixed, Route: Oral/Nasal
#3 H5N2	A/TY/MN/3689-1551 LPAI Sex: Mixed, Age: 4 weeks, Breed: Turkey / mixed, Route: Oral/Nasal
#9 H5N2	A/CK/Penn/1370/1/1983 LPAI Sex: Mixed, Age: 4 weeks, Breed: Turkey / mixed, Route: Oral/Nasal
#a10 H5N3	A/TY/Ca/35621/84 LPAI Sex: Mixed, Age: 4 weeks, Breed: Turkey mixed, Route: Oral Nasal
#2 H7N7	A/CK/Victoria/32972/1985 HPAI Sex: female, Age: Adult, Breed: Leghorn, Route: Oral Nasal
#7 H7N3	A/CK/Au/3634/1992 HPAI Sex: mixed, Age: 4 weeks, Breed: Turkey mixed, Route: Oral Nasal
#8 H7N1	A/TY/ON/18-2-2000 LPAI Sex: mixed, Age: 4 weeks, Breed: Turkey mixed, Route: Oral Nasal
#a13 H7N8	A/Magpie/Robin/China/28710/93 LPAI Sex: mixed, Age: 4 weeks, Breed: Turkey mixed, Route: Oral Nasal
#15 H7N1	A/TY/ON/18-2/2000 LPAI Sex: female, Age: Adult, Breed: CK leghorn, Route: Oral Nasal
#18 H7N8	A/Mag/Rob/China/28710/93 LPAI Sex: female, Age: Adult, Breed: CK leghorn, Route: Oral Nasal
#22 H7N3	A/CK/Aust/3634/92 HPAI Sex: female, Age: Adult, Breed: CK leghorn, Route: Oral Nasal

Table 4. Diagnostic Performance of Q-FMIA Highlighting ROC Accuracy

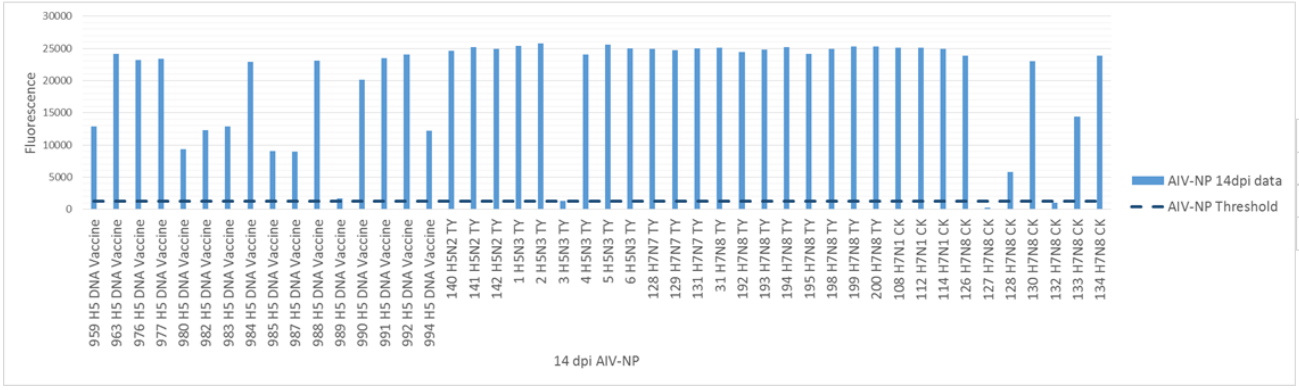
Quadriplex FMIA Diagnostic Performance based on ROC Accuracy	Quadriplex AIV-NP	Quadriplex AIV-NP	Quadriplex APMV-1-NC	Quadriplex AIV-H5	Quadriplex AIV-H5	Quadriplex AIV-H7	Quadriplex AIV-H7
# Samples used to Determine Diagnostic Performance	868	322	827	627	377	322	383
Relative to Gold Standard	IDEXX ELISA	AGID	IDEXX ELISA	HI	H5 cELISA	HI	H7 cELISA
Chickens	636	100	596	397	377	89	383
Turkeys	232	222	231	230	0	233	0
# Positives on Gold Standards	313	199	53	139	155	75	1
# Negatives on Gold Standards	555	123	774	488	222	247	382
ROC threshold	**1253**	**1253**	**3595**	**1585**	**1585**	**2584**	**2585**
Sensitivity	95.2%	99.5%	71.7%	70.5%	69.7%	96.0%	100.0%
Specificity	95.0%	91.9%	98.8%	94.3%	99.5%	93.5%	91.6%
ROC accuracy	**95.0%**	**97.8%**	**97.1%**	**89.0%**	**87.3%**	**94.1%**	**91.6%**
True Positives	298	198	38	98	108	72	1
True Negatives	527	113	765	460	221	231	350
False Positives	28	10	9	28	1	16	32
False Negatives	15	1	15	41	47	3	0



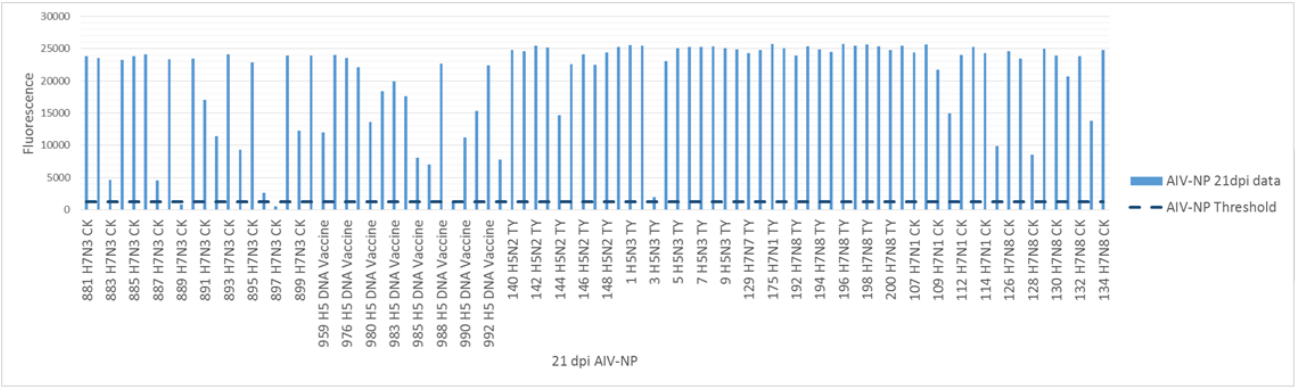
	ELISA Result	Luminex Result
Positives	0	0
Negatives	104	104
Total	104	104



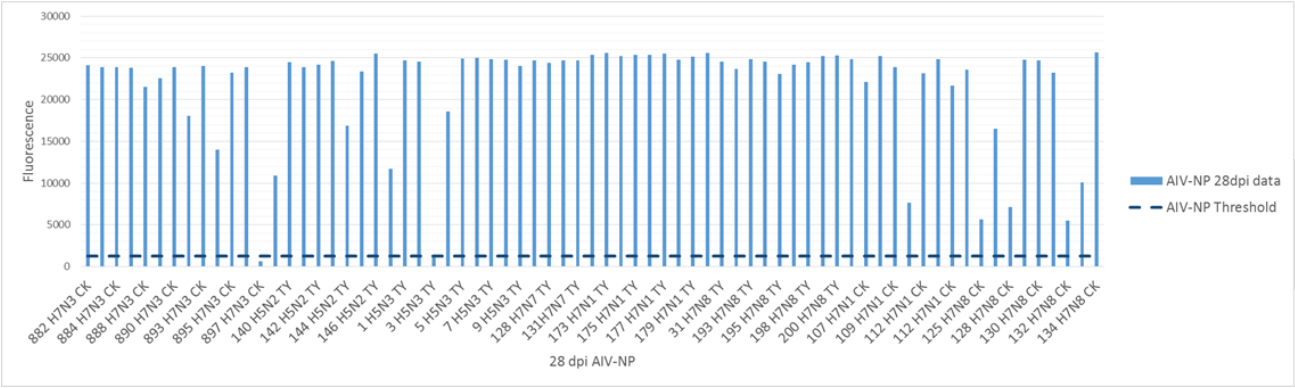
	ELISA Result	Luminex Result
Positives	66	56
Negatives	20	30
Total	**86**	**86**



	ELISA Result	Luminex Result
Positives	40	44
Negatives	6	2
Total	**46**	**46**

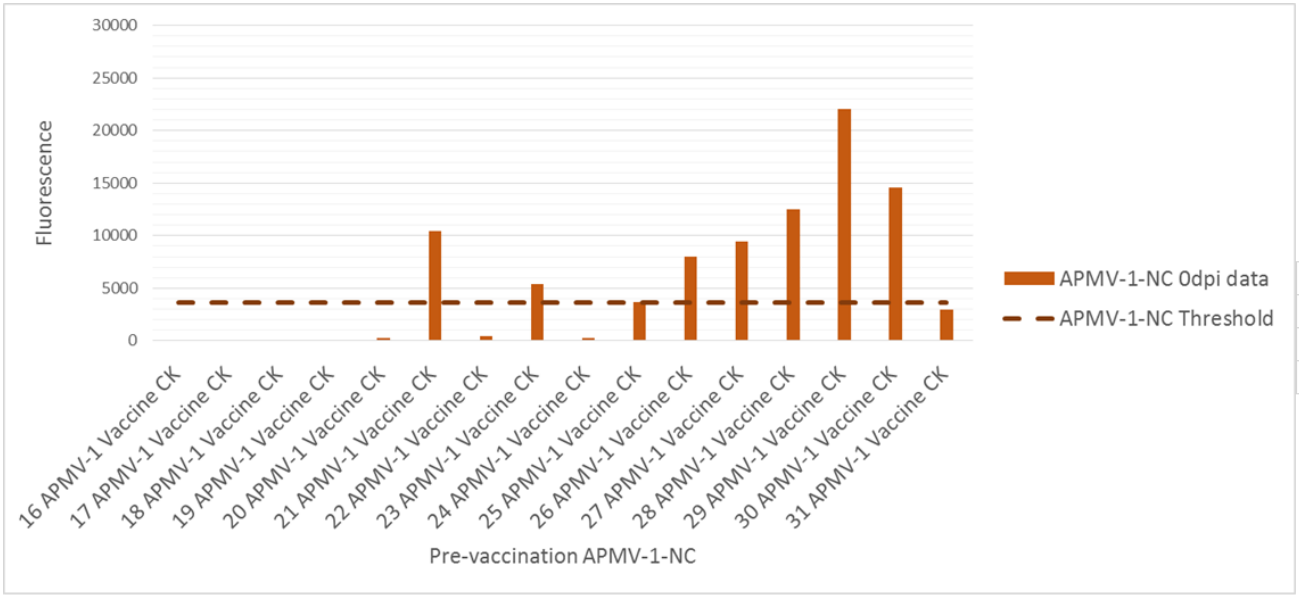


	ELISA Result	Luminex Result
Positives	81	84
Negatives	6	3
Total	**87**	**87**

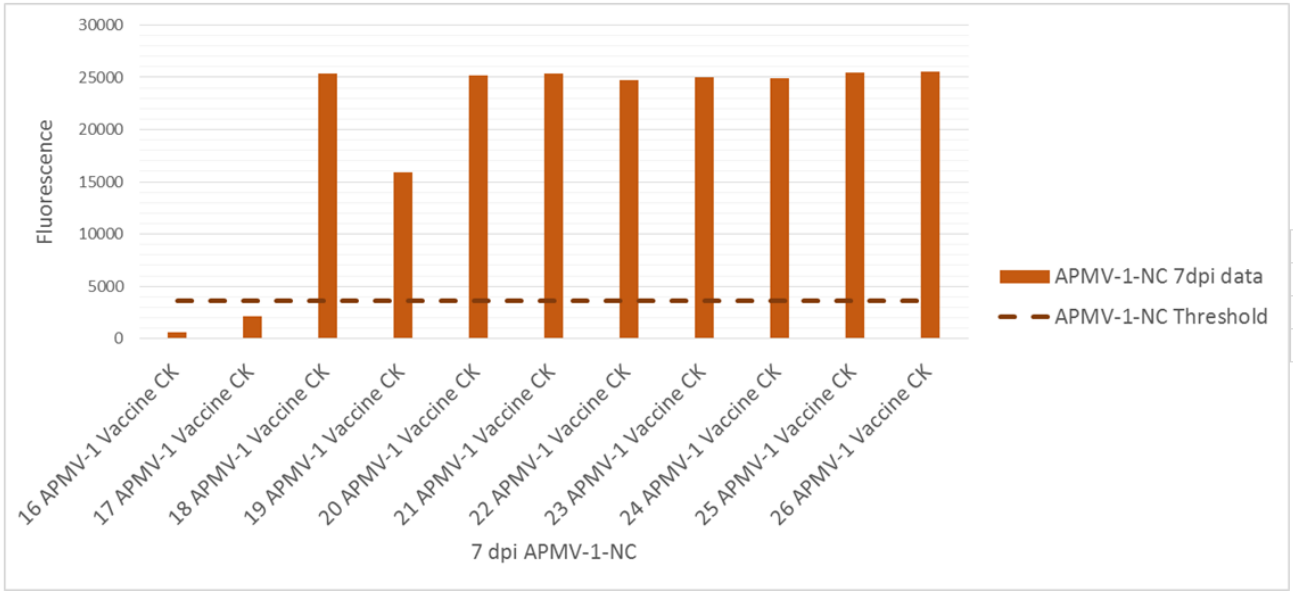


	ELISA Result	Luminex Result
Positives	63	69
Negatives	8	2
Total	**71**	**71**

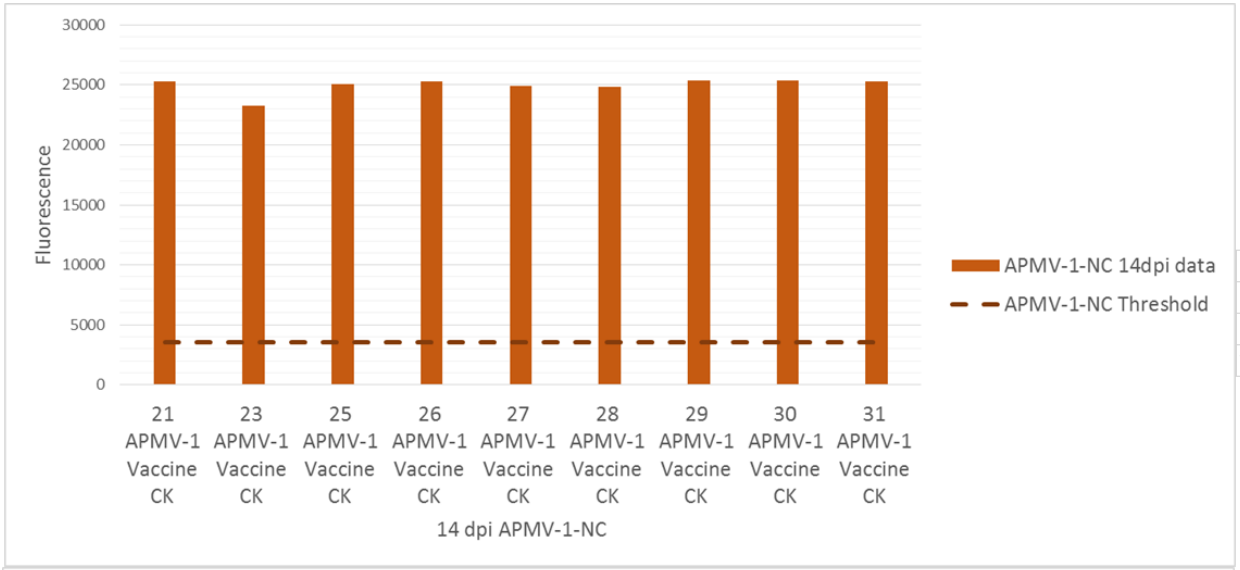
Figure 15. Time course data of serum sets tested with the AIV-NP bead to show the progression of antibody responses over time.



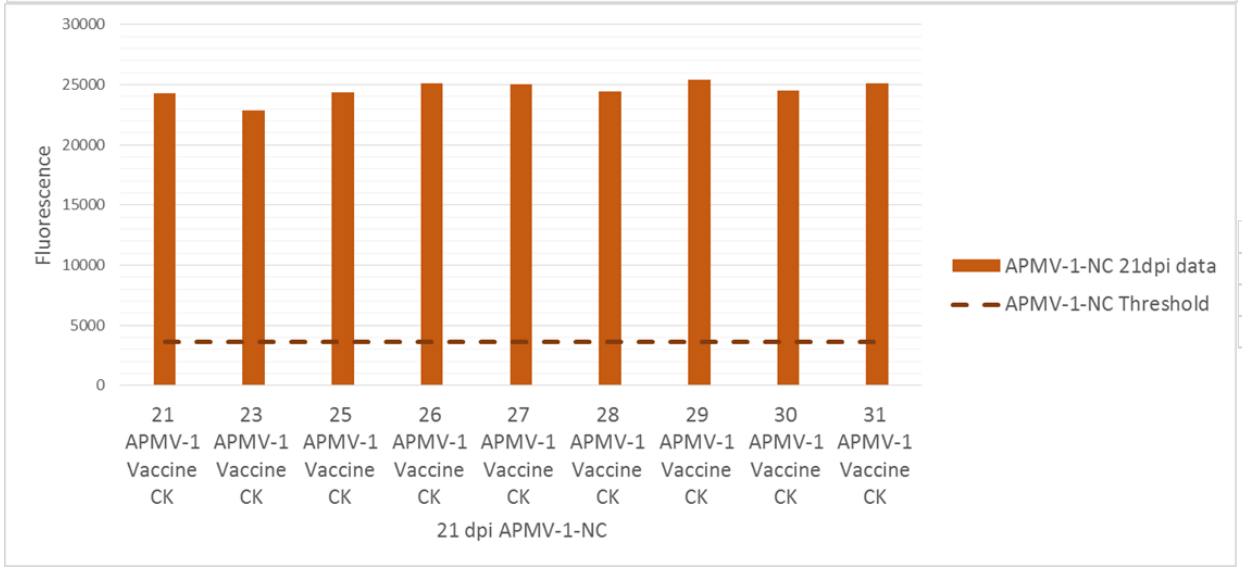
	ELISA Result	Luminex Result
Positives	6	8
Negatives	10	8
Total	16	16



	ELISA Result	Luminex Result
Positives	11	9
Negatives	0	2
Total	11	11

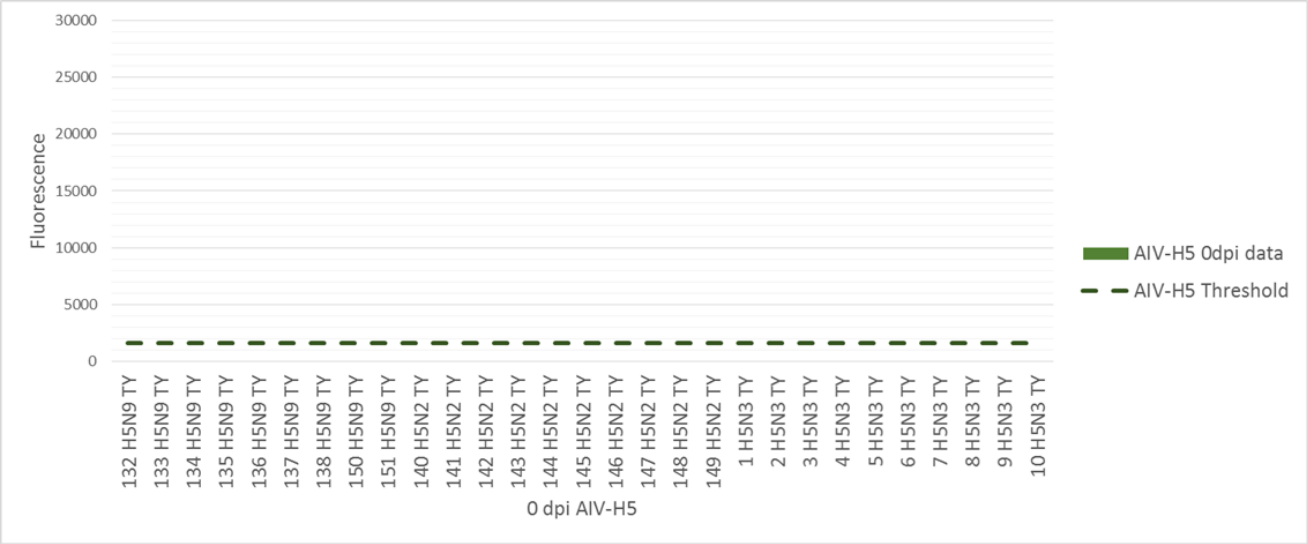


	ELISA Result	Luminex Result
Positives	9	9
Negatives	0	0
Total	9	9

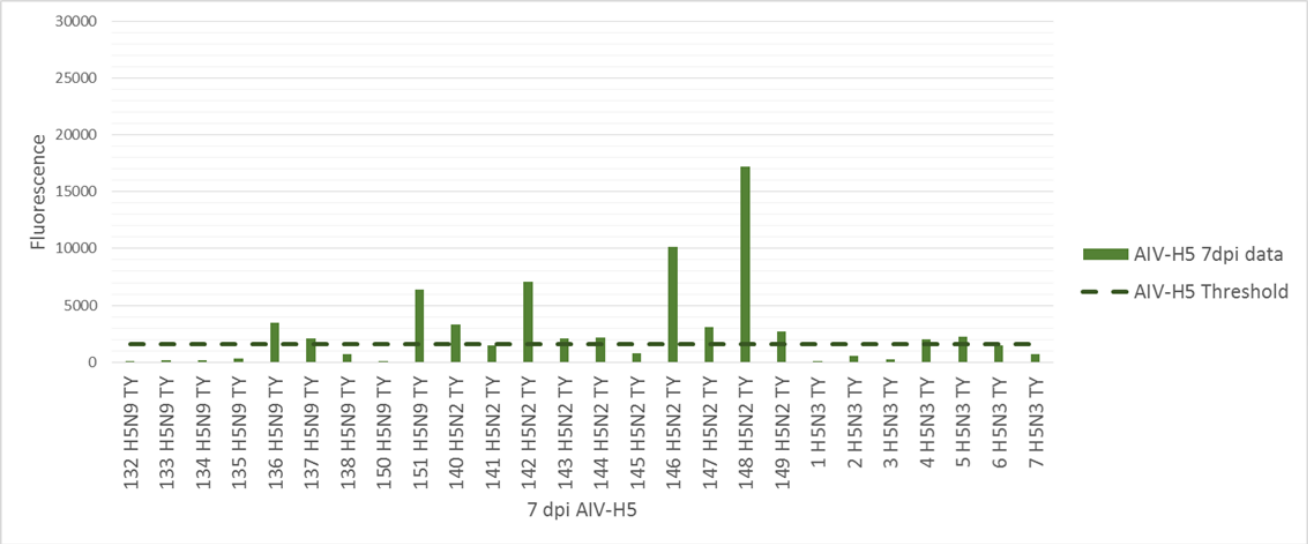


	ELISA Result	Luminex Result
Positives	9	9
Negatives	0	0
Total	9	9

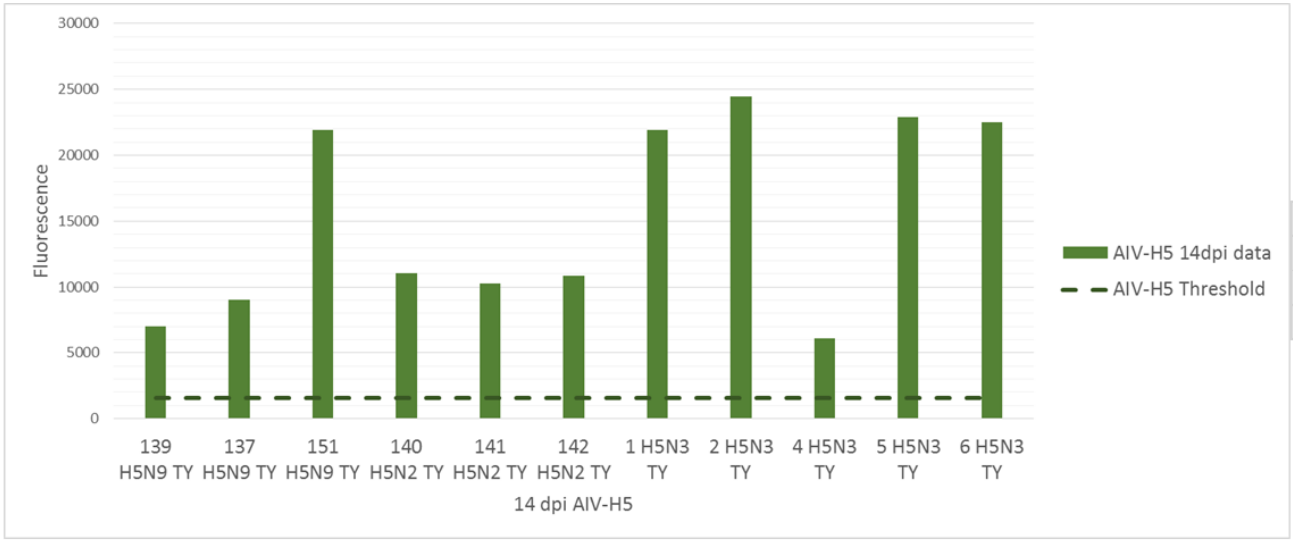
Figure 16. Time course data of serum sets tested with the APMV-1-NC bead to show the progression of antibody responses over time.



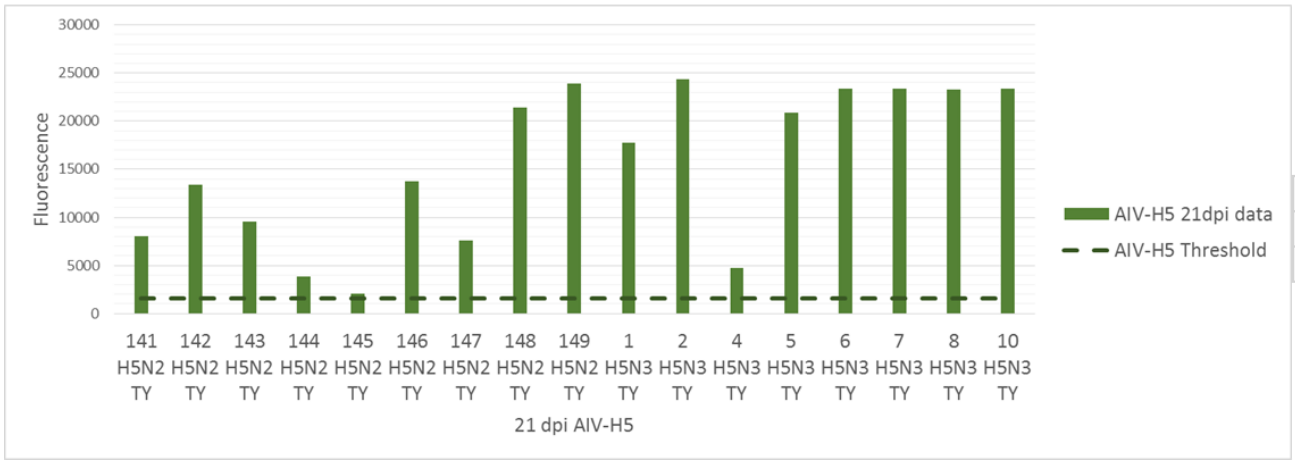
	HI Results	Luminex Result
Positives	0	0
Negatives	29	29
Total	**29**	**29**



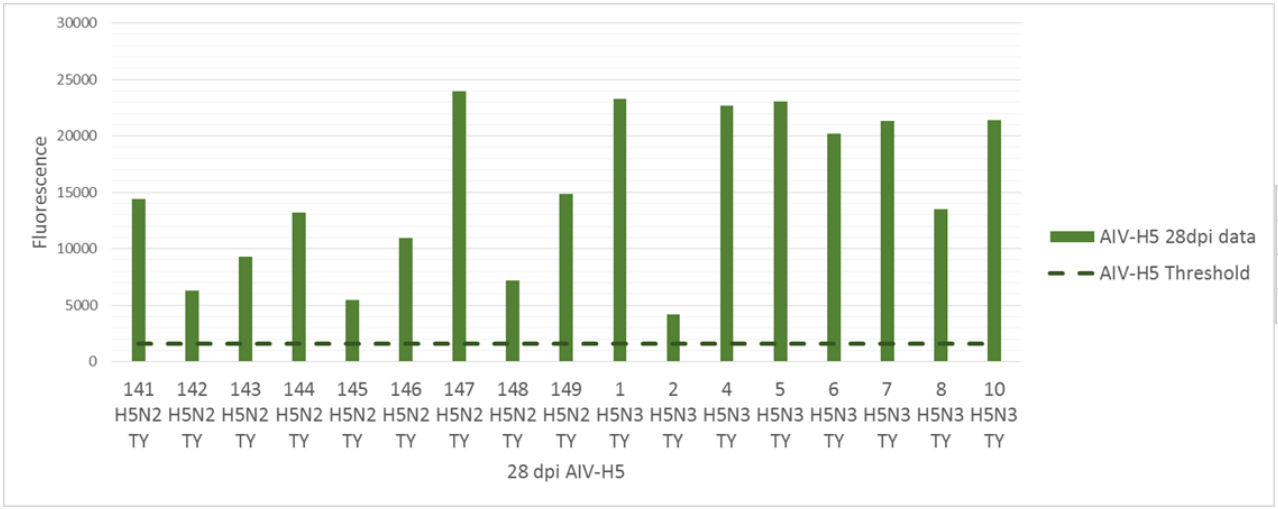
	HI Results	Luminex Result
Positives	5	14
Negatives	22	13
Total	**27**	**27**



	HI Results	Luminex Result
Positives	8	11
Negatives	3	0
Total	**11**	**11**

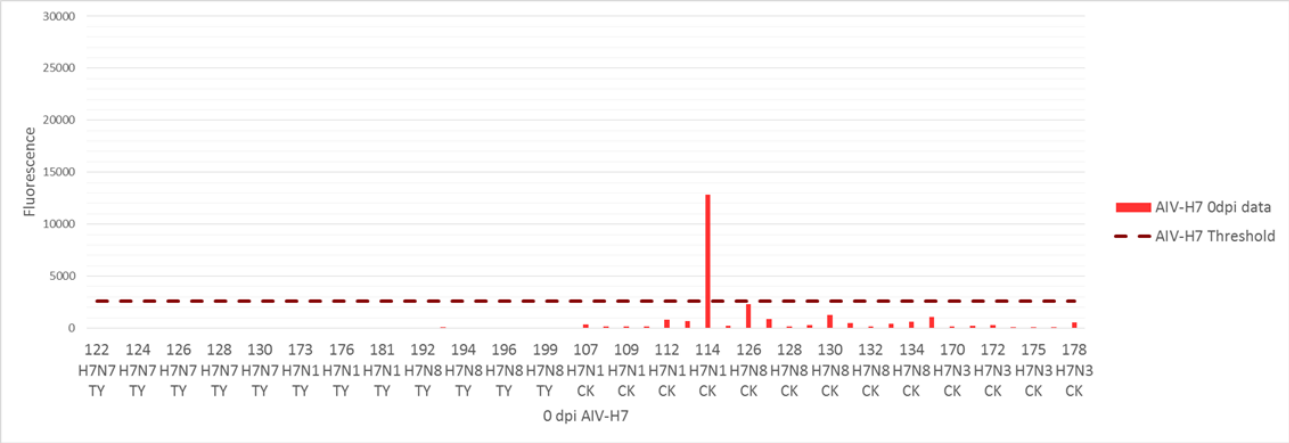


Positives	16	17
Negatives	1	0
Total	**17**	**17**

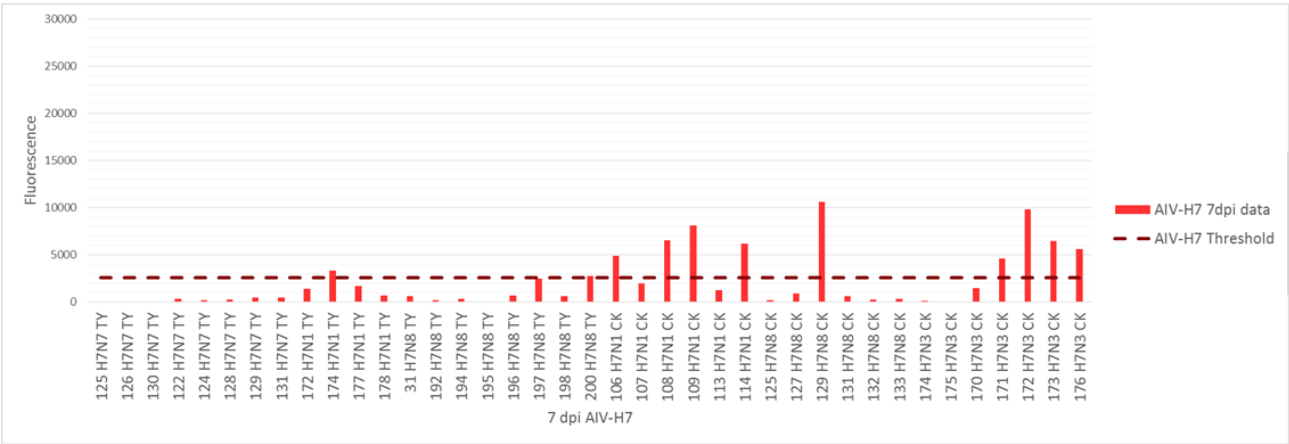


	HI Results	Luminex Result
Positives	17	17
Negatives	0	0
Total	**17**	**17**

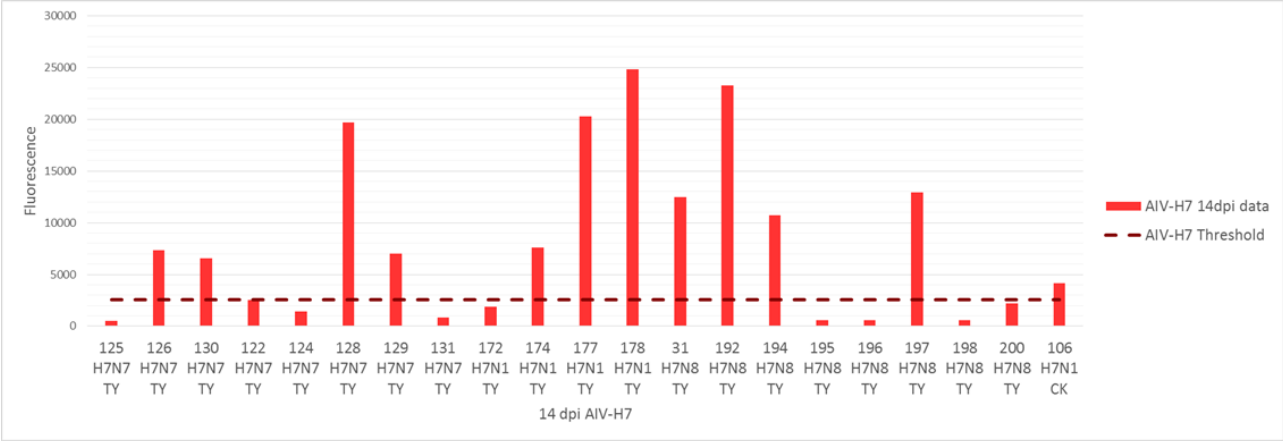
Figure 17. Time course data of serum sets tested with the AIV-H5 bead to show the progression of antibody responses over time.



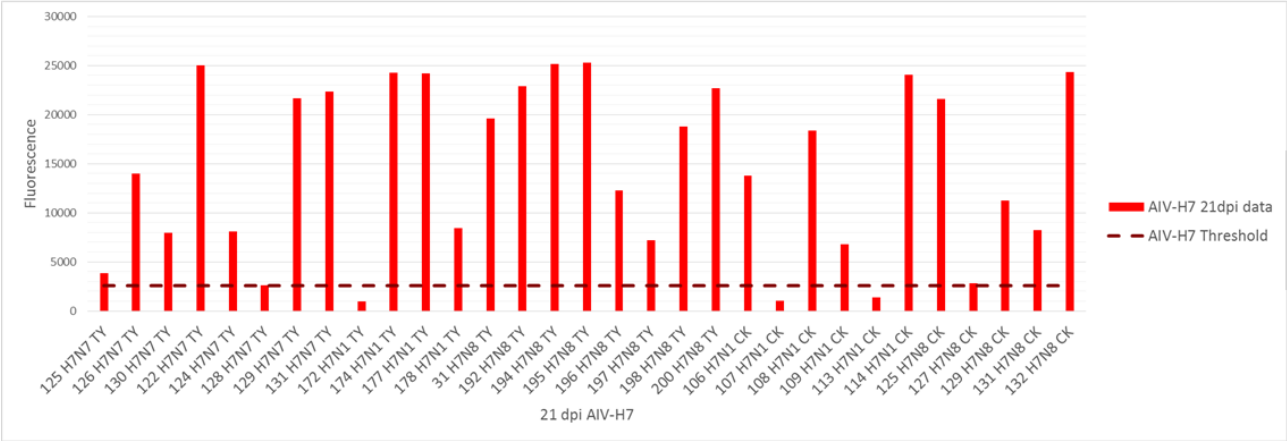
	HI Results	Luminex Result
Positives	0	1
Negatives	49	48
Total	**49**	**49**



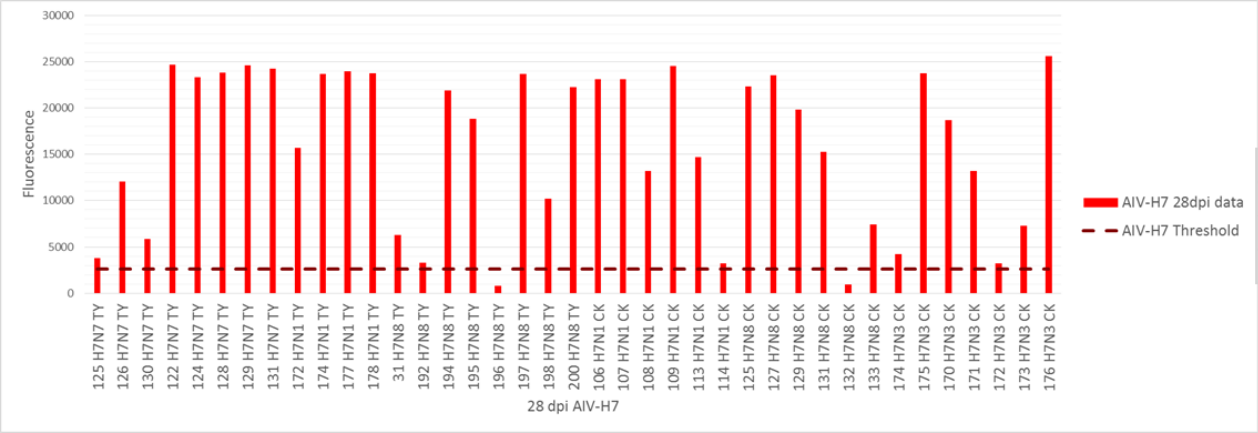
	HI Results	Luminex Result
Positives	7	11
Negatives	35	31
Total	**42**	**42**



	HI Results	Luminex Result
Positives	8	12
Negatives	13	9
Total	**21**	**21**

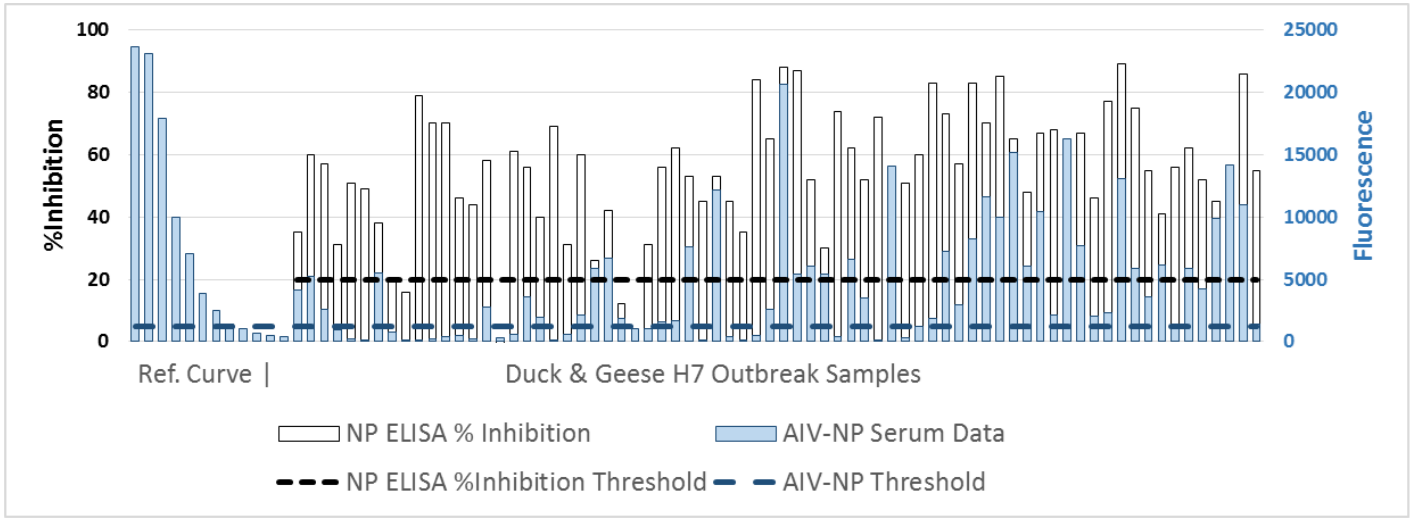


	HI Results	Luminex Result
Positives	25	28
Negatives	6	3
Total	**31**	**31**

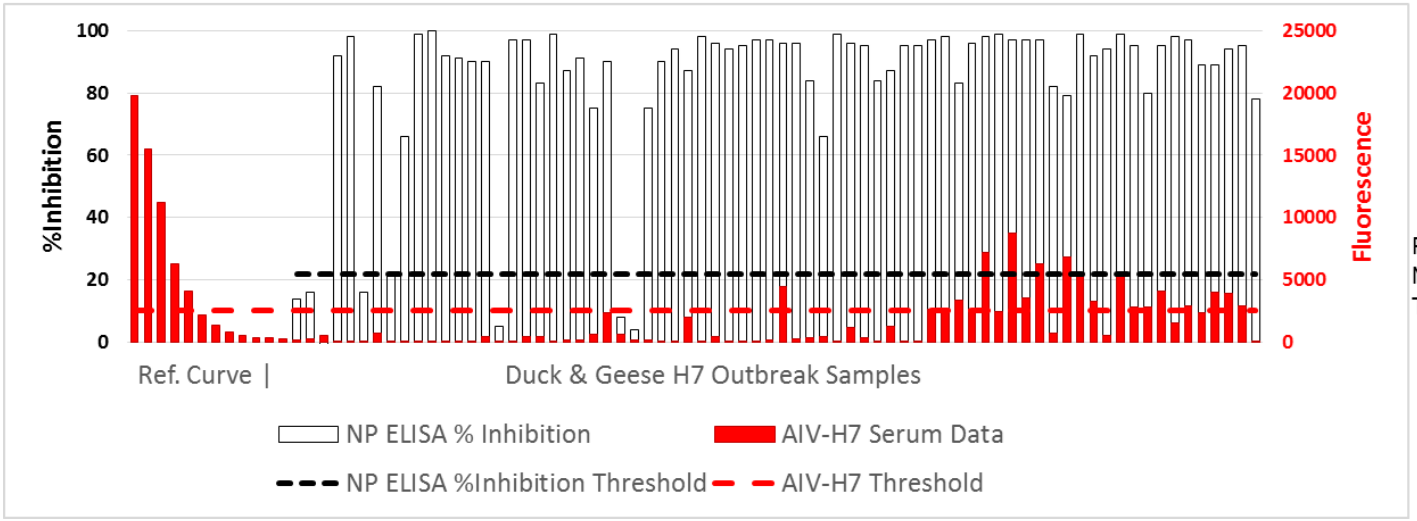


	HI Results	Luminex Result
Positives	37	37
Negatives	2	2
Total	**39**	**39**

Figure 18. Time course data of serum sets tested with the AIV-H7 bead to show the progression of antibody responses over time.



	ELISA Results	Luminex Results
Positives	66	47
Negatives	6	25
Total	72	72



	ELISA Results	Luminex Results
Positives	64	19
Negatives	8	53
Total	72	72

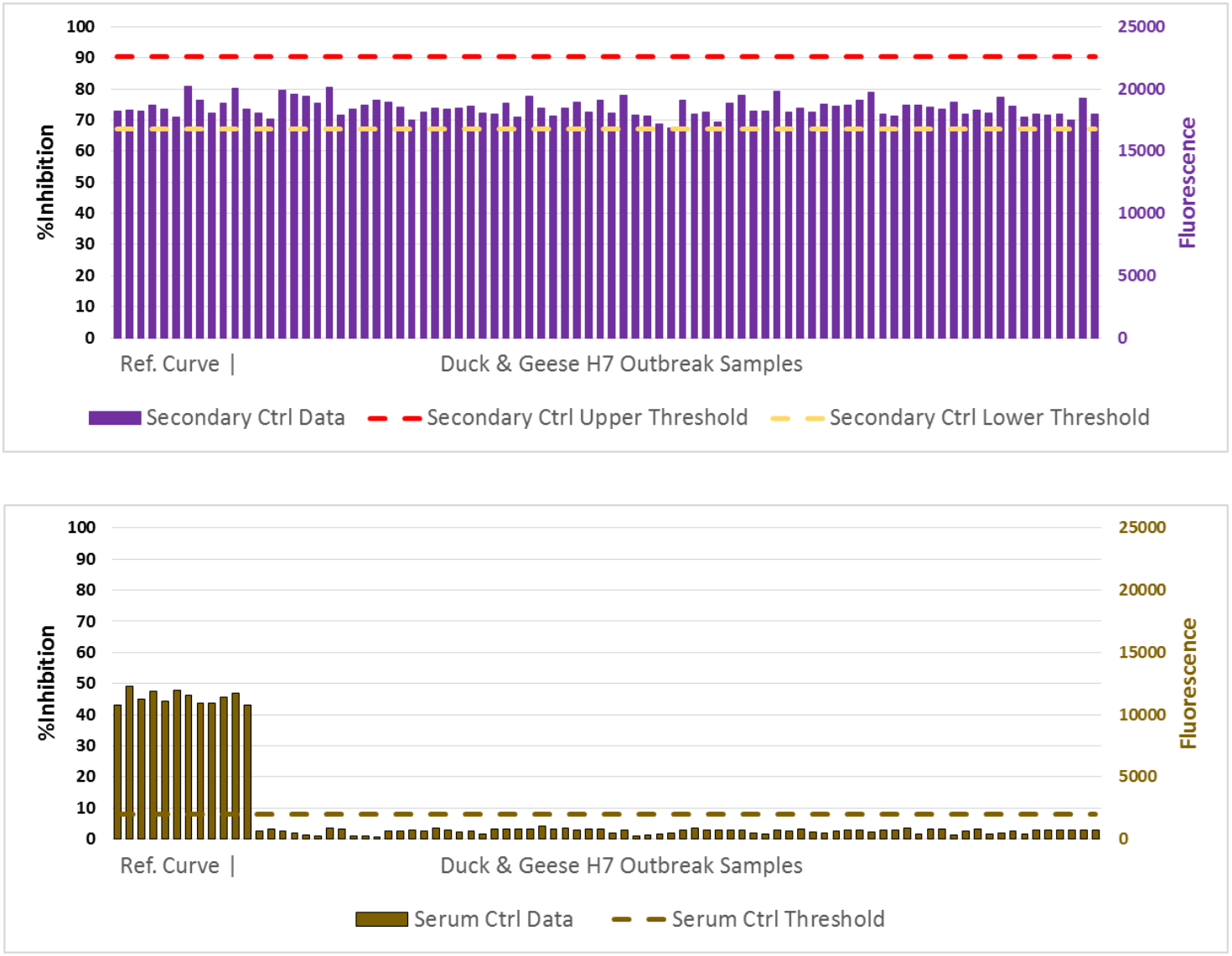


Figure 19. Internal control realization of Duck and Goose Serum Incompatibility with H7 Positive Outbreak sera as compared to the NP ELISA. Serum does not register in the appropriate range. APMV-1-NC and AIV-H5 signals were all negative and are not shown.

Table 5. Threshold Value and Analytical Sensitivity and Specificity Comparison Between Duplex and Q-FMIA

(Highlighting Q-FMIA **ROC – Accuracy** Based Threshold Value)

	Duplex AIV-NP	Quadriplex AIV-NP	Duplex APMV-1-NC	Quadriplex APMV-1-NC	Quadriplex AIV-H5	Quadriplex AIV-H7
# sera used for threshold determination	2376	1709	1495	1690	1340	1628
Chickens	2000	1307	1495	1280	1148	1288
Turkeys	376	402	0	410	192	340
# Positives on Gold Standards	333	256	328	308	55	106
# Negatives on Gold Standards	2043	1453	1167	1382	1285	1522
ROC threshold	**1835**	**1253**	**2284**	**3595**	**1585**	**2585**
Sensitivity	99.7%	98.4%	95.4%	90.1%	92.7%	71.7%
Specificity	97.3%	98.1%	98.5%	99.2%	99.4%	98.9%
ROC accuracy	**97.6%**	**98.2%**	**97.8%**	**97.6%**	**99.1%**	**97.2%**
True Positives	332	252	313	273	51	76
True Negatives	1987	1426	1149	1371	1277	1506
False Positives	56	27	18	11	8	16
False Negatives	1	4	15	30	4	30

*Table 6. Threshold value and Analytical Sensitivity and Specificity comparison between Duplex and Q-FMIA (Highlighting Q-FMIA **ROC Sensitivity and Specificity** based threshold values)*

	Duplex AIV-NP	Quadriplex AIV-NP	Duplex APMV-1-NC	Quadriplex APMV-1-NC	Quadriplex AIV-H5	Quadriplex AIV-H7
# sera used for threshold determination	2376	1709	1495	1690	1340	1628
Chickens	2000	1307	1495	1280	1148	1288
Turkeys	376	402	0	410	192	340
# Positives on Gold Standards	333	256	328	308	55	106
# Negatives on Gold Standards	2043	1453	1167	1382	1285	1522
ROC threshold	**1835**	**1253**	**2284**	**2228**	**979**	**396**
Sensitivity	**99.7%**	**98.4%**	**95.4%**	**91.7%**	**98.2%**	**91.5%**
Specificity	**97.3%**	**98.1%**	**98.5%**	**98.4%**	**98.9%**	**91.4%**
ROC accuracy	97.6%	98.2%	97.8%	97.2%	98.9%	91.4%
True Positives	332	252	313	278	54	97
True Negatives	1987	1426	1149	1360	1271	1391
False Positives	56	27	18	22	14	131
False Negatives	1	4	15	25	1	9

Table 7. Q-FMIA performance results with alternate threshold values
*(Highlighting Q-FMIA **ROC Sensitivity and Specificity** based threshold values)*

Quadriplex FMIA Diagnostic Performance based on ROC Sensitivity and Specificity	Quadriplex AIV-NP	Quadriplex AIV-NP	Quadriplex APMV-1-NC	Quadriplex AIV-H5	Quadriplex AIV-H5	Quadriplex AIV-H7	Quadriplex AIV-H7
# Samples used to Determine Diagnostic Performance	868	322	827	627	377	322	389
Relative to Gold Standard	NP-ELISA / IDEXX ELISA	AGID	IDEXX ELISA	HI	H5 cELISA	HI	H7 cELISA
Chickens	636	100	596	397	377	89	383
Turkeys	232	222	231	230	0	233	0
# Positives on Gold Standards	313	199	47	139	155	75	1
# Negatives on Gold Standards	555	123	780	488	222	247	388
ROC threshold	**1253**	**1253**	**2228**	**979**	**979**	**396**	**396**
Sensitivity	**95.2%**	**99.5%**	**91.5%**	**79.9%**	**81.9%**	**100.0%**	**100.0%**
Specificity	**95.0%**	**91.9%**	**97.9%**	**91.0%**	**99.5%**	**73.7%**	**86.6%**
ROC accuracy	95.0%	97.8%	96.9%	88.5%	92.3%	79.8%	86.7%
True Positives	298	198	43	111	127	75	1
True Negatives	527	113	764	444	221	182	337
False Positives	28	10	16	44	1	65	51
False Negatives	15	1	4	28	28	0	0

Table 8. H5N2 infected Turkeys with full Gold Standard and Q-FMIA comparison Time Course

	Gold Standard Methods					Quadriplex FMIA Method					
	H5 HI Result with homologous virus >8 units = positive	H7 HI Result with H7N3 virus >8 units = positive	NP-ELISA % inhibition results >25 = Positive	AGID results	APMV-1 IDEXX results >0.2 =positive	AIV-NP Bead Luminex Value (MFI) >1253 = positive	AIV-H5 bead Luminex value (MFI) >1585 = positive	AIV-H7 bead Luminex value (MFI) >2585 = positive	APMV-1-NC bead Luminex value (MFI) >3595 = positive	Internal Serum Ctrl bead Luminex value(MFI) >2,000 = positive	Detection Antibody Ctrl bead Luminex value(MFI) >16,787 = positive
#140 0dpi H5N2 TY LPAI	N	N	-15	N	0.049	152	43	42	490	3497	20286
141 0dpi H5N2 TY	N	N	-11	N	0.036	188	37	40	710	2864	19959
142 0dpi H5N2 TY	N	N	-9	N	0.146	139	59	48	985	2927	20023
143 0dpi H5N2 TY	N	N	-6	N	0.033	134	39	38	1238	2800	20165
144 0dpi H5N2 TY	N	N	-11	N	0.033	188	41	39	376	2628	18861
145 0dpi H5N2 TY	N	N	-8	N	0.192	132	69	47	2051	3570	20740
146 0dpi H5N2 TY	N	N	-9	N	0.096	113	32	41	2486	3618	20223
147 0dpi H5N2 TY	N	N	-8	N	0.096	313	32	44	1432	3522	19956
148 0dpi H5N2 TY	N	N	-10	N	0.117	113	33	35	3462	3591	20227
149 0dpi H5N2 TY	N	N	-9	N	0.057	123	41	51	1064	3645	20092
140 7dpi H5N2 TY	N	N	26	P	0.079	18523	3347	68	229	3055	19870
141 7dpi H5N2 TY	N	N	47	P	0.079	10980	1484	42	335	2837	18631
142 7dpi H5N2 TY	N	N	45	P	0.030	16374	7108	58	443	2882	19778
143 7dpi H5N2 TY	256	N	37	P	0.030	16867	2101	64	678	3082	20400
144 7dpi H5N2 TY	N	N	28	P	-0.001	12152	2190	51	276	2878	19321
145 7dpi H5N2 TY	N	N	24	P	-0.001	9004	807	55	729	3386	21120
146 7dpi H5N2 TY	N	N	38	Susp	0.038	22499	10180	57	1132	3355	19873
147 7dpi H5N2 TY	N	N	32	P	-0.003	14676	3088	61	732	3804	19656
148 7dpi H5N2 TY	N	N	40	Susp	0.194	24004	17169	96	1976	3386	20375
140 14dpi H5N2 TY	8	N	37	P	0.133	24634	11054	162	213	3178	20480
141 14dpi H5N2 TY	16	N	70	P	0.045	25190	10265	95	405	3268	20443
142 14dpi H5N2 TY	4/N	N	31	P	0.045	24919	10865	78	353	3128	19861
143 14dpi H5N2 TY	16	N	61	P	0.005	24781	8079	35	146	3049	20915
144 14dpi H5N2 TY	8	N	62	P	0.173	24633	13407	236	178	3295	20289
145 14dpi H5N2 TY	16	N	49	P	-0.030	25445	9530	142	274	3427	20373
146 14dpi H5N2 TY	4/N	N	66	P	-0.008	25154	3850	138	373	2806	20438
147 14dpi H5N2 TY	16	N	36	P	0.083	14703	2110	68	378	3049	18142
148 14dpi H5N2 TY	16	N	70	N	0.094	22549	13743	78	197	2852	19176
149 14dpi H5N2 TY	8	N	29	P	0.094	24158	7614	99	247	3385	19142
150 14dpi H5N2 TY	32	N	12	N	0.014	22471	21417	108	425	3447	20772
151 14dpi H5N2 TY	128	N	52	Susp	0.012	24447	23928	171	223	3209	20026
140 28dpi H5N2 TY	32	N	53	P	0.095	25241	14393	250	419	3628	19848
141 28dpi H5N2 TY	16	N	51	P	0.066	24497	6279	279	244	2875	20585
142 28dpi H5N2 TY	16	N	42	P	-0.016	23899	9288	122	228	2755	18589
143 28dpi H5N2 TY	16	N	38	N	0.028	24192	13215	120	176	2921	18954
144 28dpi H5N2 TY	8	N	41	P	0.036	24615	5474	164	223	3286	18475
145 28dpi H5N2 TY	64	N	25	P	0.122	16881	10962	83	265	3088	19450
146 28dpi H5N2 TY	64	N	21	N	-0.002	23364	23943	99	199	3008	18411
147 28dpi H5N2 TY	16	N	58	P	-0.019	25519	7212	376	193	3705	19726

Table 9. H7N8 infected Chickens with full Gold Standard and Q-FMIA comparison Time Course

	Gold Standard Methods					Quadriplex FMIA Method					
	H5 HI Result >8 units = positive	H7 HI Result with homologous virus >8 units = positive	NP-ELISA % inhibition results >25 = Positive	AGID Results	APMV-1 IDEXX results >0.2 =positive	AIV-NP Bead Luminex Value (MFI) >1253 = positive	AIV-H5 bead Luminex value (MFI) >1585 = positive	AIV-H7 bead Luminex value (MFI) >2585 = positive	APMV-1-NC bead Luminex value (MFI) >3595 = positive	Internal Serum Ctrl bead Luminex value(MFI) >2,000 = positive	Detection Antibody Ctrl bead Luminex value(MFI) >16,787 = positive
#125 0dpi H7N8 CK	N	N	1	N	0.011	156	140	248	336	9513	20428
126 0dpi H7N8 CK	N	N	3	N	0.143	296	1369	2327	736	10216	20859
127 0dpi H7N8 CK	N	N	4	N	0.157	385	1318	893	566	9740	20412
128 0dpi H7N8 CK	N	N	-2	N	0.082	123	125	141	246	6994	20902
129 0dpi H7N8 CK	N	N	-3	N	0.082	292	305	321	406	9823	21139
130 0dpi H7N8 CK	N	N	2	N	0.157	227	900	1257	759	9149	20610
131 0dpi H7N8 CK	N	N	-2	N	0.110	180	354	484	290	7532	20614
132 0dpi H7N8 CK	N	N	-3	N	0.110	139	166	157	186	8662	20832
133 0dpi H7N8 CK	N	N	8	N	0.036	693	261	425	846	10368	20791
134 0dpi H7N8 CK	N	N	-5	N	0.036	239	128	615	345	9219	21190
125 7dpi H7N8 CK	N	N	-6	N	0.014	120	127	190	252	9443	21041
127 7dpi H7N8 CK	N	N	-1	N	0.022	330	1073	911	539	9485	20600
129 7dpi H7N8 CK	N	16	80	P	0.291	25210	1627	10618	4304	9331	21222
131 7dpi H7N8 CK	N	N	-4	N	0.084	136	350	662	239	9651	20822
132 7dpi H7N8 CK	N	N	-6	N	0.130	233	180	280	239	9330	21028
133 7dpi H7N8 CK	N	N	11	N	0.115	474	208	341	504	9745	21267
126 14dpi H7N8 CK	N	16	40	P	0.047	23877	609	10714	660	9908	20346
127 14dpi H7N8 CK	N	N	3	N	0.057	217	580	575	387	9302	21280
128 14dpi H7N8 CK	N	N	11	N	-0.001	5784	67	607	277	7712	21001
130 14dpi H7N8 CK	N	16	54	P	0.028	22980	1103	12948	1481	9571	20869
132 14dpi H7N8 CK	N	N	3	N	0.142	1003	133	614	372	9032	21422
133 14dpi H7N8 CK	N	8/N	16	Susp	0.100	14366	255	2229	752	9449	19576
134 14dpi H7N8 CK	N	N	46	P	0.027	23846	350	4160	1089	9551	20378
125 21dpi H7N8 CK	N	N	15	Susp	0.001	9918	87	1028	214	8961	20325
126 21dpi H7N8 CK	N	64	40	P	0.069	24549	578	18361	398	10212	20765
127 21dpi H7N8 CK	N	32	50	P	0.108	23453	618	6789	438	8934	19541
128 21dpi H7N8 CK	N	4/N	15	P	0.118	8511	111	1379	231	8207	20300
129 21dpi H7N8 CK	N	256	83	P	0.145	24935	763	24097	533	9592	20868
130 21dpi H7N8 CK	N	64	39	Susp	0.117	23954	624	21589	540	9730	21323
132 21dpi H7N8 CK	N	16	18	P	-0.006	20631	58	2840	236	8738	20305
131 21dpi H7N8 CK	N	16	40	P	0.031	23856	474	11271	487	9031	20432
133 21dpi H7N8 CK	N	64	31	P	0.119	13793	204	8235	615	9270	20136
134 21dpi H7N8 CK	N	256	86	P	0.215	24767	2128	24326	7554	9397	20480
125 29dpi H7N8 CK	N	N	7	N	0.002	5685	98	962	212	8297	20584
127 29dpi H7N8 CK	N	16	20	P	0.075	16517	736	7394	410	10277	20841
128 29dpi H7N8 CK	N	4/N	4	Susp	0.075	7123	294	4202	406	8853	20809
129 29dpi H7N8 CK	N	256	74	P	0.044	24763	306	23789	319	9360	20405
130 29dpi H7N8 CK	N	64	36	Susp	0.003	24723	1895	18720	679	9271	20140
131 29dpi H7N8 CK	N	16	39	P	0.041	23190	277	13232	308	8478	20405
132 29dpi H7N8 CK	N	8	5	P	0.061	5492	88	3199	210	8577	20784
133 29dpi H7N8 CK	N	16	12	P	0.012	10067	158	7294	378	9186	21553

Table 10. NDV vaccinated Chickens with full Gold Standard and Q-FMIA comparison Time Course

	Gold Standard Methods			Quadriplex FMIA Method					
	AIV IDEXX ELISA % inhibition results <0.5 = Positive	NDV HI Results >8 units = positive	APMV-1 IDEXX results >0.2 =positive	AIV-NP Bead Luminex Value (MFI) >1253 = positive	AIV-H5 bead Luminex value (MFI) >1585 = positive	AIV-H7 bead Luminex value (MFI) >2585 = positive	APMV-1-NC bead Luminex value (MFI) >3595 = positive	Internal Serum Ctrl bead Luminex value(MFI) >2,000 = positive	Detection Antibody Ctrl bead Luminex value(MFI) >16,787 = positive
16 CK NDVvacc prebleed	N	0	N	153	76	99	121	12528	20757
17 CK NDVvacc prebleed	N	0	N	141	82	123	72	10975	20692
18 CK NDVvacc prebleed	N	0	N	124	56	37	51	11389	21682
19 CK NDVvacc prebleed	N	0	N	120	43	46	47	11277	20158
20 CK NDVvacc prebleed	N	0	N	368	100	182	252	12052	19421
21 CK NDVvacc prebleed	N	16	N	176	209	492	10475	13621	21075
22 CK NDVvacc prebleed	N	0	N	217	133	256	467	13437	20775
23 CK NDVvacc prebleed	N	0	N	298	129	341	5394	11978	20388
24 CK NDVvacc prebleed	N	0	N	168	89	132	223	12672	19213
25 CK NDVvacc prebleed	N	16	N	290	84	109	3694	13434	21193
26 CK NDVvacc prebleed	N	64	P	150	104	155	7986	13144	20297
27 CK NDVvacc prebleed	N	64	P	149	82	72	9423	13848	19798
28 CK NDVvacc prebleed	N	128	P	315	156	438	12513	12371	20000
29 CK NDVvacc prebleed	N	64	P	188	123	159	22011	12544	21868
30 CK NDVvacc prebleed	N	256	P	535	4751	194	14619	12717	22094
31 CK NDVvacc prebleed	N	8	P	185	177	185	2966	13348	21735
17 CK NDVvacc PMV-1 columbia 6dpi	N	16	P	186	73	74	563	10775	19948
20 CK NDVvacc PMV-1 columbia 6dpi	N	128	P	467	169	223	2175	11330	21041
21 CK NDVvacc PMV-1 columbia 7dpi	N	4096	P	715	233	430	25351	13927	18646
23 CK NDVvacc PMV-1 columbia 7dpi	N	2048	P	1342	391	234	15902	8436	22291
25 CK NDVvacc PMV-1 columbia 7dpi	N	4096	P	574	169	185	25180	14464	20707
26 CK NDVvacc PMV-1 columbia 7dpi	N	2048	P	230	161	174	25398	12984	20255
27 CK NDVvacc PMV-1 columbia 7dpi	N	4096	P	404	123	98	24748	13506	19503
28 CK NDVvacc PMV-1 columbia 7dpi	N	4096	P	235	129	234	24976	13020	20487
29 CK NDVvacc PMV-1 columbia 7dpi	N	512	P	178	94	117	24935	11767	19609
30 CK NDVvacc PMV-1 columbia 7dpi	N	4096	P	506	5020	284	25472	12594	21194
31 CK NDVvacc PMV-1 columbia 7dpi	N	0	P	1064	391	316	25537	14317	21484
21 CK NDVvacc PMV-1 columbia 14dpi	N	4096	P	330	130	150	25314	13319	20357
23 CK NDVvacc PMV-1 columbia 14dpi	N	4096	P	256	89	101	23249	12067	20871
25 CK NDVvacc PMV-1 columbia 14dpi	N	4096	P	208	100	112	25022	13317	17823
26 CK NDVvacc PMV-1 columbia 14dpi	N	4096	P	153	107	110	25287	13546	20470
27 CK NDVvacc PMV-1 columbia 14dpi	N	4096	P	634	108	91	24936	12805	19883
28 CK NDVvacc PMV-1 columbia 14dpi	N	4096	P	165	120	114	24858	14008	20907
29 CK NDVvacc PMV-1 columbia 14dpi	N	4096	P	168	111	108	25374	12785	21086
30 CK NDVvacc PMV-1 columbia 14dpi	N	4096	P	200	1968	90	25375	12677	19740
31 CK NDVvacc PMV-1 columbia 14dpi	N	0	P	164	137	103	25314	12198	19079
21 CK NDVvacc PMV-1 columbia 23dpi	N	4096	P	166	110	179	24298	12295	18827
23 CK NDVvacc PMV-1 columbia 23dpi	N	512	P	255	120	161	22870	10744	21165
25 CK NDVvacc PMV-1 columbia 23dpi	N	4096	P	261	125	241	24394	13541	20246
26 CK NDVvacc PMV-1 columbia 23dpi	N	4096	P	144	73	65	25117	12195	19502
27 CK NDVvacc PMV-1 columbia 23dpi	N	4096	P	1090	111	100	25022	13289	20597
28 CK NDVvacc PMV-1 columbia 23dpi	N	4096	P	171	86	83	24470	12925	20347
29 CK NDVvacc PMV-1 columbia 23dpi	N	4096	P	155	72	113	25379	12382	20909
30 CK NDVvacc PMV-1 columbia 23dpi	N	4096	P	224	2045	86	24549	12598	20706
31 CK NDVvacc PMV-1 columbia 23dpi	N	0	P	173	134	118	25138	13147	20246

5.0 General Discussion

The project has produced two functional serological assays with the Q-FMIA built on the foundation of the Duplex format of the assay. The optimizations that were made during development of the duplex were positive additions that were carried over seamlessly to the Q-FMIA.

There were a few methodological changes that were realized since the beginning of duplex assay optimization and were also put into practice in the Q-FMIA to the same beneficial effect. The first of which was a sample dilution change from 1 in 100; to 1 in 500 of our 1% Donkey serum + PBS dilution solution which made a large improvement of the assay's abilities to mitigate background signal from field samples in the early stages of development. This change greatly increased the sensitivity of the assay by allowing a reduction of the number of calculated false positives. Another major change in the assay protocol was the addition of a two-hour blocking step with 5% Donkey serum + PBS between aliquoting the beads for use and before allowing them into contact with test sera. This reduced background signal of the beads and also helped to prevent false positive reactions. The transition from the use of a manual magnetic plate for bead washing, to an automated magnetic plate washer also reduced the time required to complete the assay, as well as reducing the possibility of contamination between wells of the plate.

Choosing Baculovirus as the expression system for the recombinant proteins of the assay has advantages and disadvantages. The Baculovirus expression system is beneficial because it allows the proteins to have the proper folding and conformational epitopes compared to the exclusively linear epitopes from proteins produced in bacterial culture. However despite the benefits, there is some evidence shown in the Western blot results that suggest that some remnants of Baculovirus protein were isolated along with the desired protein. This small amount of contaminating protein could have an adverse effect on the binding of test sera antibodies to the protein-coupled beads. This contamination,

could also be the source of increased background signal in some samples and even be a factor in the reduced overall sensitivity of the assay.

Specific Pathogen Free sera from chickens was utilized during the threshold determination, and optimal operating range analysis. This sera was expected to have zero fluorescent signal detected by the assay but still produced a minimal level of background signal when tested. This reactivity suggests that very low levels of background signal noise are to be expected from any sera tested on the assay, even if it is known to be negative for antibodies against AIV or NDV.

Threshold determination was performed with unique field sera based on the results of gold standard methods, to determine a single acceptable threshold value. Other methods of threshold determination allow for multiple threshold values to be calculated, or three categories of variables (positive, questionable, and negative). These different methods allow threshold values to range between a high-sensitivity model and high-specificity model. This allows the same assay to be used under different circumstances, where the decision of which method to use will depend on the planned use of the assay and may differ on a case-by-case basis (OIE 2008).

The threshold value behaves like a moving slider along a line of values; moving it one way increases the sensitivity while decreasing the specificity, and moving the threshold the opposite direction affects the values conversely. We found during analysis that using the thresholds maximizing sensitivity and specificity resulted in high quantities of false positives, because large amounts of specificity were sacrificed to gain assay sensitivity. Determining the optimal threshold value to be suitable for the widest range of sera in the widest range of scenarios, the threshold values were chosen based on the value with the highest associated ROC accuracy. This was opposite to the way that thresholds were chosen for the duplex assay, because of the limited experience we had with

interpreting ROC data. The duplex assay data was also analyzed together with all field and experimental sera mixed to generate the threshold values without a separate test of assay performance.

In the following tables, the duplex analytical sensitivities and specificities are compared side-by-side with the Quadriplex values showing the differences between them (Table 5). It is important to note that the Duplex threshold values are based on optimal sensitivity and specificity, while the Q-FMIA values are highlighting ROC-Accuracy. In the alternate scenario, the Q-FMIA threshold values have been adjusted to mirror the methods used for calculating the duplex threshold values which highlight sensitivity and specificity over ROC-accuracy (Table 6). With the exception of the AIV-NP threshold which remains the same, the remaining Q-FMIA threshold values are notably decreased from the final values. These thresholds are based on the level of signal where the highest level of sensitivity and specificity occur together.

The AIV-H7 bead experiences the greatest changes while adjusting the threshold value. This is possibly due to the AIV-H7 bead having the lowest number of positive samples available for testing. With limited samples, even a single numeric increase in the number of false positives or false negatives can greatly affect the sensitivity and specificity percentages. The thresholds based on ROC-accuracy are better than those based on sensitivity and specificity for the AIV-H7 bead. The threshold can be adjusted in an attempt to minimize these values, but without additional sera for testing, it is impossible to eliminate both the false positives and false negatives in the AIV-H7 analyte while maintaining the ability to test field and experimental sera effectively. The sensitivity and specificity highlighted threshold did have an effect on diagnostic performance as well (Table 7). However, if the assay were to be used solely with field sera, or experimental sera, the threshold could be adjusted for these specialized cases and would perform more ideally. It is also completely feasible to use a combination of threshold values, or some analytes to be based heavily on ROC-accuracy while others are based on DSe or DSp, but for the purpose of the analysis, we have kept each method separate for simplification.

The thresholds based on ASe and ASp were very low, and close to the background signal of some field sera which caused a high number of false positives. Threshold values very near the assay background level may increase the sensitivity of the assay, but will decrease the specificity. For this reason we chose to use the ROC-Accuracy based threshold values because they exhibited higher diagnostic specificity. Despite the somewhat mediocre sensitivity of the assay, when compared to the other gold standard methods, the Q-FMIA shows very good performance with test sera. Here we show three different combinations of samples being tested with the Q-FMIA compared to the gold standard methods. Firstly an H5N2 challenged group of Turkeys (Table 8), followed by an H7N8 challenged group of Chickens (Table 9), and finally an NDV vaccination of Chickens (Table 10). Even though the sensitivity of the assay is low in the AIV-H5 and AIV-H7 beads, the overall ability of the assay to correctly differentiate positive samples from negatives samples is acceptable.

While testing the limits of the internal assay controls we used a set of "H7 influenza outbreak" duck and geese sera which was tested on an H7 cELISA (Figure 19). The luminex results did not agree with the H7 cELISA that was used for confirmation and revealed that the limit of the Q-FMIA's secondary detection antibody did not include sera from ducks or geese as the serum control showed results greatly reduced from the acceptable value. As discussed earlier, the chicken and turkey immunoglobulins are similar enough to be detected by the same antibody, but the duck and goose immunoglobulins are too different.

As the two assays were built upon the same framework, the challenges faced during the development of the duplex were mirrored during the development of the Q-FMIA. A large realization was that the assay is only as good as the serum you choose to test on it. And the accumulated data from the gold standard methods to which the assay is compared also makes a huge impact. Producing results from the gold standard methods that is reliable is the most important aspect of threshold determination.

6.0 General Conclusions

The duplex assay and the quadriplex assay were both developed and optimized to be functional serological assays that can successfully detect, identify, and differentiate between antibodies produced as a result of influenza infection or Newcastle disease infection/vaccination in poultry.

The assay performs at a level which is comparable to other currently used serological assays, with the added benefits of being able to detect multiple analyses simultaneously, and rapidly.

The assay threshold values calculated via ROC analysis are flexible and can be adjusted depending on the type of sera the assay will be testing. The current assay thresholds are suitable for use with both field sera as well as experimental sera despite its seemingly low values of sensitivity.

Assay development, and the statistical analysis carried out during the process is completely dependent on the quality of results generated from the gold standard methods and which methods are chosen to represent the gold standard. The quality of the serum samples also plays a large role.

We proved that the development of an assay with the ability to detect and sub-type these viruses is possible with the use of recombinantly generated proteins from the Baculovirus system, and that a duplex assay could be successfully scaled up to a quadriplex with internal controls without having to completely re-work the entire protocol. The luminex beads are completely independent of one another and allow for seamless addition of analytes to expand the assay without any difficulty of cross-reactivity between beads.

We also conclude that the diagnostic sensitivity and specificity calculated here are merely our best estimates and require further testing for these values to be considered fully accurate.

7.0 Future Directions

Future directions of the assay should involve additional testing of large sets of samples from different birds without repeated time-course samples containing both positive and negative serum samples to increase the diagnostic sensitivity and specificity of the assay. Once the assay has acceptable levels of DSe and DSp, the next step should be to evaluate the Reproducibility of the assay by having the assay performed by at least two other technicians at the host lab. The reagents involved in the test should be reproduced by these technicians to ensure the protocols are accurate and functional. The next step should include testing of Reproducibility of the assay by sending the reagents and a set of identical standard samples to at least 3 labs in distinctly different regions or countries where the assay would be put into use. This would test the assay's ability to perform with the random variables introduced in the different laboratory environments. Only after these steps would the assay be considered truly validated for the intended purpose and be suitable for use with actual surveillance samples, although continually monitoring the assay to ensure it retains the original level of performance is important.

It may also be worthwhile to change the format of the assay from a direct-detection of antibody method to a competitive format involving a developed monoclonal antibody which would allow the testing of a broader range of sera. This would require a large amount of work and the entire assay would have to be re-optimized and validated again from the ground up.

Possible advancements to the assay would involve the addition of new bead analytes with the ability to detect antibody responses to other known avian diseases, or additional sub-types of AIV or APMV. Just as the H5 and H7 beads were added to the assay, new additions could be made to enhance the multiplex ability of the assay. If expanded broadly enough, future avian serology tests may consist of only a few tests which would effectively screen for all known diseases important in the poultry

industry. It would also be interesting to test a wide range of NDV strains to make sure the APMV-1-NC bead is similar enough to detect all strains. If there is a chance that the vaccines are no longer as effective against some strains / serotypes of NDV, then it is possible that the APMV-1-NC bead cannot detect them all either.

8.0 Full Thesis Bibliography

Abdelwhab, E. M., Veits, J., & Mettenleiter, T. C. (2014). Prevalence and control of H7 avian influenza viruses in birds and humans. *Epidemiology and Infection, 142*(5), 896-920. doi:10.1017/S0950268813003324; 10.1017/S0950268813003324

Abolnik, C. (2014). A current review of avian influenza in pigeons and doves (columbidae). *Veterinary Microbiology, 170*(3-4), 181-196. doi:10.1016/j.vetmic.2014.02.042; 10.1016/j.vetmic.2014.02.042

Aldous, E. W., & Alexander, D. J. (2001). Detection and differentiation of newcastle disease virus (avian paramyxovirus type 1). *Avian Pathology, 30*(2), 117-128. doi:10.1080/03079450120044515

Alexander, D. J. (Ed.). (1988). *NDV - an avian paramyxovirus structure*. Boston, MA: Kluwer Academic Publishers.

Alexander, D. J. (1988). *Newcastle disease virus - an avian paramyxovirus.* Boston, MA: Kluwer Academic Publishers.

Alexander, D. J. (1988). Newcastle disease: Methods of spread. *Newcastle disease* (pp. 256-272). Boston, MA: Kluwer Academic Publishers.

Alexander, D. J. (1993). In McFerran J. B., McNulty M. S. (Eds.), *Paramyxovirus infection* (3rd ed.). Amsterdam: Elsevier Science Publishing Company.

Alexander, D. J., & Brown, I. H. (2009). History of highly pathogenic avian influenza. *Rev. Sci. Tech. Off. Epiz., 28*(1), 19-38.

Alexander, D. J., & Chettle, N. J. (1978). Relationship of parakeet/Netherlands/ 449/75 virus to other avian paramyxoviruses. *Research in Veterinary Science,* (25), 105-106.

Alexander, D. J., & Collins, M. S. (1981). The structural polypeptides of avian paramyxoviruses. *Archives of Virology,* (67), 309-323.

Alexander, D. J., & Senne, D. A. (Eds.). (2008). *Newcastle disease, other avian paramyxoviruses, and pneumovirus infections* (12th ed.). Ames, Iowa: Blackwell Publishing.

Alexander, D. J., & Allan, W. H. (1974). Newcastle disease virus pathotypes. *Avian Pathology: Journal of the W.V.P.A, 3*(4), 269-278. doi:10.1080/03079457409353840

Alexander, D. J. (2000). A review of avian influenza in different bird species. *Veterinary Microbiology, 74*(1–2), 3-13. doi:10.1016/S0378-1135(00)00160-7

Anbarasu, D., Raja, C. P., & Raja, A. (2013). Multiplex analysis of cytokines/chemokines as biomarkers that differentiate healthy contacts from tuberculosis patients in high endemic settings. *Cytokine, 61*(3), 747-754. doi:10.1016/j.cyto.2012.12.031; 10.1016/j.cyto.2012.12.031

Anderson, S., Wakeley, P., Wibberley, G., Webster, K., & Sawyer, J. (2011). Development and evaluation of a luminex multiplex serology assay to detect antibodies to bovine herpes virus 1, parainfluenza 3 virus, bovine viral diarrhoea virus, and bovine respiratory syncytial virus, with comparison to existing ELISA detection methods. *Journal of Immunological Methods, 366*(1-2), 79-88. doi:10.1016/j.jim.2011.01.010; 10.1016/j.jim.2011.01.010

Anderson, S., Wakeley, P., Wibberley, G., Webster, K., & Sawyer, J. (2011). Development and evaluation of a luminex multiplex serology assay to detect antibodies to bovine herpes virus 1, parainfluenza 3 virus, bovine viral diarrhoea virus, and bovine respiratory syncytial virus, with comparison to existing ELISA detection methods. *Journal of Immunological Methods, 366*(1-2), 79-88. doi:10.1016/j.jim.2011.01.010; 10.1016/j.jim.2011.01.010

Andersson, G., Ekre, H. P., Alm, G., & Perlmann, P. (1989). Monoclonal antibody two-site ELISA for human IFN-gamma adaptation for determinations in human serum or plasma. *Journal of Immunological Methods, 125*(1-2), 89-96.

Balsari, A., & Caruso, A. (1997). Natural antibodies to IL-2. *Biotherapy (Dordrecht, Netherlands), 10*(1), 25-28.

Bar-Shira, E., Sklan, D., & Friedman, A. (2003). Establishment of immune competence in the avian GALT during the immediate post-hatch period. *Developmental and Comparative Immunology, 27*(2), 147-157.

Basile, A. J., Horiuchi, K., Panella, A. J., Laven, J., Kosoy, O., Lanciotti, R. S., Biggerstaff, B. J.- *Multiplex microsphere immunoassays for the detection of IgM and IgG to arboviral diseases* - Public Library of Science.

Basile, A. J., Horiuchi, K., Panella, A. J., Laven, J., Kosoy, O., Lanciotti, R. S., Biggerstaff, B. J. (2013). Multiplex microsphere immunoassays for the detection of IgM and IgG to arboviral diseases. *PloS One, 8*(9), e75670. doi:10.1371/journal.pone.0075670; 10.1371/journal.pone.0075670

Belzhelarskaia, S. N. (2011). Baculovirus expression systems for recombinant protein production in insect and mammalian cells. *Molekuliarnaia Biologiia, 45*(1), 142-159.

Bencina, M., Cizelj, I., Bercic, R. L., Narat, M., Bencina, D., & Dovc, P. (2014). Shared epitopes of avian immunoglobulin light chains. *Veterinary Immunology and Immunopathology, 158*(3-4), 175-181. doi:10.1016/j.vetimm.2014.01.003; 10.1016/j.vetimm.2014.01.003

Benson, E. R., Alphin, R. L., Rankin, M. K., Caputo, M. P., Hougentogler, D. P., & Johnson, A. L. (2012). Mass emergency water-based foam depopulation of poultry. *Avian Diseases, 56*(4 Suppl), 891-896.

Bergmann, I. E., Neitzert, E., Malirat, V., Ortiz, S., Colling, A., Sanchez, C., & Correa Melo, E. (2003). Rapid serological profiling by enzyme-linked immunosorbent assay and its use as an epidemiological indicator of foot-and-mouth disease viral activity. *Archives of Virology, 148*(5), 891-901. doi:10.1007/s00705-002-0965-5

Berhane, Y., Joseph, T., Kehler, H., Hisanaga, T., Embury-Hyatt, C., Diederich, S., Pasick, J. (2014). Characterization of a low pathogenic avian influenza H5N2 virus isolated from a turkey breeder flock in manitoba, canada. *Avian Diseases, 58*(1), 1-7.

Berhane, Y., Kehler, H., Handel, K., Hisanaga, T., Xu, W., Ojkic, D., & Pasick, J. (2012). Molecular and antigenic characterization of reassortant H3N2 viruses from turkeys with a unique constellation of pandemic H1N1 internal genes. *PloS One, 7*(3), e32858. doi:10.1371/journal.pone.0032858; 10.1371/journal.pone.0032858

Biagini, R., Schlottmann, S., Sammons, D., Smith, J., Snawder, J., Striley, C., Weissman, D. (2003). Method for simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides. *Clinical and Diagnostic Laboratory Immunology, 10*(5), 744-750.

Boscato, L. M., & Stuart, M. C. (1988). Heterophilic antibodies: A problem for all immunoassays. *Clinical Chemistry, 34*(1), 27-33.

Bozza, F. A., Salluh, J. I., Japiassu, A. M., Soares, M., Assis, E. F., Gomes, R. N., Bozza, P. T. (2007). Cytokine profiles as markers of disease severity in sepsis: A multiplex analysis. *Critical Care (London, England), 11*(2), R49. doi:10.1186/cc5783

Briand, F. X., Massin, P., & Jestin, V. (2014). Characterisation of a type 1 avian paramyxovirus belonging to a divergent group. *Veterinary Microbiology, 168*(1), 25-33. doi:10.1016/j.vetmic.2013.10.005; 10.1016/j.vetmic.2013.10.005

Briand, F. X., Henry, A., Brown, P., Massin, P., Jestin, V. (2013). Complete Genome Sequence of a Newcastle Disease Virus Strain Belonging to a Recently Identified Genotype. *Genome Announcements.* 1(1)e00100-12. Doi:10.1128/genomeA.00100-12

Brown, J. D., Luttrell, M. P., Berghaus, R. D., Kistler, W., Keeler, S. P., Howey, A., Stallknecht, D. E. (2010). Prevalence of antibodies to type a influenza virus in wild avian species using two serologic assays. *Journal of Wildlife Diseases, 46*(3), 896-911. doi:10.7589/0090-3558-46.3.896

Campitelli, L., Mogavero, E., De Marco, M. A., Delogu, M., Puzelli, S., Frezza, F., Donatelli, I. (2004). Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in italy. *Virology, 323*(1), 24-36. doi:10.1016/j.virol.2004.02.015

Cappelle, J., Gaidet, N., Iverson, S. A., Takekawa, J. Y., Newman, S. H., Fofana, B., & Gilbert, M. (2011). Characterizing the interface between wild ducks and poultry to evaluate the potential of transmission of avian pathogens. *International Journal of Health Geographics, 10*, 60-072X-10-60. doi:10.1186/1476-072X-10-60; 10.1186/1476-072X-10-60

Cappucci, D. T.,Jr, Johnson, D. C., Brugh, M., Smith, T. M., Jackson, C. F., Pearson, J. E., & Senne, D. A. (1985). Isolation of avian influenza virus (subtype H5N2) from chicken eggs during a natural outbreak. *Avian Diseases, 29*(4), 1195-1200.

Capua, I., & Alexander, D. J. (2004). Human health implications of avian influenza viruses and paramyxoviruses. *European Journal of Clinical Microbiology & Infectious Diseases : Official*

Publication of the European Society of Clinical Microbiology, 23(1), 1-6. doi:10.1007/s10096-003-1059-3

Capua, I., & Alexander, D. J. (2004). Human health implications of avian influenza viruses and paramyxoviruses. *European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology, 23*(1), 1-6. doi:10.1007/s10096-003-1059-3

Capua, I., & Alexander, D. J. (2007). Avian influenza infections in birds--a moving target. *Influenza and Other Respiratory Viruses, 1*(1), 11-18.

Capua, I., & Cattoli, G. (2007). Diagnosing avian influenza infection in vaccinated populations by systems for differentiating infected from vaccinated animals (DIVA). *Developments in Biologicals, 130*, 137-143.

Cen, H., Windler, S. L., Rice, L. S., Zhang, A., & Zhou, H. (2013). Multiplex epitope detection: A new method overcomes limitations of antibody arrays. *Proteomics, 13*(10-11), 1696-1700. doi:10.1002/pmic.201200381; 10.1002/pmic.201200381

Chappell, L., Killian, M. L., & Spackman, E. (2014). Detection of influenza A antibodies in avian serum samples by ELISA. *Methods in Molecular Biology (Clifton, N.J.), 1161*, 151-167. doi:10.1007/978-1-4939-0758-8_14; 10.1007/978-1-4939-0758-8_14

Charlton, B., Crossley, B., & Hietala, S. (2009). Conventional and future diagnostics for avian influenza. *Comparative Immunology, Microbiology and Infectious Diseases, 32*(4), 341-350. doi:10.1016/j.cimid.2008.01.009; 10.1016/j.cimid.2008.01.009

Chen, C., Tsen, H., Lin, C., Yu, B., Chen, C. (2012). Oral administration of a combination of select lactic acid bacteria strains to reduce the salmonella invasion and inflammation of broiler chicks. *Poultry Science, 91*(9), 2139-2147. doi:10.3382/ps.2012-02237

Chen, T., Lee, F., Lin, Y., Pan, C., Shih, C., Lee, M., & Tsai, H. (2013). Development of a luminex assay for the detection of swine antibodies to non-structural proteins of foot-and-mouth disease virus. *Journal of Immunological Methods, 396*(1), 87-95. doi:http://dx.doi.org/10.1016/j.jim.2013.08.002

Chiu, Y. C., Chu, W. Y., Tsao, Z., & Wang, C. H. (2012). Antigen-capture enzyme-linked immunosorbent assays for detection of different H5 avian influenza A virus. *Journal of Veterinary Diagnostic Investigation : Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc., 24*(4), 716-718. doi:10.1177/1040638712446629

Chiu, Y. C., Chu, W. Y., Tsao, Z., & Wang, C. H. (2012). Antigen-capture enzyme-linked immunosorbent assays for detection of different H5 avian influenza A virus. *Journal of Veterinary Diagnostic Investigation : Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc., 24*(4), 716-718. doi:10.1177/1040638712446629; 10.1177/1040638712446629

Choi, J. W., Kim, J. K., Seo, H. W., Cho, B. W., Song, G., & Han, J. Y. (2010). Molecular cloning and comparative analysis of immunoglobulin heavy chain genes from phasianus colchicus, meleagris gallopavo, and coturnix japonica. *Veterinary Immunology and Immunopathology, 136*(3-4), 248-256. doi:10.1016/j.vetimm.2010.03.014; 10.1016/j.vetimm.2010.03.014

Chowdhury, F., Williams, A., & Johnson, P. (2009). Validation and comparison of two multiplex technologies, luminex and mesoscale discovery, for human cytokine profiling. *Journal of Immunological Methods, 340*(1), 55-64. doi:10.1016/j.jim.2008.10.002; 10.1016/j.jim.2008.10.002

Chowdhury, F., Williams, A., & Johnson, P. (2009). Validation and comparison of two multiplex technologies, luminex® and mesoscale discovery, for human cytokine profiling. *Journal of Immunological Methods, 340*(1), 55-64. doi:http://dx.doi.org.proxy1.lib.umanitoba.ca/10.1016/j.jim.2008.10.002

Christensen, J., Stryhn, H., Vallieres, A., & El Allaki, F. (2011). A scenario tree model for the Canadian notifiable avian influenza surveillance system and its application to estimation of probability of freedom and sample size determination. *Preventive Veterinary Medicine, 99*(2-4), 161-175. doi:10.1016/j.prevetmed.2011.01.005; 10.1016/j.prevetmed.2011.01.005

Claes, G., Vangeluwe, D., Van der Stede, Y., van den Berg, T., Lambrecht, B., & Marche, S. (2012). Evaluation of four enzyme-linked immunosorbent assays for the serologic survey of avian influenza in wild bird species. *Avian Diseases, 56*(4 Suppl), 949-954.

Clavijo, A., Hole, K., Li, M., Collingnon, B. (2006). Simultaneous detection of antibodies to foot-and-mouth disease non-structural proteins 3ABC, 3D, 3A and 3B by a multiplexed luminex assay to differentiate infected from vaccinated cattle. *Vaccine, 24*(10), 1693-1704.

Comin, A., Toft, N., Stegeman, A., Klinkenberg, D., & Marangon, S. (2013). Serological diagnosis of avian influenza in poultry: Is the haemagglutination inhibition test really the 'gold standard'? *Influenza and Other Respiratory Viruses, 7*(3), 257-264. doi:10.1111/j.1750-2659.2012.00391.x; 10.1111/j.1750-2659.2012.00391.x

Costa-Hurtado, M., Afonso, C. L., Miller, P. J., Spackman, E., Kapczynski, D. R., Swayne, D. E., Pantin-Jackwood, M. (2014). Virus interference between H7N2 low pathogenic avian influenza virus and lentogenic newcastle disease virus in experimental co-infections in chickens and turkeys. *Veterinary Research, 45*, 1-9716-45-1. doi:10.1186/1297-9716-45-1; 10.1186/1297-9716-45-1

Curran, J. M., Robertson, I. D., Ellis, T. M., & Selleck, P. W. (2014). Evaluation of avian influenza serologic and virologic diagnostic methods in wild anseriformes and charadriiformes. *Avian Diseases, 58*(1), 53-59.

Dai, Y., Liu, M., Cheng, X., Shen, X., Wei, Y., Zhou, S., Ding, C. (2013). Infectivity and pathogenicity of Newcastle disease virus strains of different avian origin and different virulence for mallard ducklings. *Avian Diseases, 57*(1), 8-14.

Davidson, I., Nagar, S., Haddas, R., Ben-Shabat, M., Golender, N., Lapin, E., Perk, S. (2010). Avian influenza virus H9N2 survival at different temperatures and pHs. *Avian Diseases, 54*(1 Suppl), 725-728.

de Jager, W., Prakken, B. J., Bijlsma, J. W. J., Kuis, W., & Rijkers, G. T. (2005). Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. *Journal of Immunological Methods, 300*(1–2), 124-135. doi:http://dx.doi.org.proxy1.lib.umanitoba.ca/10.1016/j.jim.2005.03.009

De Marco, M. A., Valentini, A., Foni, E., Savarese, M. C., Cotti, C., Chiapponi, C., Delogu, M. (2014). Is there a relation between genetic or social groups of mallard ducks and the circulation of low pathogenic avian influenza viruses? *Veterinary Microbiology, 170*(3-4), 418-424. doi:10.1016/j.vetmic.2014.03.001; 10.1016/j.vetmic.2014.03.001

de Wit, J. J., van de Sande, H. W., Counotte, G. H., & Wellenberg, G. J. (2007). Analyses of the results of different test systems in the 2005 global proficiency testing schemes for infectious bursal disease virus and Newcastle disease virus antibody detection in chicken serum. *Avian Pathology : Journal of the W.V.P.A, 36*(2), 177-183. doi:10.1080/03079450601105676

Deregt, D., Furukawa-Stoffer, T. L., Tokaryk, K. L., Pasick, J., Hughes, K. M., Hooper-McGrevy, K., Baxi, M. K. (2006). A microsphere immunoassay for detection of antibodies to avian influenza virus. *Journal of Virological Methods, 137*(1), 88-94. doi:10.1016/j.jviromet.2006.06.004

Deregt, D., Furukawa-Stoffer, T. L., Tokaryk, K. L., Pasick, J., Hughes, K. M., Hooper-McGrevy, K., Baxi, M. K. (2006). A microsphere immunoassay for detection of antibodies to avian influenza virus. *Journal of Virological Methods, 137*(1), 88-94. doi:10.1016/j.jviromet.2006.06.004

Deregt, D., Furukawa-Stoffer, T. L., Tokaryk, K. L., Pasick, J., Hughes, K. M., Hooper-McGrevy, K., Baxi, M. K. (2006). A microsphere immunoassay for detection of antibodies to avian influenza virus. *Journal of Virological Methods, 137*(1), 88-94. doi:10.1016/j.jviromet.2006.06.004

Desvaux, S., Garcia, J. M., Nguyen, T. D., Reid, S. A., Bui, N. A., Roger, F., Ellis, T. (2012). Evaluation of serological tests for H5N1 avian influenza on field samples from domestic poultry populations in Vietnam: Consequences for surveillance. *Veterinary Microbiology, 156*(3-4), 277-284. doi:10.1016/j.vetmic.2011.11.010; 10.1016/j.vetmic.2011.11.010

Dias, D., Van Doren, J., Schlottmann, S., Kelly, S., Puchalski, D., Ruiz, W., Esser, M. T. (2005). Optimization and validation of a multiplexed luminex assay to quantify antibodies to neutralizing epitopes on human papillomaviruses 6, 11, 16, and 18. *Clinical and Diagnostic Laboratory Immunology, 12*(8), 959-969. doi:10.1128/CDLI.12.8.959-969.2005

Diel, D. G., da Silva, L. H., Liu, H., Wang, Z., Miller, P. J., & Afonso, C. L. (2012). Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, 12*(8), 1770-1779. doi:10.1016/j.meegid.2012.07.012; 10.1016/j.meegid.2012.07.012

Dorjee, S., Poljak, Z., Revie, C. W., Bridgland, J., McNab, B., Leger, E., & Sanchez, J. (2013). A review of simulation modelling approaches used for the spread of zoonotic influenza viruses in animal and human populations. *Zoonoses and Public Health, 60*(6), 383-411. doi:10.1111/zph.12010; 10.1111/zph.12010

Drummond, J. E., Shaw, E. E., Antonello, J. M., Green, T., Page, G. J., Motley, C. O., Casimiro, D. R. (2008). Design and optimization of a multiplex anti-influenza peptide immunoassay. *Journal of Immunological Methods, 334*(1–2), 11-20. doi:http://dx.doi.org.proxy1.lib.umanitoba.ca/10.1016/j.jim.2008.01.017

Duan, L., Campitelli, L., Fan, X. H., Leung, Y. H., Vijaykrishna, D., Zhang, J. X., Guan, Y. (2007). Characterization of low-pathogenic H5 subtype influenza viruses from Eurasia: Implications for the origin of highly pathogenic H5N1 viruses. *Journal of Virology, 81*(14), 7529-7539. doi:10.1128/JVI.00327-07

Dunbar, S. A. (2006). Applications of luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. *Clinica Chimica Acta; International Journal of Clinical Chemistry, 363*(1-2), 71-82. doi:10.1016/j.cccn.2005.06.023

Fagerland, J. A., & Arp, L. H. (1993). Structure and development of bronchus-associated lymphoid tissue in conventionally reared broiler chickens. *Avian Diseases, 37*(1), 10-18.

Fan, J., Upadhye, S., & Worster, A. (2006). Understanding receiver operating characteristic (ROC) curves. *Canadian Journal of Emergency Medicine, 8*(1), 19-20.

Fawcett, T. (2006). An introduction to ROC analysis. *Pattern Recognition Letters,* (27), 861-874.

Fhied, C., Kanangat, S., & Borgia, J. A. (2014). Development of a bead-based immunoassay to routinely measure vimentin autoantibodies in the clinical setting. *Journal of Immunological Methods,* doi:10.1016/j.jim.2014.03.011; 10.1016/j.jim.2014.03.011

Fries, A. C., Nolting, J. M., Bowman, A. S., Killian, M. L., Wentworth, D. E., & Slemons, R. D. (2014). Genomic analyses detect Eurasian-lineage H10 and additional H14 influenza A viruses recovered from waterfowl in the central united states. *Influenza and Other Respiratory Viruses,* doi:10.1111/irv.12250; 10.1111/irv.12250

Fulton, R. J., McDade, R. L., Smith, P. L., Kienker, L. J., & Kettman, J. R.,Jr. (1997). Advanced multiplexed analysis with the FlowMetrix system. *Clinical Chemistry, 43*(9), 1749-1756.

Garcia, J. M., Lagarde, N., Ma, E. S., de Jong, M. D., & Peiris, J. S. (2010). Optimization and evaluation of an influenza A (H5) pseudotyped lentiviral particle-based serological assay. *Journal of Clinical Virology : The Official Publication of the Pan American Society for Clinical Virology, 47*(1), 29-33. doi:10.1016/j.jcv.2009.10.009; 10.1016/j.jcv.2009.10.009

Gauger, P. C., & Vincent, A. L. (2014). Serum virus neutralization assay for detection and quantitation of serum-neutralizing antibodies to influenza a virus in swine. *Methods in Molecular Biology (Clifton, N.J.), 1161*, 313-324. doi:10.1007/978-1-4939-0758-8_26; 10.1007/978-1-4939-0758-8_26

Ghumman, J. S., & Bankowski, R. A. (1976). In vitro DNA synthesis in lymphocytes from turkeys vaccinated with LaSota, TC, and inactivated Newcastle disease vaccines. *Avian Diseases, 20*(1), 18-31.

Giavedoni, L. D. (2005). Simultaneous detection of multiple cytokines and chemokines from nonhuman primates using luminex technology. *Journal of Immunological Methods, 301*(1–2), 89-101. doi:http://dx.doi.org.proxy1.lib.umanitoba.ca/10.1016/j.jim.2005.03.015

Gimenez-Lirola, L. G., Jiang, Y. H., Sun, D., Hoang, H., Yoon, K. J., Halbur, P. G., & Opriessnig, T. (2014). Simultaneous detection of antibodies against apx toxins ApxI, ApxII, ApxIII, and ApxIV in pigs with known and unknown actinobacillus pleuropneumoniae exposure using a multiplexing liquid array platform. *Clinical and Vaccine Immunology : CVI, 21*(1), 85-95. doi:10.1128/CVI.00451-13; 10.1128/CVI.00451-13

Glick, B. (1985). The ontogeny and microenvironment of the avian thymus and bursa of fabricius: Contribution of specialized cells to the avian immune response. *Advances in Veterinary Science and Comparative Medicine, 30*, 67-90.

Gordon, R. F., & McDade, R. L. (1997). Multiplexed quantification of human IgG, IgA, and IgM with the FlowMetrix system. *Clinical Chemistry, 43*(9), 1799-1801.

Greiner, M., & Gardner, I. A. (2000). Epidemiologic issues in the validation of veterinary diagnostic tests. *Preventive Veterinary Medicine, 45*(1-2), 3-22.

Guionie, O., Guillou-Cloarec, C., Courtois, D., Bougeard, B. S., Amelot, M., & Jestin, V. (2010). Experimental infection of muscovy ducks with highly pathogenic avian influenza virus (H5N1) belonging to clade 2.2. *Avian Diseases, 54*(1 Suppl), 538-547.

Hadge, D., & Ambrosius, H. (1986). Evolution of low molecular weight immunoglobulins. V. degree of antigenic relationship between the 7S immunoglobulins of mammals, birds, and lower vertebrates to the turkey IgY. *Developmental and Comparative Immunology, 10*(3), 377-385.

Hajian-Tilaki, K. O., Hanley, J. A., Joseph, L., & Collet, J. P. (1997). A comparison of parametric and nonparametric approaches to ROC analysis of quantitative diagnostic tests. *Medical Decision Making : An International Journal of the Society for Medical Decision Making, 17*(1), 94-102.

Hall, C. (2004). Impact of avian influenza on U.S. poultry trade relations-2002: H5 or H7 low pathogenic avian influenza. *Annals of the New York Academy of Sciences, 1026*, 47-53. doi:10.1196/annals.1307.006

Hanson, R. P., Brandly, C. A. (1958). Newcastle disease. *Annals of the New York Academy of Sciences, 70*(3), 585-597.

Hanson, R. P., Spalatin, J., & Jacobson, G. S. (1973). The viscerotropic pathotype of Newcastle disease virus. *Avian Diseases, 17*(2), 354-361.

Harder, T. C., Siebert, U., Wohlsein, P., & Vahlenkamp, T. (2013). Influenza A virus infections in marine mammals and terrestrial carnivores. *Berliner Und Munchener Tierarztliche Wochenschrift, 126*(11-12), 500-508.

Hassantoufighi, A., Zhang, H., Sandbulte, M., Gao, J., Manischewitz, J., King, L., Eichelberger, M. C. (2010). A practical influenza neutralization assay to simultaneously quantify hemagglutinin and neuraminidase-inhibiting antibody responses. *Vaccine, 28*(3), 790-797. doi:10.1016/j.vaccine.2009.10.066; 10.1016/j.vaccine.2009.10.066

Havlickova, M., Jirincova, H., Maresova, V., Dzupova, O., & Limberkova, R. (2012). Microneutralization assay in the diagnosis of influenza infection. [Mikroneutralizace v diagnostice chripkove infekce] *Epidemiologie, Mikrobiologie, Imunologie : Casopis Spolecnosti Pro Epidemiologii a Mikrobiologii Ceske Lekarske Spolecnosti J.E.Purkyne, 61*(1-2), 9-14.

Henzler, D. J., Kradel, D. C., Davison, S., Ziegler, A. F., Singletary, D., DeBok, P., Nesselrodt, A. (2003). Epidemiology, production losses, and control measures associated with an outbreak of avian influenza subtype H7N2 in Pennsylvania (1996-98). *Avian Diseases, 47*(3 Suppl), 1022-1036.

Hill, N. J., Takekawa, J. Y., Cardona, C. J., Meixell, B. W., Ackerman, J. T., Runstadler, J. A., & Boyce, W. M. (2012). Cross-seasonal patterns of avian influenza virus in breeding and wintering migratory birds: A flyway perspective. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.), 12*(3), 243-253. doi:10.1089/vbz.2010.0246; 10.1089/vbz.2010.0246

Hirst, G. K. (1941). The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science (New York, N.Y.), 94*(2427), 22-23. doi:10.1126/science.94.2427.22

Hitchman, R. B., Locanto, E., Possee, R. D., & King, L. A. (2011). Optimizing the baculovirus expression vector system. *Methods (San Diego, Calif.), 55*(1), 52-57. doi:10.1016/j.ymeth.2011.06.011; 10.1016/j.ymeth.2011.06.011

Hitchman, R. B., Possee, R. D., & King, L. A. (2009). Baculovirus expression systems for recombinant protein production in insect cells. *Recent Patents on Biotechnology, 3*(1), 46-54.

Hoffmann, E., Stech, J., Guan, Y., Webster, R. G., & Perez, D. R. (2001). Universal primer set for the full-length amplification of all influenza A viruses. *Archives of Virology, 146*(12), 2275-2289.

Horan, P. K., Schenck, E. A., Abraham, G. N., & Kloszewski, M. D. (1979). In Nakamure R. M., Dito W. R. and Tucker E. S. (Eds.), *Fluid phase particle fluorescence analysis: Rheumatoid factor specificity evaluated by laser flow cytophotometry.* (3rd Ed.). New York: Liss, A.R.

Hsieh, P., & Robbins, P. W. (1984). Regulation of asparagine-linked oligosaccharide processing. Oligosaccharide processing in aedes albopictus mosquito cells. *The Journal of Biological Chemistry, 259*(4), 2375-2382.

Huang, M., Choi, Y., Houde, R., Lee, J., Lee, B., & Zhao, X. (2004). Effects of lactobacilli and an acidophilic fungus on the production performance and immune responses in broiler chickens. *Poultry Science, 83*(5), 788-795.

Iowa State University 2010. *High Pathogenicity Avian Influenza*. College Veterinary Medicine. Ames, Iowa.

Iqbal, M., Yaqub, T., Mukhtar, N., Shabbir, M. Z., & McCauley, J. W. (2013). Infectivity and transmissibility of H9N2 avian influenza virus in chickens and wild terrestrial birds. *Veterinary Research, 44*, 100-9716-44-100. doi:10.1186/1297-9716-44-100; 10.1186/1297-9716-44-100

Jacobson, R. H. (1998). Validation of serological assays for diagnosis of infectious diseases. *Revue Scientifique Et Technique (International Office of Epizootics), 17*(2), 469-526.

Jensen, T. H., Ajjouri, G., Handberg, K. J., Slomka, M. J., Coward, V. J., Cherbonnel, M., Jorgensen, P. H. (2013). An enzyme-linked immunosorbent assay for detection of avian influenza virus subtypes H5 and H7 antibodies. *Acta Veterinaria Scandinavica, 55*(1), 84-0147-55-84. doi:10.1186/1751-0147-55-84; 10.1186/1751-0147-55-84

Jin, L. Z., Ho, Y. W., Ali, M. A., Abdullah, N., Ong, K. B., & Jalaludin, S. (1996). Adhesion of lactobacillus isolates to intestinal epithelial cells of chicken. *Letters in Applied Microbiology, 22*(3), 229-232.

Jin, M., Wang, G., Zhang, R., Zhao, S., Li, H., Tan, Y., & Chen, H. (2004). Development of enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection of antibodies to avian influenza virus. *Avian Diseases, 48*(4), 870-878.

Jourdain, E., Gauthier-Clerc, M., Bicout, D. J., & Sabatier, P. (2007). Bird migration routes and risk for pathogen dispersion into western mediterranean wetlands. *Emerging Infectious Diseases, 13*(3), 365-372. doi:10.3201/eid1303.060301

Kajiwara, E., Shigeta, A., Horiuchi, H., Matsuda, H., & Furusawa, S. (2003). Development of peyer's patch and cecal tonsil in gut-associated lymphoid tissues in the chicken embryo. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science, 65*(5), 607-614.

Kaliannan, K., Seawright, G. L., & Derbyshire, J. B. (1975). Adequacy of commercial lentogenic vaccines against Canadian strains of viscerotropic Newcastle disease virus. *Canadian Journal of Comparative Medicine. Revue Canadienne De Medecine Comparee, 39*(2), 139-145.

Kapczynski, D. R., Afonso, C. L., & Miller, P. J. (2013). Immune responses of poultry to Newcastle disease virus. *Developmental and Comparative Immunology,* doi:10.1016/j.dci.2013.04.012; 10.1016/j.dci.2013.04.012

Kapczynski, D. R., Afonso, C. L., & Miller, P. J. (2013). Immune responses of poultry to Newcastle disease virus. *Developmental and Comparative Immunology, 41*(3), 447-453. doi:10.1016/j.dci.2013.04.012; 10.1016/j.dci.2013.04.012

Katz, J. M., Hancock, K., & Xu, X. (2011). Serologic assays for influenza surveillance, diagnosis and vaccine evaluation. *Expert Review of Anti-Infective Therapy, 9*(6), 669-683. doi:10.1586/eri.11.51; 10.1586/eri.11.51

Katz, J. M., Hancock, K., & Xu, X. (2011). Serologic assays for influenza surveillance, diagnosis and vaccine evaluation. *Expert Rev Anti Infect Ther, 9*(6), 669-683. doi:10.1586/eri.11.51

Keeler, S. P., Dalton, M. S., Cressler, A. M., Berghaus, R. D., & Stallknecht, D. (2014). Abiotic factors affecting persistence of avian influenza virus in surface water from waterfowl habitats. *Applied and Environmental Microbiology,* doi:10.1128/AEM.03790-13

Kessler, N., Aymard, M., & Calvet, A. (1979). Study of a new strain of paramyxoviruses isolated from wild ducks: Antigenic and biological properties. *Journal of General Virology,* (43), 273-282.

Kim, H.R., Lee, K.K., Kwon, Y.K., Kang, M.S., Moon, O.K., Park, C.K. (2012). Comparison of serum treatments to remove nonspecific inhibitors from chicken sera for the hemagglutination inhibition test with inactivated H5N1 and H9N2 avian influenza A virus subtypes. *Journal of Veterinary Diagnostic Investigation,* 24(5), 954-8.

Kim, S. J., & Spradbrow, P. B. (1978). Administration of a vaccine prepared from the Australian V4 strain of Newcastle disease virus by aerosol and drinking water. *Australian Veterinary Journal, 54*(10), 486-489.

Kiseleva, I. V., Bazhenova, E. A., Larionova, N. V., Fedorova, E. A., Dubrovina, I. A., Isakova-Sivak, I. N., & Rudenko, L. G. (2013). Peculiarity of reassortment of current wild type influenza viruses with master donor viruses for live influenza vaccine. *Voprosy Virusologii, 58*(5), 26-31.

Klasing, K. C. (2007). Nutrition and the immune system. *British Poultry Science, 48*(5), 525-537. doi:10.1080/00071660701671336

Klasing, K. (1998). Nutritional modulation of resistance to infectious diseases. *Poultry Science, 77*(8), 1119-1125.

Knight, P. R., Sreekumar, A., Siddiqui, J., Laxman, B., Copeland, S., Chinnaiyan, A., & Remick, D. G. (2004). Development of a sensitive microarray immunoassay and comparison with standard enzyme-linked immunoassay for cytokine analysis. *Shock (Augusta, Ga.), 21*(1), 26-30. doi:10.1097/01.shk.0000101668.49265.19

Kuroda, K., Geyer, H., Geyer, R., Doerfler, W., & Klenk, H. D. (1990). The oligosaccharides of influenza virus hemagglutinin expressed in insect cells by a baculovirus vector. *Virology, 174*(2), 418-429.

Kuroda, K., Hauser, C., Rott, R., Klenk, H. D., & Doerfler, W. (1986). Expression of the influenza virus haemagglutinin in insect cells by a baculovirus vector. *The EMBO Journal, 5*(6), 1359-1365.

Kuroda, K., Veit, M., & Klenk, H. D. (1991). Retarded processing of influenza virus hemagglutinin in insect cells. *Virology, 180*(1), 159-165.

Ladman, B. S., Rosenberger, S. C., Rosenberger, J. K., Pope, C. R., & Gelb, J.,Jr. (2008). Virulence of low pathogenicity H7N2 avian influenza viruses from the delmarva peninsula for broiler and leghorn chickens and turkeys. *Avian Diseases, 52*(4), 623-631.

Langenhorst, R. J., Lawson, S., Kittawornrat, A., Zimmerman, J. J., Sun, Z., Li, Y., Fang, Y. (2012). Development of a fluorescent microsphere immunoassay for detection of antibodies against porcine reproductive and respiratory syndrome virus using oral fluid samples as an alternative to serum-based assays. *Clinical and Vaccine Immunology : CVI, 19*(2), 180-189. doi:10.1128/CVI.05372-11; 10.1128/CVI.05372-11

Lee, C. C., Zhu, H., Huang, P. Y., Peng, L., Chang, Y. C., Yip, C. H., Guan, Y. (2014). The emergence and evolution of avian H5N2 influenza viruses in chickens in Taiwan. *Journal of Virology,* doi:10.1128/JVI.00139-14

Lee, S. H., Lillehoj, H. S., Park, D. W., Jang, S. I., Morales, A., Garcia, D., Lillehoj, E. P. (2009). Induction of passive immunity in broiler chickens against eimeria acervulina by hyperimmune egg yolk immunoglobulin Y. *Poultry Science, 88*(3), 562-566. doi:10.3382/ps.2008-00340; 10.3382/ps.2008-00340

Leijon, M., Ullman, K., Thyselius, S., Zohari, S., Pedersen, J. C., Hanna, A., Belak, S. (2011). Rapid PCR-based molecular pathotyping of H5 and H7 avian influenza viruses. *Journal of Clinical Microbiology, 49*(11), 3860-3873. doi:10.1128/JCM.01179-11; 10.1128/JCM.01179-11

Lenhoff, R. J., Naraghi-Arani, P., Thissen, J. B., Olivas, J., Carillo, A. C., Chinn, C., McBride, M. T. (2008). Multiplexed molecular assay for rapid exclusion of foot-and-mouth disease. *Journal of Virological Methods, 153*(1), 61-69. doi:10.1016/j.jviromet.2008.06.015; 10.1016/j.jviromet.2008.06.015

Li, X., Lu, D., Sheng, Z., Chen, K., Guo, X., Jin, M., & Han, H. (2012). A fast and sensitive immunoassay of avian influenza virus based on label-free quantum dot probe and lateral flow test strip. *Talanta, 100*, 1-6. doi:10.1016/j.talanta.2012.08.041; 10.1016/j.talanta.2012.08.041

Lillehoj, H. S., & Lee, K. W. (2012). Immune modulation of innate immunity as alternatives-to-antibiotics strategies to mitigate the use of drugs in poultry production. *Poultry Science, 91*(6), 1286-1291. doi:10.3382/ps.2012-02374

Lin, K., Wang, C., Murtaugh, M. P., & Ramamoorthy, S. (2011). Multiplex method for simultaneous serological detection of porcine reproductive and respiratory syndrome virus and porcine circovirus type 2. *Journal of Clinical Microbiology, 49*(9), 3184-3190. doi:10.1128/JCM.00557-11

Linna, T. J., Hemmingsson, E., Back, R. (1969). A method for local labelling of the thymus and of the bursa of fabricius of the chicken with tritiated thymidine. *Acta Pathologica Et Microbiologica Scandinavica, 77*(3), 557-558.

Linster, M., van Boheemen, S., de Graaf, M., Schrauwen, E. J., Lexmond, P., Manz, B., Herfst, S. (2014). Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus. *Cell, 157*(2), 329-339. doi:10.1016/j.cell.2014.02.040; 10.1016/j.cell.2014.02.040

Lipkind, M., Weisman, Y., Shihmanter, E., & Shoham, D. (1982). Isolation of yucaipa-like avian paramyxovirus from a wild mallard duck (anas platyrhinchos) wintering in Israel. *Veterinary Records,* (110), 15-16.

Liu, Q., Chen, H., Huang, J., Chen, Y., Gu, M., Wang, X., Liu, X. (2014). A nonpathogenic duck-origin H9N2 influenza A virus adapts to high pathogenicity in mice. *Archives of Virology,* doi:10.1007/s00705-014-2062-y

Lung, O., Beeston, A., Ohene-Adjei, S., Pasick, J., Hodko, D., Hughes, K. B., Deregt, D. (2012). Electronic microarray assays for avian influenza and Newcastle disease virus. *Journal of Virological Methods, 185*(2), 244-253. doi:10.1016/j.jviromet.2012.07.005; 10.1016/j.jviromet.2012.07.005

Lupiani, B., Mozisek, B., Mason, P. W., Lamichhane, C., & Reddy, S. M. (2010). Simultaneous detection of avian influenza virus NP and H5 antibodies in chicken sera using a fluorescence microsphere immunoassay. *Avian Diseases, 54*(1 Suppl), 668-672.

Lupiani, B., Mozisek, B., Mason, P. W., Lamichhane, C., & Reddy, S. M. (2010). Simultaneous detection of avian influenza virus NP and H5 antibodies in chicken sera using a fluorescence microsphere immunoassay. *Avian Diseases, 54*(1 Suppl), 668-672.

Makkay, A. M., Krell, P. J., & Nagy, E. (1999). Antibody detection-based differential ELISA for NDV-infected or vaccinated chickens versus NDV HN-subunit vaccinated chickens. *Veterinary Microbiology, 66*(3), 209-222.

Makkay, A. M., Krell, P. J., & Nagy, E. (1999). Antibody detection-based differential ELISA for NDV-infected or vaccinated chickens versus NDV HN-subunit vaccinated chickens. *Veterinary Microbiology, 66*(3), 209-222.

Martins, T. B. (2002). Development of internal controls for the luminex instrument as part of a multiplex seven-analyte viral respiratory antibody profile. *Clinical and Diagnostic Laboratory Immunology, 9*(1), 41-45.

Martins, T. B. (2002). Development of internal controls for the luminex instrument as part of a multiplex seven-analyte viral respiratory antibody profile. *Clinical and Diagnostic Laboratory Immunology, 9*(1), 41-45.

Maxion, R. A., & Roberts, R. R. (2004). *Proper use of ROC curves in Intrusion/anomaly detection.* (Technical Report). University of Newcastle upon Tyne: School of Computing Science.

McFerran, J. B., & McCracken, R. M. (1988). Newcastle disease. In D. J. Alexander (Ed.), *Newcastle disease* (pp. 161-183). Boston, MA: Kluwer Academic Publishers.

McHugh, T. M., Viele, M. K., Chase, E. S., & Recktenwald, D. J. (1997). The sensitive detection and quantitation of antibody to HCV by using a microsphere-based immunoassay and flow cytometry. *Cytometry, 29*(2), 106-112.

McNabb, L., Barr, J., Crameri, G., Juzva, S., Riddell, S., Colling, A., Lunt, R. (2014). Henipavirus microsphere immuno-assays for detection of antibodies against hendra virus. *Journal of Virological Methods, 200*, 22-28. doi:10.1016/j.jviromet.2014.01.010; 10.1016/j.jviromet.2014.01.010

Meulemans, G. (1988). Control by vaccination. In D. J. Alexander (Ed.), *Newcastle disease* (pp. 318-332). Boston, MA: Kluwer Academic Publishers.

Miller, P. J., Alfonso, C. L., Spackman, E., Scott, M. A., Pedersen, J. C., Senne, D. A., Swayne, D. E. (2010). Evidence for a new avian paramyxovirus serotype 10 detected in rockhopper penguins from the falkland islands. *Journal of Virology, 21*(84), 11496-11504.

Miller, P. J., & Torchetti, M. K. (2014). Newcastle disease virus detection and differentiation from avian influenza. *Methods in Molecular Biology (Clifton, N.J.), 1161*, 235-239. doi:10.1007/978-1-4939-0758-8_19; 10.1007/978-1-4939-0758-8_19

Molesti, E., Milani, A., Terregino, C., Cattoli, G., & Temperton, N. J. (2013). Comparative serological assays for the study of h5 and h7 avian influenza viruses. *Influenza Research and Treatment, 2013*, 286158. doi:10.1155/2013/286158; 10.1155/2013/286158

Molesti, E., Milani, A., Terregino, C., Cattoli, G., & Temperton, N. J. (2013). *Comparative serological assays for the study of H5 and H7 avian influenza viruses*

Mondal, S., Xing, Z., & Cardona, C. (2013). A comparison of virulence of influenza A virus isolates from mallards in experimentally inoculated turkeys. *Avian Diseases, 57*(4), 790-796.

Monne, I., Fusaro, A., Nelson, M. I., Bonfanti, L., Mulatti, P., Hughes, J., Cattoli, G. (2014). Emergence of a highly pathogenic avian influenza virus from a low-pathogenic progenitor. *Journal of Virology, 88*(8), 4375-4388. doi:10.1128/JVI.03181-13; 10.1128/JVI.03181-13

More, S.J., Copeman, D.B. (1991). Antigen detection ELISAs: pretreatment of serum to reduce interference by specific host antibodies. *Tropical Medicine and Parasitology*, 42(2), 91-4.

Moro de Sousa, R. L., Montassier, H. J., & Pinto, A. A. (2000). Detection and quantification of antibodies to Newcastle disease virus in ostrich and rhea sera using a liquid phase blocking enzyme-linked immunosorbent assay. *Clinical and Diagnostic Laboratory Immunology, 7*(6), 940-944.

Munro, S. B., Kuypers, J., & Jerome, K. R. (2013). Comparison of a multiplex real-time PCR assay with a multiplex luminex assay for influenza virus detection. *Journal of Clinical Microbiology, 51*(4), 1124-1129. doi:10.1128/JCM.03113-12; 10.1128/JCM.03113-12

Murphy, C. I., & Piwnica-Worms, H. (2001). Overview of the baculovirus expression system. *Current Protocols in Neuroscience. Chapter 4*, Unit 4.18. doi:10.1002/0471142301.ns0418s10; 10.1002/0471142301.ns0418s10

Mutinelli, F., Capua, I., Terregino, C., & Cattoli, G. (2003). Clinical, gross, and microscopic findings in different avian species naturally infected during the H7N1 low- and high-pathogenicity avian influenza epidemics in italy during 1999 and 2000. *Avian Diseases, 47*(3 Suppl), 844-848.

Mutinelli, F., Capua, I., Terregino, C., & Cattoli, G. (2003). Clinical, gross, and microscopic findings in different avian species naturally infected during the H7N1 low- and high-pathogenicity avian influenza epidemics in Italy during 1999 and 2000. *Avian Diseases, 47*(3 Suppl), 844-848.

Nickbakhsh, S., Matthews, L., Bessell, P. R., Reid, S. W., & Kao, R. R. (2011). Generating social network data using partially described networks: An example informing avian influenza control in the british poultry industry. *BMC Veterinary Research, 7*, 66-6148-7-66. doi:10.1186/1746-6148-7-66; 10.1186/1746-6148-7-66

Nickbakhsh, S., Matthews, L., Dent, J. E., Innocent, G. T., Arnold, M. E., Reid, S. W., & Kao, R. R. (2013). Implications of within-farm transmission for network dynamics: Consequences for the spread of avian influenza. *Epidemics, 5*(2), 67-76. doi:10.1016/j.epidem.2013.03.001; 10.1016/j.epidem.2013.03.001

Nielsen, K., Yu, W. L., Lin, M., Davis, S. A., Elmgren, C., Mackenzie, R., Pasick, J. (2007). Prototype single step lateral flow technology for detection of avian influenza virus and chicken antibody to avian influenza virus. *Journal of Immunoassay & Immunochemistry, 28*(4), 307-318. doi:10.1080/15321810701603443

Niqueux, E., Guionie, O., Schmitz, A., Hars, J., & Jestin, V. (2010). Presence of serum antibodies to influenza A subtypes H5 and N1 in swans and ibises in french wetlands, irrespective of highly pathogenic H5N1 natural infection. *Avian Diseases, 54*(1 Suppl), 502-508.

Niqueux, E., Picault, J. P., Amelot, M., Allee, C., Lamande, J., Guillemoto, C., Jestin, V. (2014). Quantitative transmission characteristics of different H5 low pathogenic avian influenza viruses in muscovy ducks. *Veterinary Microbiology, 168*(1), 78-87. doi:10.1016/j.vetmic.2013.10.020; 10.1016/j.vetmic.2013.10.020

Oliver, K. G., Kettman, J. R., & Fulton, R. J. (1998). Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clinical Chemistry, 44*(9), 2057-2060.

Olson, S. H., Parmley, J., Soos, C., Gilbert, M., Latorre-Margalef, N., Hall, J. S., Joly, D. (2014). Sampling strategies and biodiversity of influenza a subtypes in wild birds. *PloS One, 9*(3), e90826. doi:10.1371/journal.pone.0090826; 10.1371/journal.pone.0090826

O'Shaughnessy, L., & Doyle, S. (2011). Purification of proteins from baculovirus-infected insect cells. *Methods in Molecular Biology (Clifton, N.J.), 681*, 295-309. doi:10.1007/978-1-60761-913-0_16; 10.1007/978-1-60761-913-0_16

Palese, P., & Schulman, J. L. (1976). Mapping of the influenza virus genome: Identification of the hemagglutinin and the neuraminidase genes. *Proceedings of the National Academy of Sciences of the United States of America, 73*(6), 2142-2146.

Pan, Q., Liu, A., Zhang, F., Ling, Y., Ou, C., Hou, N., & He, C. (2012). Co-infection of broilers with ornithobacterium rhinotracheale and H9N2 avian influenza virus. *BMC Veterinary Research, 8*, 104-6148-8-104. doi:10.1186/1746-6148-8-104; 10.1186/1746-6148-8-104

Park, J. K., Lee, D. H., Cho, C. H., Yuk, S. S., To, E. O., Kwon, J. H., Song, C. S. (2014). Supplementation of oil-based inactivated H9N2 vaccine with M2e antigen enhances resistance against heterologous H9N2 avian influenza virus infection. *Veterinary Microbiology, 169*(3-4), 211-217. doi:10.1016/j.vetmic.2014.01.001; 10.1016/j.vetmic.2014.01.001

Parmley, E. J., Bastien, N., Booth, T. F., Bowes, V., Buck, P. A., Breault, A., Leighton, F. A. (2008). Wild bird influenza survey, Canada, 2005. *Emerging Infectious Diseases, 14*(1), 84-87. doi:10.3201/eid1401.061562; 10.3201/eid1401.061562

Parmley, E. J., Soos, C., Breault, A., Fortin, M., Jenkins, E., Kibenge, F., Leighton, F. A. (2011). Detection of low pathogenic avian influenza viruses in wild ducks from Canada: Comparison of two sampling methods. *Journal of Wildlife Diseases, 47*(2), 466-470. doi:10.7589/0090-3558-47.2.466

Pasick, J., Berhane, Y., Hisanaga, T., Kehler, H., Hooper-McGrevy, K., Handel, K., Leighton, F. (2010). Diagnostic test results and pathology associated with the 2007 Canadian H7N3 highly pathogenic avian influenza outbreak. *Avian Diseases, 54*(1 Suppl), 213-219.

Pasick, J., Berhane, Y., Kehler, H., Hisanaga, T., Handel, K., Robinson, J., Leighton, F. (2010). Survey of influenza A viruses circulating in wild birds in Canada 2005 to 2007. *Avian Diseases, 54*(1 Suppl), 440-445.

Pasick, J., Berhane, Y., Kehler, H., Hisanaga, T., Handel, K., Robinson, J., Leighton, F. (2010). Survey of influenza A viruses circulating in wild birds in Canada 2005 to 2007. *Avian Diseases, 54*(1 Suppl), 440-445.

Pasick, J., Pedersen, J., & Hernandez, M. S. (2012). Avian influenza in North America, 2009-2011. *Avian Diseases, 56*(4 Suppl), 845-848.

Pasick, J., Handel, K., Robinson, J., Copps, J., Ridd, D., Hills, K., Czub, S. (2005). Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. *Journal of General Virology, 86*(3), 727-731. doi:10.1099/vir.0.80478-0

Pawar, S. D., Kale, S. D., Rawankar, A. S., Koratkar, S. S., Raut, C. G., Pande, S. A., Mishra, A. C. (2012). Avian influenza surveillance reveals presence of low pathogenic avian influenza viruses in poultry

during 2009-2011 in the west Bengal state, India. *Virology Journal, 9*, 151-422X-9-151. doi:10.1186/1743-422X-9-151; 10.1186/1743-422X-9-151

Pearson, J. E., & Knowles, R. C. (1984). Standardization of the equine infectious anemia immunodiffusion test and its application to the control of the disease in the United States. *Journal of the American Veterinary Medical Association, 184*(3), 298-301.

Perez-Ramirez, E., Rodriguez, V., Sommer, D., Blanco, J. M., Acevedo, P., Heffels-Redmann, U., & Hofle, U. (2010). Serologic testing for avian influenza viruses in wild birds: Comparison of two commercial competition enzyme-linked immunosorbent assays. *Avian Diseases, 54*(1 Suppl), 729-733.

Pinette, M.M., Rodriguez-Lecompte, J.C., Pasick, J., Ojkic, D., Leith, M., Suderman, M., Berhane, Y. (2014). Development of a Duplex Fluorescent Microsphere Immunoassay (FMIA) for the detection of antibody responses to influenza A and Newcastle disease viruses. *Journal of Immunological Methods*, 405, 167-77.

Rankin, M. K., Alphin, R. L., Benson, E. R., Johnson, A. L., Hougentogler, D. P., & Mohankumar, P. (2013). Comparison of water-based foam and carbon dioxide gas emergency depopulation methods of turkeys. *Poultry Science, 92*(12), 3144-3148. doi:10.3382/ps.2013-03341; 10.3382/ps.2013-03341

Rauch, P., Zellmer, A., Dankbar, N., Specht, C., & Sperling, D. (2005). Optimization of assays: Interference in immunoassays recognize and avoid. [„Assayoptimierung: Störeffekte bei Immunoassays erkennen und vermeiden“ published in No. 4 / 2005 - Vol. 6] *Laborwelt, 6*(4), 2-7.

Rebel, J. M., Peeters, B., Fijten, H., Post, J., Cornelissen, J., & Vervelde, L. (2011). Highly pathogenic or low pathogenic avian influenza virus subtype H7N1 infection in chicken lungs: Small differences in

general acute responses. *Veterinary Research, 42*, 10-9716-42-10. doi:10.1186/1297-9716-42-10; 10.1186/1297-9716-42-10

Rendic, D., Wilson, I. B. H., & Paschinger, K. (2008). The glycosylation capacity of insect cells. *Croatica Chemica Acta, 81*(1), 7-21.

Rendic, D., Linder, A., Paschinger, K., Borth, N., Wilson, I. B., & Fabini, G. (2006). Modulation of neural carbohydrate epitope expression in drosophila melanogaster cells. *The Journal of Biological Chemistry, 281*(6), 3343-3353. doi:10.1074/jbc.M508334200

Reperant, L. A., van Amerongen, G., van de Bildt, M. W., Rimmelzwaan, G. F., Dobson, A. P., Osterhaus, A. D., & Kuiken, T. (2008). Highly pathogenic avian influenza virus (H5N1) infection in red foxes fed infected bird carcasses. *Emerging Infectious Diseases, 14*(12), 1835-1841. doi:10.3201/eid1412.080470; 10.3201/eid1412.080470

Reynolds, D. L., & Maraqa, A. D. (1999). A rapid virus neutralization assay for Newcastle disease virus with the swine testicular continuous cell line. *Avian Diseases, 43*(3), 564-571.

Ritchey, M. B., Palese, P., & Schulman, J. L. (1976). Mapping of the influenza virus genome. III. identification of genes coding for nucleoprotein, membrane protein, and nonstructural protein. *Journal of Virology, 20*(1), 307-313.

Rodríguez-Lecompte, J. C., Yitbarek, A., Brady, J., Sharif, S., Cavanagh, M. D., Crow, G., Camelo-Jaimes, G. (2012). The effect of microbial-nutrient interaction on the immune system of young chicks after early probiotic and organic acid administration. *Journal of Animal Science, 90*(7), 2246-2254. doi:10.2527/jas.2011-4184

Rodriguez-Sanchez, B., Sanchez-Vizcaino, J. M., Uttenthal, A., Rasmussen, T. B., Hakhverdyan, M., King, D. P., Belak, S. (2008). Improved diagnosis for nine viral diseases considered as notifiable by the world organization for animal health. *Transboundary and Emerging Diseases, 55*(5-6), 215-225. doi:10.1111/j.1865-1682.2008.01037.x; 10.1111/j.1865-1682.2008.01037.x

Ryan, J. T., & Rose, T. M. (2013). Development of whole-virus multiplex luminex-based serological assays for diagnosis of infections with kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 homologs in macaques. *Clinical and Vaccine Immunology : CVI, 20*(3), 409-419. doi:10.1128/CVI.00673-12; 10.1128/CVI.00673-12

Salk, J. E. (1944). A simplified procedure for titrating hemagglutinating capacity of influenza-virus and the corresponding antibody. *The Journal of Immunology, 49*(2), 87-98.

Samji, T. (2009). Influenza A: Understanding the viral life cycle. *The Yale Journal of Biology and Medicine, 82*(4), 153-159.

Schmidt, N. J., Dennis, J., & Lennette, E. H. (1976). Plaque reduction neutralization test for human cytomegalovirus based upon enhanced uptake of neutral red by virus-infected cells. *Journal of Clinical Microbiology, 4*(1), 61-66.

Schmitz, A., Le Bras, M. O., Guillemoto, C., Pierre, I., Rose, N., Bougeard, S., & Jestin, V. (2013). Evaluation of a commercial ELISA for H5 low pathogenic avian influenza virus antibody detection in duck sera using bayesian methods. *Journal of Virological Methods,* doi:10.1016/j.jviromet.2013.05.002; 10.1016/j.jviromet.2013.05.002

Seal, B. S., Crawford, J. M., Sellers, H. S., Locke, D. P., & King, D. J. (2002). Nucleotide sequence analysis of the Newcastle disease virus nucleocapsid protein gene and phylogenetic relationships among the paramyxoviridae. *Virus Research, 83*(1-2), 119-129.

Seideman, J., & Peritt, D. (2002). A novel monoclonal antibody screening method using the luminex-100™ microsphere system. *Journal of Immunological Methods, 267*(2), 165-171. doi:http://dx.doi.org.proxy1.lib.umanitoba.ca/10.1016/S0022-1759(02)00168-0

Shalaby, A. A., Slemons, R. D., & Swayne, D. E. (1994). Pathological studies of A/chicken/Alabama/7395/75 (H4N8) influenza virus in specific-pathogen-free laying hens. *Avian Diseases, 38*(1), 22-32.

Shi, J., Gao, L., Zhu, Y., Chen, T., Liu, Y., Dong, L., Shu, Y. (2014). Investigation of avian influenza infections in wild birds, poultry and humans in eastern dongting lake, china. *PloS One, 9*(4), e95685. doi:10.1371/journal.pone.0095685; 10.1371/journal.pone.0095685

Shortridge, K. F., Alexander, D. J., & Collins, M. S. (1980). Isolation and properties of viruses from poultry in Hong Kong which represent a new (sixth) distinct group of avian paramyxoviruses. *Research in Veterinary Science,* (49), 255-262.

Sivay, M. V., Sayfutdinova, S. G., Sharshov, K. A., Alekseev, A. Y., Yurlov, A. K., Runstadler, J., & Shestopalov, A. M. (2012). Surveillance of influenza A virus in wild birds in the Asian portion of Russia in 2008. *Avian Diseases, 56*(3), 456-463.

Smirnov, Y. A., Lipatov, A. S., Gitelman, A. K., Okuno, Y., Van Beek, R., Osterhaus, A. D., & Claas, E. C. (1999). An epitope shared by the hemagglutinins of H1, H2, H5, and H6 subtypes of influenza A virus. *Acta Virologica, 43*(4), 237-244.

Smith, P. C., & Stewart, W. C. (1978). Agar-gel immunodiffusion assay for pseudorabies virus antibody. *Journal of Clinical Microbiology, 7*(5), 423-425.

Smith, P. L., WalkerPeach, C. R., Fulton, R. J., & DuBois, D. B. (1998). A rapid, sensitive, multiplexed assay for detection of viral nucleic acids using the FlowMetrix system. *Clinical Chemistry, 44*(9), 2054-2056.

Snyder, D. B., Marquardt, W. W., Yancey, F. S., & Savage, P. K. (1985). An enzyme-linked immunosorbent assay for the detection of antibody against avian influenza virus. *Avian Diseases, 29*(1), 136-144.

Spalatin, J., & Hanson, R. P., (1975). Epizootiology of Newcastle disease in waterfowl. *Avian Diseases, 19*(3), 573-582.

Stallknecht, D. E. (2007). Impediments to wildlife disease surveillance, research, and diagnostics. *Current Topics in Microbiology and Immunology, 315*, 445-461.

Staples, E., Ingram, R. J., Atherton, J. C., & Robinson, K. (2013). Optimising the quantification of cytokines present at low concentrations in small human mucosal tissue samples using luminex assays. *Journal of Immunological Methods, 394*(1-2), 1-9. doi:10.1016/j.jim.2013.04.009; 10.1016/j.jim.2013.04.009

Sutton, T. C., Finch, C., Shao, H., Angel, M., Chen, H., Capua, I., Perez, D. R. (2014). Airborne transmission of highly pathogenic H7N1 influenza in ferrets. *Journal of Virology,* doi:10.1128/JVI.02765-13

Swayne, D. E., & Spackman, E. (2013). Current status and future needs in diagnostics and vaccines for high pathogenicity avian influenza. *Developments in Biologicals, 135*, 79-94. doi:10.1159/000325276; 10.1159/000325276

Thayer, S. G., Villegas, P., & Fletcher, O. J. (1987). Comparison of two commercial enzyme-linked immunosorbent assays and conventional methods for avian serology. *Avian Diseases, 31*(1), 120-124.

Toffan, A., Olivier, A., Mancin, M., Tuttoilmondo, V., Facco, D., Capua, I., & Terregino, C. (2010). Evaluation of different serological tests for the detection of antibodies against highly pathogenic avian influenza in experimentally infected ostriches (struthio camelus). *Avian Pathology : Journal of the W.V.P.A, 39*(1), 11-15. doi:10.1080/03079450903431390; 10.1080/03079450903431390

Toyoda, T., Sakaguchi, T., Imai, K., Inocencio, N. M., Gotoh, B., Hamaguchi, M., & Nagai, Y. (1987). Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. *Virology, 158*(1), 242-247.

Tumova, B., Robinson, J. H., & Easterday, B. C. (1979). A hitherto unreported paramyxovirus of turkeys. *Research in Veterinary Science,* (27), 135-140.

Upadhyay, C., Ammayappan, A., & Vakharia, V. N. (2009). Detection of NP, N3 and N7 antibodies to avian influenza virus by indirect ELISA using yeast-expressed antigens. *Virology Journal, 6*, 158-422X-6-158. doi:10.1186/1743-422X-6-158; 10.1186/1743-422X-6-158

van Brunschot, S. L., Bergervoet, J. H. W., Pagendam, D. E., de Weerdt, M., Geering, A. D. W., Drenth, A., & van, d. V. (2014). Development of a multiplexed bead-based suspension array for the detection and discrimination of *pospiviroid* plant pathogens. *Plos One, 9*(1), e84743.

van Gageldonk, P. G., van Schaijk, F. G., van der Klis, F. R., & Berbers, G. A. (2008). Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to bordetella pertussis, diphtheria and tetanus. *Journal of Immunological Methods, 335*(1-2), 79-89. doi:10.1016/j.jim.2008.02.018; 10.1016/j.jim.2008.02.018

van Ginkel, F. W., Gulley, S. L., Lammers, A., Hoerr, F. J., Gurjar, R., & Toro, H. (2012). Conjunctiva-associated lymphoid tissue in avian mucosal immunity. *Developmental and Comparative Immunology, 36*(2), 289-297. doi:10.1016/j.dci.2011.04.012; 10.1016/j.dci.2011.04.012

Vasin, A. V., Temkina, O. A., Egorov, V. V., Klotchenko, S. A., Plotnikova, M. A., & Kiselev, O. I. (2014). Molecular mechanisms enhancing the proteome of influenza A viruses: An overview of recently discovered proteins. *Virus Research, 185C*, 53-63. doi:10.1016/j.virusres.2014.03.015; 10.1016/j.virusres.2014.03.015

Vickers, M. L., & Hanson, R. P. (1982). Newcastle disease virus in waterfowl in Wisconsin. *Journal of Wildlife Diseases, 18*(2), 149-158.

Vignali, D. A. (2000). Multiplexed particle-based flow cytometric assays. *Journal of Immunological Methods, 243*(1-2), 243-255.

Wagner, R., Liedtke, S., Kretzschmar, E., Geyer, H., Geyer, R., & Klenk, H. D. (1996). Elongation of the N-glycans of fowl plague virus hemagglutinin expressed in spodoptera frugiperda (Sf9) cells by coexpression of human beta 1,2-N-acetylglucosaminyltransferase I. *Glycobiology, 6*(2), 165-175.

Wallerstrom, S., Lagerqvist, N., Temperton, N. J., Cassmer, M., Moreno, A., Karlsson, M., Falk, K. I. (2014). Detection of antibodies against H5 and H7 strains in birds: Evaluation of influenza

pseudovirus particle neutralization tests. *Infection Ecology & Epidemiology, 4*, 10.3402/iee.v4.23011. eCollection 2014 Jan 15. doi:10.3402/iee.v4.23011; 10.3402/iee.v4.23011

Wang, L., Pan, C., Severinghaus, L. L., Liu, L., Chen, C., Pu, C., En, Want, C. (2008). Simultaneous detection and differentiation of Newcastle disease and avian influenza viruses using oligonucleotide microarrays. *Veterinary Microbiology, 127*, 217-226.

Wang, L. S., Leung, Y. Y., Chang, S. K., Leight, S., Knapik-Czajka, M., Baek, Y., Clark, C. M. (2012). Comparison of xMAP and ELISA assays for detecting cerebrospinal fluid biomarkers of Alzheimer's disease. *Journal of Alzheimer's Disease : JAD, 31*(2), 439-445. doi:10.3233/JAD-2012-120082; 10.3233/JAD-2012-120082

Watkins, B. A., & Kratzer, F. H. (1984). Drinking water treatment with a commercial preparation of a concentrated lactobacillus culture for broiler chickens. *Poultry Science, 63*(8), 1671-1673.

Watkins, B. A., Miller, B. F., & Neil, D. H. (1982). In vivo inhibitory effects of lactobacillus acidophilus against pathogenic escherichia coli in gnotobiotic chicks. *Poultry Science, 61*(7), 1298-1308.

Watkins, B. A., Miller, B. F., & Neil, D. H. (1982). In vivo inhibitory effects of lactobacillus acidophilus against pathogenic escherichia coli in gnotobiotic chicks. *Poultry Science, 61*(7), 1298-1308.

Watson, D. S., Reddy, S. M., Brahmakshatriya, V., & Lupiani, B. (2009). A multiplexed immunoassay for detection of antibodies against avian influenza virus. *Journal of Immunological Methods, 340*(2), 123-131. doi:10.1016/j.jim.2008.10.007

World Organization for Animal Health (OIE). (2008). *Manual of diagnostic tests and vaccines for terrestrial animals* (Sixth Edition Ed.). Paris, France: OIE.

Wright, P. F. (1998). International standards for test methods and reference sera for diagnostic tests for antibody detection. *Revue Scientifique Et Technique (International Office of Epizootics), 17*(2), 527-549.

Wu, H. S., Yang, J. R., Liu, M. T., Yang, C. H., Cheng, M. C., & Chang, F. Y. (2014). Influenza A (H5N2) virus antibodies in humans after contact with infected poultry, Taiwan, 2012. *Emerging Infectious Diseases, 20*(5), 857-860. doi:10.3201/eid2005.131393; 10.3201/eid2005.131393

Wu, R., Hu, S., Xiao, Y., Li, Z., Shi, D., & Bi, D. (2007). Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus. *Veterinary Research Communications, 31*(5), 631-641. doi:10.1007/s11259-007-3510-x

Yang, C. M., Cao, G. T., Ferket, P. R., Liu, T. T., Zhou, L., Zhang, L., Chen, A. G. (2012). Effects of probiotic, clostridium butyricum, on growth performance, immune function, and cecal microflora in broiler chickens. *Poultry Science, 91*(9), 2121-2129. doi:10.3382/ps.2011-02131; 10.3382/ps.2011-02131

Yang, M., Berhane, Y., Salo, T., Li, M., Hole, K., & Clavijo, A. (2008). Development and application of monoclonal antibodies against avian influenza virus nucleoprotein. *Journal of Virological Methods, 147*(2), 265-274. doi:10.1016/j.jviromet.2007.09.016

Yang, P., Ma, C., Shi, W., Cui, S., Lu, G., Peng, X., Wang, Q. (2012). A serological survey of antibodies to H5, H7 and H9 avian influenza viruses amongst the duck-related workers in Beijing, china. *Plos One, 7*(11), e50770.

Yu, X., Jin, T., Cui, Y., Pu, X., Li, J., Xu, J., Wang, J. (2014). Influenza H7N9 and H9N2 viruses: Coexistence in poultry linked to human H7N9 infection and genome characteristics. *Journal of Virology, 88*(6), 3423-3431. doi:10.1128/JVI.02059-13; 10.1128/JVI.02059-13

Zhang, J. L., Xie, Q. M., Ji, J., Yang, W. H., Wu, Y. B., Li, C., Bi, Y. Z. (2012). Different combinations of probiotics improve the production performance, egg quality, and immune response of layer hens. *Poultry Science, 91*(11), 2755-2760. doi:10.3382/ps.2012-02339; 10.3382/ps.2012-02339

Zhang, Y., Birru, R., & Di, Y. P. (2014). Analysis of clinical and biological samples using microsphere-based multiplexing luminex system. *Methods in Molecular Biology (Clifton, N.J.), 1105*, 43-57. doi:10.1007/978-1-62703-739-6_4; 10.1007/978-1-62703-739-6_4

Zhou, E. M., Chan, M., Heckert, R. A., Riva, J., & Cantin, M. F. (1998). Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein. *Avian Diseases, 42*(3), 517-522.

Zhu, W. F., Gao, R. B., Wang, D. Y., Yang, L., Zhu, Y., & Shu, Y. L. (2013). A review of H7 subtype avian influenza virus. *Bing Du Xue Bao = Chinese Journal of Virology / [Bian Ji, Bing Du Xue Bao Bian Ji Wei Yuan Hui], 29*(3), 245-249.

Zhu, W. F., Gao, R. B., Wang, D. Y., Yang, L., Zhu, Y., & Shu, Y. L. (2013). A review of H7 subtype avian influenza virus. *Bing Du Xue Bao = Chinese Journal of Virology / [Bian Ji, Bing Du Xue Bao Bian Ji Wei Yuan Hui], 29*(3), 245-249.

Zweig, M. H., & Campbell, G. (1993). Receiver-operating characteristic (ROC) plots: A fundamental evaluation tool in clinical medicine. *Clinical Chemistry, 39*(4), 561-577.