EVALUATION OF AVIAN EMBRYOS AND CELL CULTURE FOR ISOLATION AND PROPAGATION OF LOW PATHOGENICITY AVIAN INFLUENZA VIRUSES

by

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(Under the Direction of David E. Swayne)

ABSTRACT

Growth parameters of low pathogenicity avian influenza viruses were examined in eight cell cultures and in three types of avian embryos. Virus detection was compared for samples collected from experimentally infected birds, using real-time reverse transcriptase polymerase chain reaction, embryonating chicken eggs, and five cell culture systems. Virus isolation from wild bird samples was compared using embryonating chicken, duck, and turkey eggs, as well as two cell culture systems. Virus isolation success was lowest using cell culture. Virus isolation was similar in embryonating chicken, duck, and turkey eggs. Embryonating chickens eggs had the highest virus isolation efficiency for avian influenza virus samples with cycle threshold of less than or equal to thirty seven. Embryonating turkey and duck eggs produced one hundred percent association between detection by real-time reverse transcription polymerase chain reaction and virus isolation up to cycle threshold of thirty six for avian paramyxovirus serotype 1.

INDEX WORDS: Low pathogenicity avian influenza, Cell culture, Avian embryos, Real-time reverse transcription polymerase chain reaction

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CHAPTER 1

INTRODUCTION

Thesis is arranged in the manuscript style with a literature survey and all subsequent chapters distinguishing a separate body of work. Each chapter contains an introduction. Methods and materials are followed by results, discussions, acknowledgements, and references. Tables and figures are located at the end of each chapter and are referenced in the table of contents.

The goal was to develop an optimized laboratory procedure to obtain the highest number of AI virus isolates from wild bird surveillance samples. The experiment was divided into three main objectives:

Objective one was to determine which cell line or primary culture with and without supplemental trypsin would provide the highest yield when inoculated with a diverse selection of LPAI viruses. As well as which avian embryo, chicken, duck or turkey, would also provide the best yield when inoculated with a diverse selection of LPAI viruses.

Objective two was to determine which combination of real-time reverse transcriptase polymerase chain reaction (RRT-PCR) assay, avian embryos, and cell culture would provide the highest detection or isolation rate from birds experimentally infected with LPAI viruses.

Objective three was to determine which combination of RRT-PCR, avian embryos, and cell culture would provide the highest AI virus isolation rate from wild bird surveillance samples.

CHAPTER 2

LITERATURE REVIEW

History

In 1878, Perroncito described a virulent disease affecting poultry and termed this disease fowl plague (Kaleta and Rulke 2008). Originally fowl plague was mistaken as fowl cholera until Rivolot and Delprato distinguished the two diseases in 1880 (Swayne and Suarez 2000). The demonstration that fowl plague was caused by a filterable agent is accredited to Centanni and Savonuzzi in 1901 (Kaleta and Rulke 2008). In 1902, Centanni described the use of embryonating chicken eggs as a biological system for propagation of fowl plague virus (i.e. high pathogenicity avian influenza virus) though it was not until 1934 that Burnet and Ferry implemented the use of embryonating eggs to titrate and propagate influenza viruses (Kaleta and Rulke 2008). In addition, they demonstrated the ability of influenza A viruses to agglutinate red blood cells. The discovery that there were variants of the original fowl plague virus (i.e. low pathogenicity avian influenza) was made by Dinter in 1949; however, it was not identified as an avian influenza virus; i.e. now termed A/chicken/Germany/49 (H10N7), until 1960 (Swayne and Halvorson 2008) . Schafer, in 1955, provided groundbreaking information that classified fowl plague virus as influenza A virus and differentiated these viruses from paramyxovirus type 1, or Newcastle Disease virus. This led to the discovery that other influenza A viruses, though not quite as pathogenic for chickens as fowl plague, were also avian influenza viruses (Swayne and Suarez 2000). The first isolation of influenza viruses from wild bird species was in 1961 from the common term (*Sterna hirundo)* in South Africa (Becker 1966). Systematic surveillance

examining influenza viruses in wild birds began in the mid 1970s; the results showed enormous pools of AI viruses that are now known to be present in the wild bird populations (Easterday, Trainer et al. 1968; Slemons, Johnson et al. 1973; Stallknecht and Brown 2008).

Etiology

Influenza A viruses are negative-strand RNA viruses with segmented genomes that belong to the *Orthomyxoviridae* family. Five genera compose this family: influenza type A, B, C, Isavirus, and Thogotovirus (Palese and Shaw 2007). Influenza A viruses can be further subdivided based on the possession of one of the sixteen antigenically distinct hemagglutinin (HA) glycoproteins (H1 through H16) and one of the nine antigenically distinct neuraminidase (NA) glycoproteins (N1 through N9) (Suarez 2008). Influenza A viruses contain eight segments of single-stranded RNA (ssRNA) that code for at least 10 different viral proteins (Lamb and Krug 1996). The HA, NA, and the membrane ion channel (M2) compose the surface proteins and are coded by segments 4, 6, and 7 respectively. The internal proteins: nucleoprotein (NP), matrix protein (M1), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and the polymerase acidic protein (PA) are encoded by segments 5, 7, 2, 1, and 3, respectively (Palese and Shaw 2007). The two non-structural proteins: nonstructural protein 1 (NS1) and nonstructural proteins 2 (NS2) are both encoded by segment 8 (O'Neill, Jaskunas et al. 1995; Suarez 2008).

The overall structure of the virus consists of a lipid membrane derived from the host cell that contains three integral membrane proteins: HA, NA, and M2 proteins (Swayne and Halvorson 2008). The HA protein, which is the most abundant surface protein, was aptly named for the ability of the virus to agglutinate erythrocytes through attachment to sialic acid receptors (alpha-2, 3 and alpha-2, 6). The HA functions in the mediation of viral attachment to host cells

by binding to sialic acid receptors and resulting penetration of the virus into the host cell cytoplasm. The HA also acts as the major antigen to which neutralizing antibodies are produced (Wiley and Skehel 1987). The HA glycoprotein is translated as an inactive homotrimer precursor. Each identical monomer, HA_0, must be proteolytically cleaved to produce the active components HA_1 and HA_2. This cleavage is necessary for the virus to become infectious (Klenk, Rott et al. 1975; Lamb and Krug 1996). The NA is a homotetramer consisting of a head domain that is enzymatically active and a stalk region that attaches to the membrane (Suarez 2008). The main function of this protein is to catalyze the release of the virus particle from the host cell. This function is essential to prevent accumulation of mature influenza viruses during their release from the infected cell (Seto and Rott 1966; Suarez 2008).

Virulence

The determination of influenza virulence is a multifaceted process. Although studies have shown that neither HA nor NA act as exclusive determinants of virulence, it has been confirmed that the HA protein plays a dominant role in determining pathogenicity (Bosch, Orlich et al. 1979; Steinhauer 1999; Perdue 2008; Swayne and Halvorson 2008). Specifically, the posttranslational cleavage of the precursor HA_0 protein into HA_1 and HA_2 is vital to multiple rounds of replication and enzymes which can perform this cleavage target specific sequences of the connecting peptide between the HA_1 and the HA_2 (Klenk, Rott et al. 1975; Bosch and Garten 1981). The HAs of highly pathogenic influenza (HPAI) viruses are highly cleavable and contain multiple basic amino acids, specifically lysine and arginine, at their cleavage site. The HPAI viruses can be cleaved by multiple proteases present in a broad spectrum of cells. This highly cleavable HA enables the virus to produce systemic infections (Stieneke, Vey et al. 1992; Klenk, Feldman et al. 1998; Steinhauer 1999). In contrast, the HA of low pathogenicity avian influenza

(LPAI) viruses are cleaved in a limited number of cell types, principally epithelial cells of the respiratory and intestinal tract. This limited susceptibility to enzymatic cleavage, which is a direct result of containing only a monobasic cleavage sequence, allows LPAI viruses to produce only localized infections (Rott, Klenk et al. 1995; Klenk, Feldman et al. 1998; Swayne and Halvorson 2008). In addition, the accessibility of proteases to the cleavage site also plays a vital role in virulence determination. Studies have shown that protease accessibility may be hindered by the presence of glycosylation located near the cleavage site. This can result in the protease being unable to gain assess to the HA_0 cleavage sight thus preventing the virus from exerting its maximum pathogenicity (Perdue, Latimer et al. 1995; Swayne, Beck et al. 1998; Perdue and Suarez 2000).

Clinical Signs

LPAI virus infections in wild birds are generally thought to be asymptomatic (Swayne and Halvorson 2008). Influenza viruses have the capacity to exhibit varying pathogenicity in experimentally infected birds, which ranges from low to highly pathogenicity depending on host and subtype. LPAI virus experimental studies with domestic ducks and geese have shown decreases in egg production, conjunctivitis, sinusitis, and lower respiratory tract lesions. Co-infection with bacteria increases the severity of clinical infection and can result in air sacculitis and pneumonia (Pantin-Jackwood and Swayne 2009). LPAI virus infections of other domestic poultry species such as chickens and turkeys will also present different signs depending on the virus strain, species, and age of host. Low mortality is normally accompanied by morbidity, along with mild to severe respiratory disease with coughing, sneezing, rales, rattles, and ocular discharge. Hens may exhibit a decrease in egg production. Birds may become lethargic and may decrease feed and water consumption (Swayne and Halvorson 2008; Pantin-Jackwood and

Swayne 2009). Additional signs of HPAI virus infection are marked depression, inactivity, dyspnea, diarrhea containing excess urates and bile, periorbital edema, decreased egg production and cyanotic combs and wattles (Swayne and Halvorson 2008). Nervous signs such as tremors of head, torticollis, and paralysis may also be present. Post mortem examinations may show ruptured hemorrhagic ova with yolk material in the body cavity, visceral urate deposition, swollen pale kidneys containing urates, petechial hemorrhages in the abdominal and pericardial fat, as well as pneumonia and necrosis in lungs (Swayne and Halvorson 2008; Pantin-Jackwood and Swayne 2009).

Transmission

Reservoirs of avian influenza can be various wild aquatic bird populations and outbreaks of both LPAI and HPAI in domestic poultry may be a direct result of cross over or introduction of AI viruses from these wild bird species. Several farming techniques, such as rearing birds on range, fields, or ponds contribute to the possibility of domestic poultry coming into close contact with infected material from infected wild birds (Swayne 2008). LPAI viruses preferentially infect and replicate in the intestinal epithelial cells of wild birds and are excreted in the feces (Slemons and Easterday 1975; Slemons and Easterday 1977; Webster, Yakhno et al. 1978). This can lead to contamination of surface water and shores. In this situation, waterborne virus is most effectively transmitted through the fecal-oral route (Hinshaw, Weber et al. 1979; Sandu and Hinshaw 1981). Influenza viruses were shown to be capable of persisting in water (Webster, Yakhno et al. 1978). Subsequent studies have shown that AI viruses can persist in water of varying temperatures for extended periods of time (Stallknecht and Shane 1990 B) and that environmental persistence was inversely proportional to both temperature and salinity

(Stallknecht and Kearney 1990 A; Brown, Goekjian et al. 2009). Combined with the movement of waterfowl and shorebirds, this lead to a mechanism for dispersal of AI viruses in nature (Halvorson 2008; Stallknecht and Brown 2008).

Ecology of North American Influenza Viruses

It is widely accepted that aquatic birds are the natural reservoirs for the gene segments of all influenza A viruses. Surveillance for *Orthomyxoviridae* in wild ducks and shorebirds has established that influenza A viruses are harbored in the Anseriformes (ducks, geese, and swans) and the Charadriiformes (gulls, terns, and waders) and that these orders constitute the major LPAI virus reservoir (Slemons, Johnson et al. 1973; Kawaoka, Chambers et al. 1988; Stallknecht and Brown 2008). Many Anseriformes and Charadriiformes perform seasonal long distance migrations and co-habitat with numerous species, which interconnect many bird populations (Stallknecht and Brown 2007). There are four major flyways in North America: the Mississippi and Central flyways, which merge between Missouri and the Gulf of Mexico, as well as the Pacific, and Atlantic flyways. The peak prevalence of AI virus in North American Anseriformes occurs in late summer to early fall when high concentrations of susceptible juvenile birds congregate in pre-migration staging sites throughout Canada and northern United States. During this time AI virus infection rates can exceed 30% in susceptible juveniles (Hinshaw, Wood et al. 1985; Stallknecht and Brown 2008). On the wintering ground, the AI virus prevalence is often lower than 1-2% (Stallknecht and Shane 1988). The peak prevalence of AI virus in North American Charadriiformes occurs in the spring when shorebirds migrating along the Atlantic coast of the United States congregate at Delaware Bay to feed on newly spawned horseshoe crab eggs. The increased bird density during this time is high which facilitates transmission of AI

viruses within the populations. This is the geographic location worldwide where consistent AI virus isolation from shorebirds has been reported (Stallknecht and Shane 1988; Hanson, Luttrell et al. 2008; Stallknecht and Brown 2008).

AI viruses have been identified from birds representing more than 100 species in 12 avian orders. All known HA subtypes (H1- H16) and NA subtypes (N1-N9) have been recovered from feral birds (Olsen, Munster et al. 2006; Stallknecht and Brown 2008); however AI virus subtype diversity with wild birds is dependent on the avian host and geographical location. Subtype diversity within populations varies year to year, with some AI virus subtypes reportedly following a two-year cycle (Krauss, Walker et al. 2004). Hemagglutinin subtypes H3, H4, and H6 and neuraminidase subtypes N2, N6, and N8 are the most common subtypes isolated for waterfowl (Stallknecht and Shane 1988; Stallknecht, Shane et al. 1990 C; Sharpe, Kawaoka et al. 1993; Krauss, Walker et al. 2004). AI viruses of the H5, H7, and H9 subtypes are more uncommon in waterfowl, though occurrences of higher prevalence of these subtypes have been reported (Hanson, Stallknecht et al. 2003; Hanson, Swayne et al. 2005; Munster, Wallensten et al. 2005). Subtype diversity is poorly understood in the Charadriiformes. However the H13 and possibly the H16 subtypes may appear to be gull associated. The prevalence of H5 and H7 viruses in Charadriiformes and Anseriformes, is generally low (Stallknecht and Brown 2008).

The first recorded isolation of influenza virus from wild birds was from a Common tern (*Sterna hirundo)* in 1961 (Becker 1966). Within the Charadriiformes, influenza viruses have been detected in small numbers from three families, with the majority in Scolopacidae (sandpipers and turnstones) and Laridae (gulls and terns) (Olsen, Munster et al. 2006; Hanson, Luttrell et al. 2008). The majority of shorebird AI virus isolations come from ruddy turnstones (*Arenaria interpres)* located at Delaware Bay (Stallknecht 1998; Stallknecht and Brown 2007).

Within the Anseriformes, the subfamily Anatinae, which consists of dabbling and diving ducks, most of the AI virus isolations have been from mallards (Stallknecht and Brown 2008). Limited numbers of AI virus has also been isolated from avian orders associated with aquatic habitats, including, Gaviiformes (loons), Podicepediformes (grebes), Procellariiformes (sheawaters, and petrels), Pelecaniformes (pelicans and cormorants), Ciconiiformes (ibis and herons), and Gruiformes (coots) (Stallknecht 1998; Stallknecht and Brown 2008).

Surveillance in North America

Since wild aquatic birds are considered the natural reservoir of AI viruses, their seasonal migratory behavior presents a method for increased transmission of AI viruses as well as global distribution (Stallknecht, Shane et al. 1990 C). Systematic surveillance for AI viruses among wild bird populations began in the late 1970s. The overall goal of AI surveillance has been to determine the prevalence and subtype diversity of AI viruses (Stallknecht and Brown 2007; Stallknecht and Brown 2008). The prevalence of AI depends on multiple factors such as host species, host age, season, and other environmental factors. The detection of AI viral strains fluctuates from year to year. Identifying migratory behavior as well as subtype diversity within wild bird populations is crucial to the understanding of AI persistence and evolution (Stallknecht and Brown 2007; Hanson, Luttrell et al. 2008; Stallknecht and Brown 2008).

Proper sample collection and processing is critical to the identification of an AI virus infection. Sampling consists mainly of tracheal or oropharyngeal swabs, cloacal swabs, and on occasion, select tissues for virus isolation. Cloacal swabs are routinely collected from wild birds simply because LPAI virus infection results in heavy intestinal replication (Slemons and Swayne 1995; Swayne, Senne et al. 1998). Samples obtained from wild birds, specifically cloacal swabs contain fecal material that may be laden with bacteria. Bacteria introduced into an isolation

system have the ability to grow in numbers that will kill embryos and cell cultures or outgrow any AI virus that might be present. Samples are collected using sterile swabs and are placed into media such as brain heart infusion (BHI), tryptose phosphate broth (TPB) or viral transport media (VTM) (Swayne, Senne et al. 1998) supplemented with antibiotics such as penicillin G, streptomycin, gentamicin, and amphotericin B (Swayne, Senne et al. 1998; Killian 2008). In viral isolation based investigations, the inability to maintain the optimum cold temperatures following specimen collection and during shipping can contribute to decreased frequency of virus recovery. Prolonged storage of experimental, clinical, and surveillance samples under less than ideal conditions reduces virus viability. The cold chain can be maintained by keeping samples out of direct sunlight and by placing samples on wet or dry ice immediately after collection (Johnson 1990; Swayne, Senne et al. 1998; Swayne and Halvorson 2008).

Diagnostic Tools

Isolation of an AI virus may be achieved by culturing the potential viral isolate using embryonating chicken eggs or cell culture. Because numerous agents can replicate in embryonating chicken eggs and cell culture, all primary isolations need to be confirmed as AI virus by using secondary tests (Swayne, Senne et al. 1998). Another widely used diagnostic tool is the detection of specific nucleic acids using molecular methods. The selection and use of a diagnostic system depends on factors such as sensitivity, specificity, speed, and availability of resources (Spackman, Suarez et al. 2008).

Embryos

Virus isolation (VI) of AI viruses using embryos is considered the "gold standard." This preferred method uses 9-to-11 day old embryonating chickens eggs (ECEs) inoculated via the chorioallantoic sac (CAS) route. However some isolates may be recovered following inoculated

into the yolk sac (YS) or onto the chorioallantoic membrane (CAM) routes when the CAS route fails (Swayne, Senne et al. 1998; Woolcock, McFarland et al. 2001; Swayne and Halvorson 2008). Although VI using ECEs is considered the most sensitive system, there remain drawbacks. ECEs are not highly specific and will support growth of a large range of other infectious agents, including bacteria, which can grow to vast numbers effectively overwhelming the possibility of successful isolation of AI virus. Even though ECEs are able to support growth of a broad range of influenza viruses, many field isolates do not grow to high titers in ECEs. In some circumstances, it is necessary to perform serial passages of samples in ECEs to achieve optimal sensitivity. The use of ECEs can be expensive and requires much forethought and planning, as the ECEs require a moderate length of incubation time prior to use and securing a commercial or laboratory source that is reliable (Spackman, Suarez et al. 2008). This removes the possibility of rapidly increasing production to meet the needs of the laboratory, something that can be accomplished with the use of cell culture systems. Conversely, the use of ECEs as a host system does not require the addition of exogenous proteases to facilitate cleavage of the HA_0 protein into HA_1 and HA_2, which allows the virus to achieve multiple rounds of infection (Klenk, Rott et al. 1975). The addition of exogenous proteases to some cell culture systems may be necessary for AI virus replication and this requires testing of the culture system to determine the optimal level of protease to add to a specific culture in order to achieve the desired balance of active AI virus with limited damage to the cell culture. In addition obtaining vast quantities of specific pathogen (SPF) ECEs or AI antibody negative conventional eggs can be difficult. When performing VI using ECEs, any infectious agent present in the original sample material will be amplified. This increases the possibility of human exposure to infectious agents and creates a need for trained personnel to determine causative agent and to eliminate cross contamination

(Spackman, Suarez et al. 2008). In addition, consistent passage or growth of influenza viruses in ECEs can lead to the selection of variants with antigenic and structural changes in the HA molecule. These negative aspects have led to the exploration of alterative AI virus isolation and propagation systems (Brand and Palese 1980; Schild and Oxford 1983).

Cell Culture

An additional reason for the continued reliance on ECEs is the concern that cell cultures were not as sensitive or as consistent for isolating AI viruses. Influenza viruses are able to replicate in a variety of primary and continuous cell cultures such as chicken embryo fibroblasts (CEF) and epithelial kidney cells such as the Madin-Darby canine kidney (MDCK) continuous cell line (Swayne, Senne et al. 1998; Swayne and Halvorson 2008). MDCK cells have been used for isolation and propagation of influenza viruses with trypsin supplemented media since it was reported 1975 (Tobita, Sugiura et al. 1975). Other cell lines such as primary chicken kidney (CK), swine kidney (SK), embryonic swine kidney (ESK), baby hamster kidney (BHK), and monkey kidney (VERO) have been tested for susceptibility to and support of influenza infections but of these cell lines, the MDCK cell line and CEF primary cultures have proven to be the most sensitive and consistent cell culture for the growth of AI viruses (Sugimura, Murakami et al. 2000; Swayne and Halvorson 2008).

The use of trypsin as an exogenous protease that facilitated viral replication of LPAI viruses in cell culture was noted, demonstrating the function of the HA glycoprotein in mediating infection (Klenk, Rott et al. 1975). The HA_0 precursor protein must be cleaved into HA_1 and HA_2 for the virus to become infectious and produce multiple replication cycles (Lamb and Krug 1996). Hemagglutinin of LPAI viruses are cleaved by trypsin-like proteases, found in respiratory and intestinal epithelial cells (Rott, Klenk et al. 1995; Klenk, Feldman et al. 1998; Swayne and

Halvorson 2008). In most cell culture isolation systems, LPAI viruses require the addition of exogenous trypsin to the medium in order to cleave the HA and produce infectious virus (Spackman, Suarez et al. 2008; Swayne and Halvorson 2008).

Many of the same concerns present with the use of ECEs are still present with the use of cell cultures. Cell cultures are not specific to which infectious agent is amplified, and as such, any potential agent present in sample materials, including bacteria, may be amplified to high levels. This requires trained personnel to avoid cross contamination with other samples, avoid exposure to infectious agents, and avoid misidentification of the agent (Spackman, Suarez et al. 2008).

Agent Identification Assays

The hemagglutination assay is a tool used to screen cell culture supernatant or allantoic fluid harvested from embryonating chicken eggs for hemagglutinating agents, such as type A influenza (Carbrey, Beard et al. 1974; Swayne and Halvorson 2008). The hemagglutination assay does not provide a definitive diagnosis, as other agents such as avian paramyxovirus also hemagglutinate erythrocytes (Swayne, Senne et al. 1998). Live and inactivated viruses are detected by the hemagglutination test but amplification of the virus in ECEs or cell culture to increase the concentration may be required before hemagglutination activity may be detected (Swayne and Halvorson 2008). Differential diagnosis may be obtained serologically using the agar gel immunodiffusion assay (AGID), the hemagglutination inhibition (HI) assay, or a commercially available immunoassay kit specific for type A influenza (Swayne and Halvorson 2008).

Serologic Assays

Serologic assays can be utilized to demonstrate the presence of AI virus antibody or antigen in samples. There are several options for serologic diagnosis and surveillance (Swayne and Halvorson 2008).

The agar gel immunodiffusion assay is an inexpensive and simple assay that does not require extensive supplies or equipment. The principle of the AGID is to visualize the immunoprecipitation reaction of AI virus nucleoprotein (NP) and matrix (M) antigen, and antibody after diffusion in an agar matrix (Beard 1970; Spackman, Suarez et al. 2008). Antigen may be prepared by concentrating virus from infective AAF by acid precipitation or by extraction from infected chorioallantoic membranes (Swayne, Senne et al. 1998). Detection of anti-NP/M antibody is frequently used to detect infection by any AI virus irregardless of HA or NA subtype, as this detects antibodies to influenza A antigens that are shared by all AI viruses (Swayne and Halvorson 2008).

The HI assay is another serologic test that can be used to detect and differentiate between the numerous hemagglutinin subtypes in serum, plasma, or egg yolk (Carbrey, Beard et al. 1974; Swayne and Halvorson 2008). The basis of the HI test is inhibition of hemagglutination with subtype-specific antibodies, as such suspected AI virus isolates can be identified with a panel of subtype specific antisera representing each HA subtype; i.e. H1-16 (Swayne, Senne et al. 1998). However, some sera may contain nonspecific inhibitors of hemagglutination that may interfere with the specificity of the HI test; i.e. mask low levels of HI activity. Pretreatment of test sera by preabsorption with chicken erythrocytes can remove the nonspecific inhibitors (Swayne and Halvorson 2008). The HI assay is relatively inexpensive, less technical than molecular tests, and can be completed within several hours. The HI assay is considered more sensitive than the AGID

and will detect AI virus antibody for a longer period post exposure than the AGID (Swayne and Halvorson 2008). The HI is routinely used for species such as chickens and turkeys but can be considered for use with other species, such as ducks (Spackman, Suarez et al. 2008).

Polymerase Chain Reaction

Many laboratories have the capacity to employ molecular techniques for detection of AI virus genome. Such techniques include reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR (RRT-PCR). Conventional diagnostic methods, such as virus isolation using ECEs and cell culture can be costly, labor intensive, and time consuming considering the demand for rapid diagnosis and control measures (Spackman, Suarez et al. 2008). Paired with the correctly defined primers, RT-PCR can produce rapid detection of influenza A virus and subtype identification (at least of H5 and H7), and allow creation of a cDNA product that can be used for nucleotide sequencing**.** Modifications of the standard RT-PCR method have been applied to reduce the time for both identification of virus subtype and sequencing. Spackman et al. (2002) used a 'real time' single step RT-PCR primer set paired with a fluorogenic hydrolysis probe to allow detection of AI viruses and differentiation of subtypes H5 and H7. The test performed well relative to virus isolation and offered a more cost effective and more rapid alternative to virus isolation (Spackman, Senne et al. 2002; Spackman, Senne et al. 2003). Although trials conducted with conventional RT-PCR to assess its sensitivity have determined this method was sufficient to detect viral RNA from clinical specimens such as swabs, RRT-PCR is an improvement over convention RT-PCR in regard to rapidity, sensitivity, reproducibility, and reduction in carry-over contamination and as such has become the method of choice for diagnosis in many laboratories (Spackman, Suarez et al. 2008).

Clinical and diagnostic samples and samples from experimentally infected animals are complex mixtures of host cells, bacteria, and other materials than can interfere with detection of AI virus by molecular methods. As such the methods by which the RNA is extracted and purified is critical and as such, must be evaluated to avoid false negative results due to inefficient extraction resulting in low quality RNA or the presence of inhibitors (Cone, Hobson et al. 1992; Buonagurio, Coleman et al. 1999; Petrich, Mahony et al. 2006). PCR inhibitors have been detected in cloacal samples from wild birds as well as in various tissue samples. The diet of domestic poultry is generally uniform which allows for more consistent RNA extraction. By contrast, samples obtained from wild birds can vary greatly because of the variety of foods they consume (Spackman and Suarez 2008). The ideal extraction method is one that will provide high quality RNA and will also produce co-extraction of PCR inhibitors. Samples, such as cloacal swabs, may contain substances that can degrade RNA once it is released from the virion. Specimens containing fecal material are generally more difficult to work with and may require processing methods that inactivate RNases and efficiently remove PCR inhibitors (Spackman and Suarez 2008). To increase confidence in PCR results, it is useful to include an internal positive control (IPC), which differentiates functioning from nonfunctional tests (Das, Spackman et al. 2006)

Molecular methods offer high sensitivity and detection when compared to the "gold standard" of isolation using ECEs. In addition, PCR based techniques also offer specificity. There are many other advantages to consider, such as limited contact with infectious material as the viral content of samples is inactivated during the first stage of processing. RRT-PCR is also extremely rapid, returning results in a fraction of the time and the cost is relatively similar to that

of ECEs. However with detection based on RNA, viable and nonviable virus will be detected and the assays require requires highly skilled trained personnel (Spackman, Suarez et al. 2008).

REFERENCES

Beard, C. W. 1970. Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion. Bulletin of the World Health Organization 42: 779-785.

Becker, W. B. 1966. The Isolation and classification of tern virus: influenza virus a/tern/southafrica/1961. Journal of Hygiene 64(3): 309-320.

Bosch, F. X. and W. Garten 1981. Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between ha1 and ha2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. Virology 113(2): 725-735.

Bosch, F. X., M. Orlich, H. D. Klenk and R. Rott 1979. The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology 95: 197-207.

Brand, C. and P. Palese 1980. Sequential passage of influenza virus in embryonated eggs or tissue culture: emergence of mutants. Virology 107: 424-433.

Brown, J. D., G. Goekjian, R. Poulson, S. Valeika and D. E. Stallknecht 2009. Avian influenza virus in water: infectivity is dependent of pH, salinity, and temperature. Veterinary Microbiology 136: 20-26.

Buonagurio, D. A., J. W. Coleman, S. A. Patiandla, B. S. Prabhakar and J. M. Tatem 1999. Direct detection of sabin poliovirus vaccine strains in stool specimens of first-dose vaccines by a sensitive reverse transcription-pcr method. Journal of Clinical Microbiology 37: 283-289.

Carbrey, E. A., C. W. Beard, R. J. Cooper, R. P. Hansen and B. S. Pomeroy 1974. Hemagglutination and hemagglutination-inhibition tests with newcastle disease virus--

microtiter technique. 17th Annual Proceedings of the American Association of Veterinary Laboratory Diagnosticians: 1-6.

Cone, R. W., A. C. Hobson and M. L. Huang 1992. Coamplified positive control detects inhibition of polymerase chain reactions. Journal of Clinical Microbiology 30: 3185-3189.

Das, A., E. Spackman, D. Senne, J. Pedersen and D. L. Suarez 2006. Development of an internal positive control for rapid diagnosis of avian influenza virus infections by real-time reverse transcription-pcr with lyophilized reagents. Journal of Clinical Microbiology 44(9): 3065-3073.

Easterday, B. C., D. O. Trainer, B. Tumova and H. G. Pereira 1968. Evidence of infection with influenza viruses in migratory waterfowl. Nature 219: 523-524.

Halvorson, D. 2008. Control of low pathogenicity avian influenza. Avian Influenza Ed. David Swayne, Blackwell Publishing (Ames, IA): 513-536.

Hanson, B. A., M. P. Luttrell, V. H. Goekjian, L. Niles, D. E. Swayne, D. A. Senne and D. E. Stallknecht 2008. Is the occurrence of avian influenza virus in charadriiformes species and location dependent? Journal of Wildlife Diseases 44(2): 351-361.

Hanson, B. A., D. E. Stallknecht, D. E. Swayne, L. A. Lewis and D. A. Senne 2003. Avian influenza viruses in minnesota ducks during 1998-2000. Avian Diseases 47: 867-871.

Hanson, B. A., D. E. Swayne, D. A. Senne, D. S. Lobpries, J. Hurst and D. E. Stallknecht 2005. Avain influenza and paramyxoviruses in wintering and resident ducks in texas. Journal of Wildlife Diseases 41: 624-629.

Hinshaw, V. S., R. G. Weber and B. Turner 1979. Water-borne transmission of influenza a viruses. Intervirology 11: 66-69.

Hinshaw, V. S., J. M. Wood, R. G. Webster, R. Deibel and B. Turner 1985. Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of north america. Bulletin of the World Health Organization 63: 711-719.

Johnson, F. B. 1990. Transport of viral specimens. Clinical Microbiology Reviews 3: 120-131.

Kaleta, E. and C. Rulke 2008. The beginning and spread of fowl plague (h7 high pathogenicity avian influenza) across europe and asia (1878-1955). Avian Influenza Ed. David E. Swayne, Blackwell Publishing, (Ames, IA): 145-189.

Kawaoka, Y., T. M. Chambers, W. L. Sladen and R. G. Webster 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from than in wild ducks? Virology 163: 247-250.

Killian, M. L. 2008. Avian influenza virus sample types, collection, and handling. Avian Influenza Virus Ed. Erica Spackman, Humana Press (Totowa, NJ): 7-12.

Klenk, H. D., A. Feldman and W. Garten 1998. Virus and host factors determining the polygenic nature of pathogenicity. In, Proceedings of the Fourth Annual Symposium on Avian Influenza: 155-161.

Klenk, H. D., R. Rott, M. Orlich and J. Blodorn 1975. Activation of influenza a viruses by trypsin treatment. Virology 68: 426-439.

Krauss, S., D. Walker, S. Paul-Pryor, l. Niles, L. Chenghong, V. S. Hinshaw and R. G. Webster 2004. Influenza a viruses in migrating wild aquatic birds in north america. Vector-borne and Zoonotic Diseases 4: 177-189.

Lamb, R. A. and R. M. Krug 1996. Orthomyxoviridae: the viruses and their replication. In, Fields Virology 3rd Ed. D.M. Knipe and P.M. Howley, Lippincott Williams + Wilkins (Philadelphia, PA): 1353-1395.

Munster, V. J., A. Wallensten, C. Baas, G. F. Rimmelzaann, M. Schutten, B. Olsen, A. D. Osterhaus and R. Fouchier, A. 2005. Mallards and highly pathogenic avian influenza ancestral viruses, northern europe. Emerging Infectious Diseases 11: 1545-1551.

O'Neill, R. E., R. Jaskunas, G. Bolbel, P. Palese and J. Moroianu 1995. Nuclear import of influenza virus rna can be mediated by viral nucleoprotein and transport factors required for protein import. Journal of Biological Chemistry 270: 22701-22704.

Olsen, B., V. J. Munster, A. Wallensten, J. Wallensten, A. D. Osterhaus and R. Fouchier, A. 2006. Global patterns of influenza a virus in wild birds. Science 312: 384-388.

Palese, P. and M. Shaw 2007. Orthomyxoviridae: the viruses and their replication. Fields Virology 5th Ed Ed. D.M. Knipe and P.M. Howely, Lippincott Williams + Wilkins (Philadelphia, PA): 1647-1689.

Pantin-Jackwood, M. J. and D. E. Swayne 2009. Pathogenesis and pathobiology of avian influenza virus infection in birds. Revue Scientifique et Technique 28(1): 113-136.

Perdue, M. 2008. Molecular determinants of pathogenicity for avian influenza viruses. Avian Influenza Ed. David E. Swayne, Blackwell Publishing (Ames, IA): 23-39.

Perdue, M. L., J. W. Latimer and J. M. Crawford 1995. A novel carbohydrate addition site on the hemagglutinin protein of a highly pathogenic h7 subtype avian influenza virus. Virology 213: 276-281.

Perdue, M. L. and D. L. Suarez 2000. Structural features of the avian influenza virus hemagglutinin that influence virulence. Veterinary Microbiology 74: 77-86.

Petrich, A., J. Mahony, S. Chong, G. Broukhanski, F. Gharabaghi, G. Johnson, L. Louie, K. Luinstra, B. Willey, P. Akhaven, L. Chui, F. Jamieson, M. Louie, T. Mazzulli, R. Tellier, M. Smieja, W. Cai, M. Chernesky and S. E. Richardso 2006. Multicenter comparison of

nucleic acid extraction methods for detection of severe acute respiratory syndrome coronavirus rna in stool specimens. Journal of Clinical Microbiology 44: 2681-2688.

Rott, R., H. D. Klenk, Y. Nagai and M. Tashiro 1995. Influenza viruses, cell enzymes, and pathogenicity. American Journal of Respiratory and Critical Care Medicine 152(2): S16-S19.

Sandu, T. and V. S. Hinshaw 1981. Influenza a virus infection in domestic ducks. Proceedings of the 1st International Symposium on Avian Influenza: 222-227.

Schild, G. C. and J. S. Oxford 1983. Evidence for host-cell selection of influenza virus antigenic variants. Nature 303: 706-709.

Seto, J. T. and R. Rott 1966. Functional significance of sialidose during influenza virus multiplication. Virology 30: 731-737.

Sharpe, G. B., Y. Kawaoka, S. M. Wright, B. Turner, V. S. Hinshaw and R. G. Webster 1993. Wild ducks are the reservoir for only a limited number of influenza a subtypes. Epidemiology and Infection 110: 161-176.

Slemons, R. D. and B. C. Easterday 1975. The natural history of type a influenza viruses in wild waterfowl. Third international wildlife disease conference: 215-224.

Slemons, R. D. and B. C. Easterday 1977. Type-a influenza viruses in the feces of migratory waterfowl. Journal of the American Veterinary Medical Association 171: 947-948.

Slemons, R. D., D. C. Johnson, J. S. Osborn and F. Hayes 1973. Type-a influenza viruses isolated from wild free-flying ducks in california. Avian Diseases 18(1): 119-124.

Slemons, R. D. and D. E. Swayne 1995. Tissue tropism and replicative properties of waterfowl origin influenza viruses in chickens. Avian Diseases 39: 521-527.

Spackman, E., D. A. Senne, L. L. Bulaga, T. J. Myers, M. L. Perdue, L. P. Garber, K. Lohman, L. T. Daum and D. L. Suarez 2003. Development of real-time rt-pcr for the detection of avian influenza virus. Avian Diseases 47: 1079-1082.

Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum and D. L. Suarez 2002. Development of a real-time reverse transcriptase pcr assay for type a influenza virus and the avian h5 and h7 hemagglutinin subtypes. Journal of Clinical Microbiology 40: 3256-3260.

Spackman, E. and D. L. Suarez 2008. Avian influenza virus rna extraction from tissue and swab material. Methods in Molecular Biology 436: 13-18.

Spackman, E., D. L. Suarez and D. A. Senne 2008. Avian influenza diagnostic and surveillance methods. Avian Influenza ED. D.E. Swayne, Blackwell Publishing (Ames, IA): 299-308.

Stallknecht, D. E. 1998. Ecology and epidemology of avian influenza viruses in wild bird populations: waterfowl, shorebirds, pelicans, cormorants, etc. Proceedings of the fourth international symposium on avain influenza: 61-69.

Stallknecht, D. E. and J. D. Brown 2007. Wild birds and the epidemiology of avian influenza Journal of Wildlife Diseases 43(S3): S15-S20.

Stallknecht, D. E. and J. D. Brown 2008. Ecology of avian influenza in wild birds. Avian Influenza Ed. D.E.. Swayne, Blackwell Publishing (Ames, IA): 43-58.

Stallknecht, D. E. and M. T. Kearney 1990 A. Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. Avian Diseases 34(2): 412-418.

Stallknecht, D. E. and S. M. Shane 1988. Host range of avian influenza virus in free-living birds. Veterinary Research Communications 12: 125-141.

Stallknecht, D. E. and S. M. Shane 1990 B. Persistence of avian influenza viruses in water. Avian Diseases 34(2): 406-411.

Stallknecht, D. E., S. M. Shane, P. J. Zwank, D. A. Senne and M. T. Kearney 1990 C. Avian influenza viruses from migratory and resident ducks of coastal louisiana. Avian Diseases 34: 398-405.

Steinhauer, D. A. 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology 258: 1-20.

Stieneke, G. A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk and W. Garten 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. The EMBO Journal 11: 2407-2414.

Suarez, D. 2008. Influenza a virus. Avian Influenza Ed. David E. Swayne, Blackwell Publishing (Ames, IA): 3-22.

Sugimura, T., Y. Murakami and T. Ogawa 2000. The susceptibility of culture cells to avain influenza viruses. Journal of Veterinary Medical Science 62: 659-660.

Swayne, D. and D. Halvorson 2008. Influenza. Diseases of Poultry 12th Ed Ed. A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.E. Swayne, Blackwell Publishing (Ames, IA): 153-184.

Swayne, D. and D. Suarez 2000. Highly pathogenic avian influenza Revue Scientifique et Technique 19: 463-482.

Swayne, D. E. 2008. Epidemiology of avian influenza in agricultural and other man-made systems. Avian Influenza Ed. David E. Swayne, Blackwell Publishing (Ames, IA): 59-85.

Swayne, D. E., J. R. Beck, M. Garcia, M. L. Perdue and M. Brugh 1998. Pathogenicity shifts in experimental avian influenza virus infections in chickens. In, Proceedings of the Fourth Annual Symposium on Avian Influenza: 171-181.

Swayne, D. E., D. A. Senne and C. W. Beard 1998. Avian Influenza, in a laboratory manual for the isolation and identification of avian pathogens. 4th Ed. ED. D.E. Swayne, J.R. Glission, M.W. Jackwood, J.E. Pearson, W.M Reed. The American Association of Avian Pathologists (Kennett Square, PA).

Tobita, K., A. Sugiura, C. Enomote and M. Furuyama 1975. Plaque assay and primary isolation of influenza a viruses in an established line of canine kidney cells (mdck) in the presence of trypsin. Medical Microbiology and Immunology 162: 9-14.

Webster, R. G., V. S. Yakhno, W. R. Hinshaw, J. Bean and K. G. Murtl 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology 84: 268-278.

Wiley, D. C. and J. J. Skehel 1987. The structure and function of the heamagglutinin membrane glycoprotein of influenza virus. Annual Review of Biochemistry 56: 365-394.

Woolcock, P. R., M. D. McFarland, S. Lai and R. P. Chin 2001. Enhanced recovery of avian infuenza virus isolates by a combination of chicken embryo inoculation methods. Avian Diseases 45: 1030-1035.

CHAPTER 3

EVALUATION AND OPTIMIZATION OF AVIAN EMBRYOS AND CELL CULTURE METHODS FOR EFFICIENT ISOLATION AND PROPAGATION OF AVIAN INFLUENZA VIRUSES[1]

[1]Moresco, K.A., D.E. Stallknecht, D.E. Swayne. *Avian Diseases*. 54: 622-626.

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INTRODUCTION

Surveillance of wild bird populations for avian influenza viruses (AI viruses) is essential to our understanding of AI virus evolution and ecology. Subsequently, efficient detection and isolation of AI viruses from surveillance samples continues to be a high priority. Currently, the use of molecular techniques such as real-time reverse transcriptase polymerase chain reaction (RRT-PCT) for the detection of viral nucleic acid has emerged as the preferred test for identification of AI viruses in wild bird samples (Spackman, Suarez et al. 2008). However the recovery and propagation of viable AI virus still depends on the use of embryonating chicken eggs (ECEs). As the "gold" standard method of isolation of AI viruses, ECEs are able to support growth of a broad spectrum of AI viruses and subtypes. However, some RRT-PCR positive surveillance samples have failed to yield AI viruses on isolation attempts in ECEs (Lee, Jung et al. 2009). This fact has prompted investigation of other species of avian embryos and potentially cell culture systems for higher isolation rates for AI viruses from surveillance samples. Virus isolation using ECEs tends to be costly and requires much forethought concerning scheduling due to the fact that embryos must be incubated 9-11 days prior to use (Spackman, Suarez et al. 2008). In addition, the persistent propagation of AI viruses in ECEs has been shown to lead to the emergence of mutations in the hemagglutinin glycoprotein (Schild and Oxford 1983). Finally, the ability of diagnostic laboratories to maintain a large volume of high quality avian embryos can be a limiting factor in virus isolation and propagation (Lee, Jung et al. 2009).

Other methods of AI virus isolation and propagation have been explored, such as the use of cell culture. Overall the MDCK cell line has been the most widely used cell culture system since 1975 when Tobita et al. reported that MDCK cells were useful for the propagation of

influenza viruses (Asaoka, Tanaka et al. 2006). Other cell lines have been tested for permissiveness to AI viruses such as embryonic swine kidney (ESK), African green monkey kidney (Vero), swine kidney (SK), hamster lung (HmLu-1), and monkey kidney (JTC-12) (Sugimura, Murakami et al. 2000; Seo, Goloubeva et al. 2001). While these cell lines are permissive to AI viruses, the sensitivity is lower than ECEs. Of all the cell lines tested, the MDCK was identified as the best for propagation of influenza viruses, but number of hemagglutinin subtypes of influenza A tested has been limited (Seo, Goloubeva et al. 2001). In this study, we evaluated different species of avian embryos for the highest yield when inoculated with a diverse selection of LPAI viruses and determined which avian embryos would produce the higher isolation rate when inoculated with wild bird surveillance samples. In addition, we evaluated which cell lines or cell cultures with and without supplemental trypsin would produce the highest yield when inoculated with a diverse selection of LPAI viruses.

METHODS AND MATERIALS

Viruses. The virus isolates used in this study are listed in Table 3.1. The viruses were obtained from the repository of the Southeast Poultry Research Laboratory (SEPRL) with original viruses provided by Dennis Senne (NVSL, Ames, IA), Robert Webster (St. Jude's, Memphis TN), David Stallknecht (University of Georgia, Athens, GA), and Richard Slemons (The Ohio State University, Columbus, OH). Individual virus strains were selected to cover all 16 hemagglutinin subtypes and eight of nine neuraminidase subtypes (N1-6, N8 and N9). The viruses were originally isolated from a variety of wild bird species or domestic waterfowl.

Cell Cultures/Cell Lines. The cell cultures selected for this study are listed in Table 3.2. The impact of passage history on replication titer was determined for one virus, A/CanadaGoose/WI/902/75 (H5N2), through passages 1-10 with and without supplemental L-

(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-trypsin in the MDCK cell line. For determination of H1 through H16 virus growth in different cell lines, cells were seeded in 48-well cell-culture plates and allowed to monolayer for 24 hours. When the monolayers were between 90 to 95% confluent, 5 replicates were inoculated with a ten fold serial dilution of a specific virus strain. Each virus was inoculated onto two plates per cell culture: one plate received TPCK-trypsin supplemented media, and the other received non-supplemented media. After four days of incubation, the cells were examined microscopically for presence of cytopathic effect (CPE). Plates were frozen at -70C, allowed to thaw and the supernatant was harvested for hemagglutination (HA) testing. The median tissue culture infectious dose ($TCID_{50}$)/ml was calculated by the Reed and Muench method using the HA pattern (Reed and Muench 1938). Ten day of age specific pathogen free (SPF) embryonating chicken eggs (ECEs) were inoculated via the chorioallantoic sac route (CAS) according to standard methods with the same tenfold serial dilution (Swayne, Senne et al. 2008). Chorioallantoic fluid was harvested on 4 days post inoculation (DPI) to test for HA activity. Using the HA pattern, the mean embryo infectious dose (EID_{50})/ml was calculated by the Reed and Muench method (Reed and Muench 1938).

Avian Embryos. The embryos selected for this study were specific pathogen free embryonating chicken (SEPRL, Athens, GA), and turkey (SEPRL, Athens, GA), and conventional embryonating duck eggs (Maple Leaf Farms, Milford, IN) eggs. All of the embryos were tested with the same H1 through H16 LPAI viruses listed in Table 3.1. The duck and turkey embryos were used on 13 days of incubation, while the chicken embryos were used on 10 days of incubation. Embryos were inoculated via the CAS route according to standard methods (Swayne, Senne et al. 2008). On 4 DPI, chorioallantoic fluid was harvested for HA testing. The

EID_{50}/ml was calculated by the Reed and Muench method using the HA pattern (Reed and Muench 1938).

In addition, the efficiency of the embryos for primary isolation of AI viruses from wild bird surveillance samples was compared. Initially, the surveillance samples were determined to be positive for AI virus matrix protein by RRT-PCR and had cycle threshold (CT) values of greater than or equal to 32. The samples were inoculated into the CAS according to the standard methods. On 4 DPI, embryos were candled for mortality and chorioallantoic fluid was harvested for HA testing. Samples with a positive HA were tested for AI virus by antigen capture test (BinaxNow® test, Inverness Medical, Scarborough, ME). If samples were determined to be negative for a hemagglutinating agent, they were reinoculated into embryos for a second and third isolation attempt.

RESULTS AND DISCUSSIONS

Cell Cultures/Cell Lines. Using a dose response test, the optimal trypsin concentration was determined for each culture. The BHK-21, QT-35, and DF-1 cell lines had greater susceptibility to trypsin toxicity; these lines received 0.1µg/ml or less. The Mv1Lu, CEF, CEK, and HD11 cultures showed moderate resistance to trypsin; these lines received 0.15µg/ml, 0.2µg/ml, 0.2µg/ml and 0.25 µg/ml, respectively. The MDCK cell line received 0.45µg /ml and was the most resistance to toxicity caused by trypsin. To determine impact of cell passage on virus replication titers, ten serial passages of the H5N2 virus was conducted in the MDCK cell line with and without supplemental trypsin (Figure 3.1). The titers ranged from undetectable by HA testing on passages four and nine, to as high as $10^{6.8}$ $TCID_{50}$/ml on passages five and seven. Also noted was lack of differences in titer between passage one and passage ten. Based on this

information, one passage of each virus in cell culture and calculating the $TCID_{50}$ would be a sufficient indicator of the cell culture's ability to support LPAI virus replication.

The $TCID_{50}$ results (Figure 3.2) for all cell cultures and lines tested with the H1 through H16 LPAI viruses allowed categorization of the cell cultures into three groups. First, the MDCK, CEK, and CEF with supplemental trypsin were placed into the group that produced $TCID_{50}$ s on average between 10^{1-3} less than the corresponding EID_{50} in ECEs. The second group consisted of the DF-1, HD11, Mv1LU, QT-35 with supplemental trypsin, and the CEF without supplemental trypsin. These cultures replicated LPAI virus on average between 10^{3-5} $TCID_{50}$ less than the EID_{50} in ECEs. The third group represents the cultures that replicated LPAI virus on average between 10^{5-7} $TCID_{50}$ less than the EID_{50} in ECEs; this group consisted of the BHK-21 and the QT-35 without supplemental trypsin. The addition of supplemental trypsin did allow for an increase in titer for certain cell lines. The CEF, DF-1, QT-35 and the Mv1Lu cultures experienced an increase ranging from $10^{0.5}$ $TCID_{50}$ /ml to as much as 10^{3} $TCID_{50}$ /ml, where as the kidney epithelial cultures, e.g. MDCK and CEK, experienced on average less than $10^{0.3}$ $TCID_{50}$/ml increase in titer with the addition of supplemental trypsin.

The passage of the H5N2 virus ten times in the MDCK cell lines produced fluctuations in titer that were interpreted as adaptation cycles to growth in the cell culture system. Most influenza A and B isolates undergo periods of adjustment or adaptation to host cells but can be converted to high growth viruses after several passages through MDCK cells (Asaoka, Tanaka et al. 2006). Two factors seem to play a role in the efficient propagation of LPAI virus in cell culture: (1) the cell culture selected for use and (2) the particular virus strain used. The MDCK cell line has been the most consistently used for culturing and propagating AI viruses. The data here indicates that the primary cultures, CEK and CEF, may also be added as efficient cultures

for AI virus propagation, although the production of these primary cultures is moderately labor intensive compared to the perpetual MDCK line. The majority of the fibroblast cell lines required the addition of supplemental trypsin to facilitate LPAI virus growth. LPAI viruses require trypsin-like enzyme cleavage of the hemagglutinin precursor protein to enable multiple replication cycles. Epithelial cell cultures contain endogenous trypsin-like enzymes and are permissive to LPAI virus growth, but fibroblastic and other mesenchymal cells lack trypsin-like enzymes and require addition of trypsin in culture for LPAI virus replication (Bosch and Garten 1981; Spackman, Suarez et al. 2008). In our study, MDCK and CEK cell cultures exhibited small increases in LPAI virus growth titers with the addition of supplemental trypsin. The addition of supplemental trypsin may not be necessary with the kidney epithelial cells, which are known to contain endogenous trypsin-like proteases.

The most inefficient cultures for LPAI virus growth were the BHK-21 and QT-35, whereas the remaining cell lines supported moderate levels of LPAI virus replication. Some of the cell lines produced titers in a more consistent fashion than others; the HD11 cell line consistently produced $TCID_{50}$s between $10^{4.8}$ $TCID_{50}$/ml and $10^{5.8}$ $TCID_{50}$/ml, whereas the Mv1Lu cell line showed varying results from a low of $10^{2.8}$ $TCID_{50}$/ml to the high of $10^{7.7}$ $TCID_{50}$/ml.

Avian Embryos. As a whole, the propagation titers on first passage for embryonating chicken, turkey, and duck eggs inoculated with H1 through H16 LPAI viruses were similar. Minor differences in titer were noted between different embryo types for individual virus strains; however the range of these differences was as low a $10^{0.1}$ EID_{50}/ml to the high of $10^{1.2}$ EID_{50}/ml. To determine if different embryos could affect AI virus isolation from surveillance swabs, two sample sets totaling 56 wild bird surveillance samples with RRT-PCR CT values of 32 or greater

for the matrix protein were inoculated into all three types of embryos. The first group, consisted of 12 samples taken from gulls within the United States, was processed using embryonating chicken and turkey embryos. No viable isolates were recovered from this sample set. The second group, consisted of 44 samples collected from various avian species in Mongolia, was processed with embryonating duck and chicken embryos. One AI virus isolate was recovered in both the duck and chicken embryos from this sample set. While the data did not identify a species of embryos that was better for isolation of AI viruses from wild bird surveillance samples, the results suggest the three types of embryonating avian eggs may be used interchangeably. However, the use of a larger sample set for isolation attempts may result in a higher number of positive isolations for one or more of the three types of avian embryos.

REFERENCES

Asaoka, N., Y. Tanaka, T. Sakai, Y. Fujii, R. Ohuchi and M. Ohuchi 2006. Low growth ability of recent influenza clinical isolates in mdck cells is due to their low receptor binding affinities. Microbes and Infection 8(2): 511-519.

Bosch, F. X. and W. Garten 1981. Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between ha1 and ha2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. Virology 113(2): 725-735.

Lee, C. W., K. Jung, S. J. Jadjao and D. L. Suarez 2009. Evaluation of chicken-origin (df-1) and quail-origin (qt-35) fibroblast cell lines for replication of avian influenza viruses. Journal of Virological Methods 153(1): 22-28.

Reed, L. J. and H. Muench 1938. A simple method for estimating fifty percent endpoints. American Journal of Hygiene 27: 493-497.

Schild, G. C. and J. S. Oxford 1983. Evidence for host-cell selection of influenza virus antigenic variants. Nature 303: 706-709.

Seo, S. H., O. Goloubeva, R. Webby and R. G. Webster 2001. Characterization of a porcine lung epithelial cell line suitable for influenza virus studies. Journal of Virology 75(19): 9517-9525.

Spackman, E., D. L. Suarez and D. A. Senne 2008. Avian influenza diagnostic and surveillance methods. Avian Influenza Ed. D.E. Swayne, Blackwell Publishing, (Ames, IA): 299-308.

Sugimura, T., Y. Murakami and T. Ogawa 2000. The susceptibility of culture cells to avian influenza viruses. Journal of Veterinary Medical Sciences 62(6): 659-660.

Swayne, D. E., D. A. Senne and C. W. Beard 2008. Avian influenza, in: a laboratory manual for the isolation and identification of avian pathogens. 5th Edition. Ed. D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson, WlM. Reed. American Association of Avian Pathologists (Jacksonville, FL): 128-134.

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TABLES AND FIGURES

Table 3.1. Virus strains and subtypes

Virus Strain	Subtype	Figure 2. Graph
A/Duck/New York/15024-21/96	H1N1	A
A/HerringGull/Delaware/677/88	H2N8	B
A/Duck/NewYork/6874/78	H3N2	C
A/Mallard/Ohio/338/86	H4N8	D
A/Canada Goose/Wisconsin/802/75	H5N2	E
A/Duck/Pennsylvania/69	H6N1	F
A/Goose/NewYork/8600-3/98	H7N2	G
A/Duck/Lousiana/B174/86	H8N4	H
A/Ruddy Turnstone/New Jersey/650658/02	H9N9	I
A/Green WingTeal/272W/87	H10N2	J
A/Ruddy Turnstone/New Jersey/650678/02	H11N4	K
A/Duck/Louisana/188D/87	H12N5	L
A/Gull/Maryland/1824/78	H13N9	M
A/Mallard/Gurjev(Russia)/263/82	H14N5	N
A/Shearwater/Western Australia/2576/79	H15N6	O
A/Black-HeadedGull/Mongolia/1756/06	H16N3	P

Table 3.2. Cell cultures and cell lines

Name	Origin	Tissue	Morphology
CEK	Chicken	Kidney	Epithelial
CEF	Chicken	Embryo	Fibroblast
BHK-21	Hamster	Kidney	Fibroblast
DF-1	Chicken	Embryo	Fibroblast
HD11	Chicken	Bone Marrow	Leukocyte
Mv1Lu	Mink	Lung	Epithelial
QT-35	Quail	Fibrosarcoma	Fibroblast
MDCK	Canine	Kidney	Epithelial

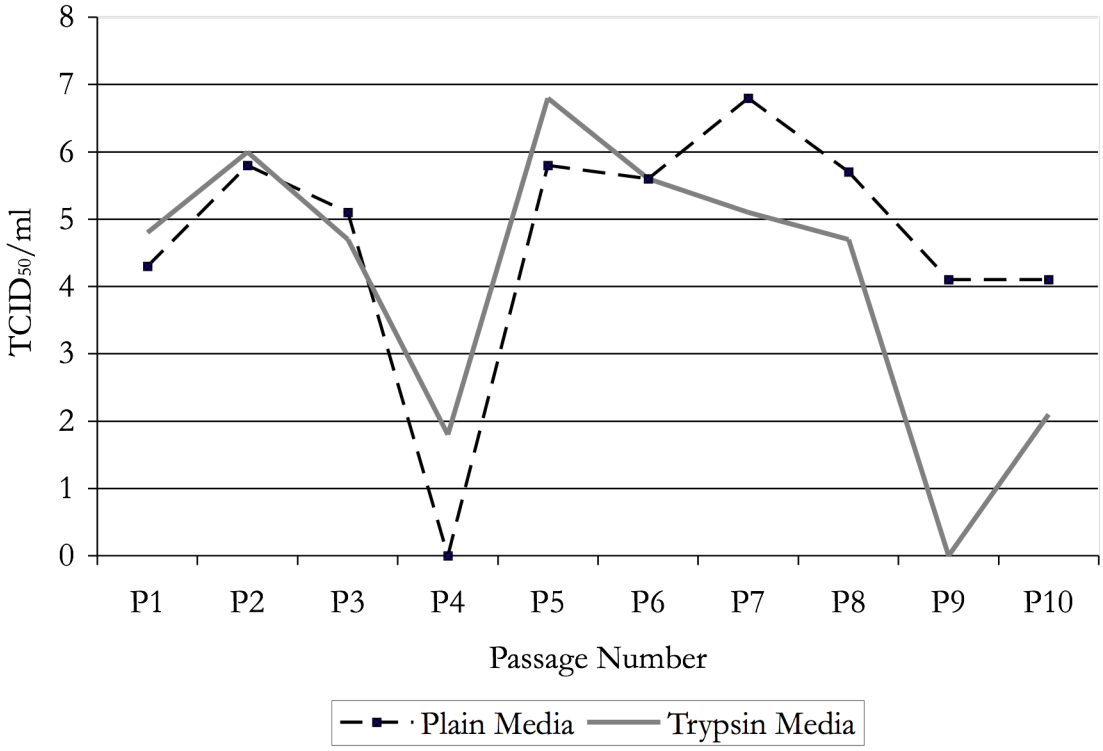
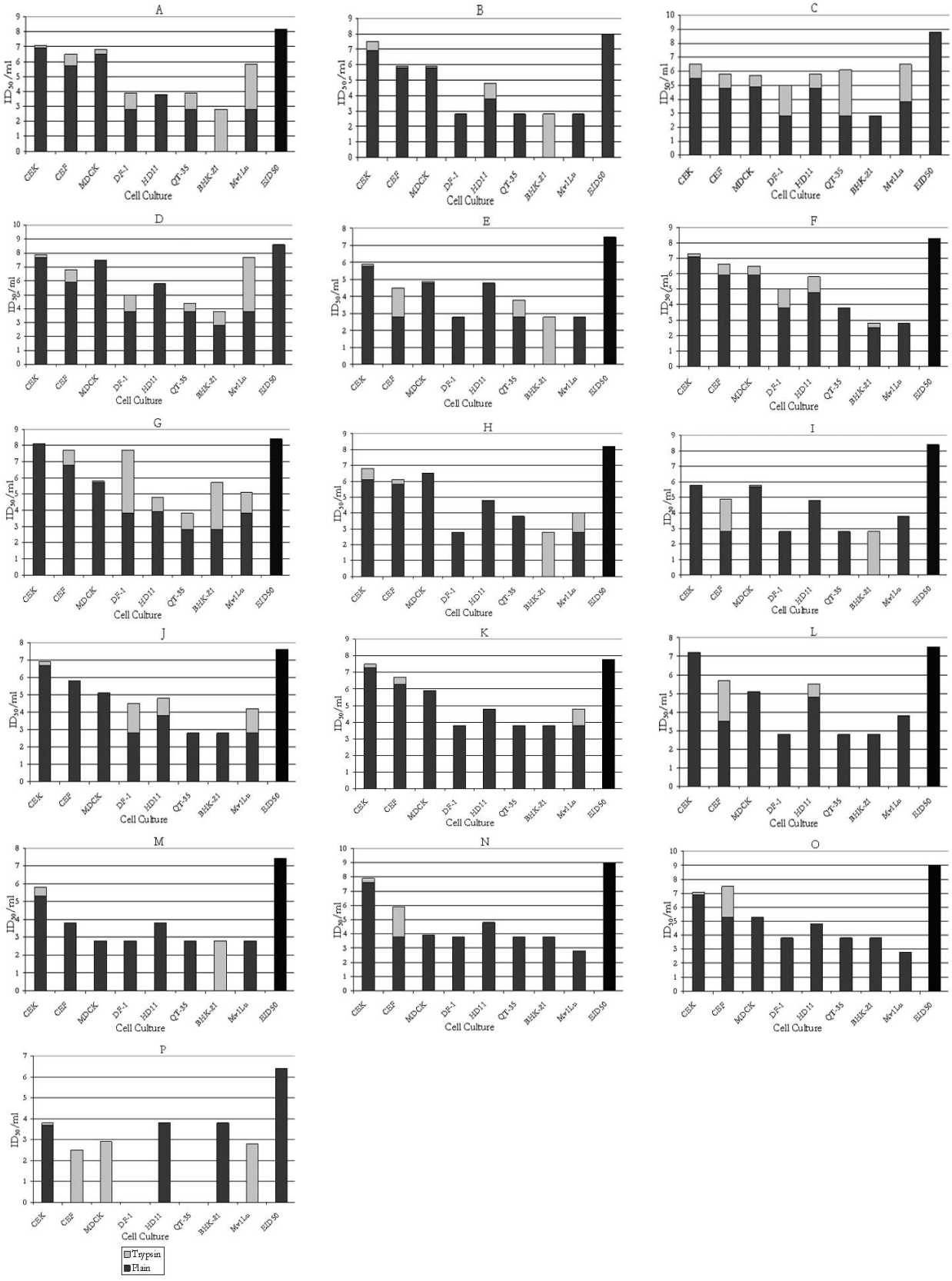


Figure 3.1. $TCID_{50}$/ml produced by MDCK cells

Cell line inoculated with A/CanadaGoose/WI/902/75 (H5N2). The vertical axis is the $TCID_{50}$/ml. The horizontal axis is the passage number. The virus was passed in the MDCK cells ten times. The solid line represents trypsin-supplemented media and the dashed line with squares represents non-supplemented media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O
P
ID50/ml
Cell Culture
CEK
CEF
MDCK
DF-1
HD11
QT-35
BHK-21
Mv1Lu
EID50
Trypsin
Plain

Figure 3.2. $TCID_{50}$s for H1-H16 in cell lines and cultures

Graphs A through P represent the selected cell lines and cell cultures inoculated with the H1 through H16 virus subtypes listed in Table 3.1. The vertical axis represents ID_{50}/ml. The horizontal axis represents all cell lines and cell cultures. The far right bar in each graph represents the EID_{50}/ml produced by ECEs, placed for comparison to the $TCID_{50}$/ml. As for the cell lines and cell cultures $TCID_{50}$/ml, the light colored bars represent the $TCID_{50}$/ml achieved without the use of supplemental trypsin. The dark bar stacked on top represents the increase in the $TCID_{50}$/ml with the addition of supplemental trypsin.

CHAPTER 4

COMPARISON OF EMBRYONATING CHICKEN EGGS, REAL TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION, AND CELL CULTURE FOR DETECTION OF AVIAN INFUENZA VIRUS FROM EXPERIMENTALLY INFECTED LAUGHING GULLS (*Leucophaeus atricilla*) AND MALLARDS (*Anas platyrhynchos*) [2]

[2] Moresco, K.A., D.E. Stallknecht, D.E. Swayne. To be submitted to *Journal of Wildlife Diseases.*

INTRODUCTION

Wild bird surveillance is an established method for studying ecology of avian influenza (AI) viruses. However, the number of AI viruses obtained depends on the bird species and type of sample collected such as oropharyngeal or cloacal sample, as well as the time of year when sampled, and location of sampling along the migration routes (Ip, Flint et al. 2008). Most AI virus isolates have originated from species in two avian orders: Anseriformes and Charadriiformes (Stallknecht and Brown 2008). Currently, embryonating chicken eggs (ECEs) and real time reverse transcriptase polymerase chain reaction (RRT-PCR) are the most common methods for detecting AI virus in surveillance samples. Both methods do have drawbacks such as being time consuming and costly, respectively. Virus isolation in ECEs can take up to three weeks to provide results and depends on the availability of high quality embryos and the number of passage attempts, while RRT-PCR requires highly trained personnel, uses costly reagents and specialized equipment (Spackman, Suarez et al. 2008). Sequential passage of viruses in ECEs has been shown to select for mutations (Brand and Palese 1980), cell culture has been explored as a possible alternative to ECEs. One of the most promising culture systems for influenza virus isolation and propagation is the Madin Darby canine kidney (MDCK) cell line first reported by Tobita *et al*. (Sugimura, Murakami et al. 2000; Asaoka, Tanaka et al. 2006), which carries with it the benefit of low cost, minimal use of technician time, and wide availability. Over the years many different cell lines have been tested for susceptibility to influenza viruses (Sugimura, Murakami et al. 2000; Lee, Jung et al. 2009). Since the time and funds spent on running a surveillance study are considerable, that investment demands the use of highly sensitive and specific assays that provide the most accurate results and identifies the highest number of influenza virus positive samples for the financial and personnel resources available.

In this study, we evaluated and compared virus isolation and detection methods for oropharyngeal and cloacal swabs samples collected from laughing gulls and mallards experimentally infected with low pathogenicity avian influenza viruses (LPAI virus) representing the most commonly occurring LPAI viruses subtypes found in North American wild aquatic birds. AI virus detection using RRT-PCR and VI using ECEs were compared for efficiency. Using VI from ECEs as a reference standard, four continuous cell lines and one primary cell culture were compared to evaluate the relative sensitivity. Detection results from ECEs, RRT-PCR, four continuous cell lines and one primary cell culture were compared to evaluate the relative sensitivity.

MATERIALS AND METHODS

Viruses: All viruses strains used in this experiment are listed in 4.1, which includes subtype and inoculum titers.

Animal Studies: Two experiments were performed consisting of 14 laughing gulls (Dr. Stallknecht, University of Georgia, Athens, GA) and 8 mallards received from a commercial source. All birds were housed with constant light and *ad libitum* access to feed and water. Each experiment included two control birds (non-infected) and three birds infected with each of the viruses listed in Table 4.1. The viruses were inoculated via choanal cleft with 100μl volume per bird. The viral challenge titers are reported in Table 4.1. Oropharyngeal and cloacal swabs were collected on 0, 1, 2, 3, 4, 7, and 10 days post inoculation (DPI). Swabs were placed in 2.5ml of brain heart infusion (BHI) broth containing a 2X concentration of antibiotics (10,000 U/ml Penicillin G, 10,000 μg/ml Streptomycin, 25 μg/ml Amphotercin B) (HyCone Laboratories, Inc, Logan, UT). Samples were stored at -70C until assayed. All birds were terminated at 10 DPI by intravenous injection of 0.5ml of 324 mg/ml sodium pentobarbital.

Real -Time Reverse Transcriptase Polymerase Chain Reaction: Oropharyngeal and cloacal samples collected from the laughing gull (n=196) and mallard (n=112) experiments were tested using RRT-PCR for detection of the AI virus matrix gene as follows: 100µl of each sample was added to 750µl of TRIzol LS® Reagent (Invitrogen, Inc., Carlsbad, CA) and 150µl of RNase-free water (Ambion, Austin, TX), 200µl of chloroform (Sigma-Aldrich, St. Louis, MO) was added to each sample. 400µl of the aqueous phase was removed and processed into high quality RNA using the Ambion RNA extraction kit (Ambion, Austin, TX). A tenfold serial dilution of each virus strain used for infection was created in BHI spanning 10^0 to 10^{-6} and RNA was extracted in the same manner as the swab material. Samples were tested by RRT-PCR against a standard curve created from each virus strain used during infection. The forward primer (MA +25), the reverse primer (MA-124), and probe (MA +64) were purchased from Integrated DNA Technologies (IDT, Skokie, IL). The RRT-PCR was performed utilizing the Qiagen One Step PCR Kit®(QIAGEN, Valencia, CA) as previously described (Spackman, Senne et al. 2002; Spackman, Senne et al. 2003).

Embryonating Chicken Eggs: All oropharyngeal and cloacal samples collected from the laughing gull and mallard experiments were tested by injection of 100µl of sample material into the chorioallantoic sac (CAS) of three ten-day-of-age specific pathogen free (SPF) ECEs (Swayne, Senne et al. 1998). The embryos were allowed to incubate for four days at 37C and on 4 DPI the embryos were candled for mortality and chilled. Allantoic fluid (AAF) was harvested for hemagglutination (HA) testing (Carbrey, Beard et al. 1974).

Additional Embryo VI: In order to be more comprehensive, additional ECEs VI attempts were conducted. Samples that were RRT-PCR+/VI HA- or RRT-PCR-/VI HA+ were inoculated into SPF ECEs for the following pathway: two CAS VI attempts followed by one

yolk sac (YS) VI attempt. The first CAS isolation attempt involved injection of 100µl of original sample material into three ten-day-of-age SPF ECEs. The embryos were allowed to incubate for four days at 37C and on 4 DPI the embryos were candled for mortality, chilled and a minimum of 1.0ml of AAF was harvested for HA testing (Carbrey, Beard et al. 1974). HA positive samples were determined to be free of anaerobic and aerobic bacteria by negative culture results in Thioglycolate (TG) broth (BD Biosciences, San Jose, CA) incubated at 37C for two days and confirmed as AI virus by antigen capture ELISA test (BinaxNow®; Binax, Inc., Scarborough, ME). When an AI virus positive result was confirmed, no further testing was performed. If samples were negative by HA, 200µl of AAF collected from the first VI attempt was injected into the CAS of three more ten-day-of-age SPF ECEs for a second passage. The same procedure was repeated during incubation, harvesting, and testing. For the third passage of CAS negative samples, 200µl of AAF was injected into the yolk sac of three six-day-of-age SPF ECEs (Woolcock, McFarland et al. 2001; Lira, Moresco et al. 2009). On 4 DPI, the embryos were candled and chilled. The YS membrane and embryo were harvested, homogenized, and the supernatant was HA tested (Carbrey, Beard et al. 1974).

Cell Culture: The cell cultures selected are listed in Table 4.2. All cell lines and cultures, except for the HD11 cell line, were propagated in Dulbecco's Modified Eagle Media (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and antibiotic/antimycotic solution (10,000 U/ml Penicillin G, 10,000 µg/ml Streptomycin, 25 µg/ml Amphotercin B) (HyCone Laboratories, Inc, Logan, UT). The HD11 cell line, a suspension cell line, was propagated in Roswell Park Memorial Institute (RPMI) media (Invitrogen, Carlsbad, CA) supplemented with 5% FBS and antibiotic/antimycotic solution (10,000 U/ml Penicillin G, 10,000 µg/ml Streptomycin, 25 µg/ml Amphotercin B). All cultures

were grown and maintained at 37C and 5% CO_2. The chicken embryo fibroblast (CEF) primary cultures were created for each VI attempt from 10 day of age SPF ECEs. All cell cultures were supplemented upon infection with the optimal L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-trypsin (Sigma-Aldrich, St Louis, MO) concentration. For the MDCK, Mv1Lu, HD11, QT-35, and CEF, the trypsin concentrations were 0.45µg /ml, 0.15µg/ml, 0.25 µg/ml, 0.1µg/ml, and 0.2µg/ml, respectively. These concentrations were previously determined using a dose response assay for LPAI virus replication.

Each cell culture was used to perform three VI attempts for each oropharyngeal and cloacal sample collected from the laughing gull and mallard experiments that were AI virus positive by ECEs VI. All cultures were seeded in three 96 well cell culture plates each with DMEM or RPMI supplemented with 5% FBS and antibiotic/antimycotic solution (10,000 U/ml Penicillin G, 10,000 µg/ml Streptomycin, 25 µg/ml Amphotercin B). The cells were allowed to grow to a monolayer overnight at 37C and 5% CO_2. They were washed twice with 1X sterile phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA) and inoculated with 20µl of original sample into each of three wells and allowed to incubate for 45 minutes at 37C. After incubation, 150µl of media supplemented with the previously mentioned TPCK-trypsin concentration and antibiotic/antimycotic solution (10,000 U/ml Penicillin G, 10,000 µg/ml Streptomycin, 25 µg/ml Amphotercin B) was added. The infected cells were allowed to incubate for four days at 37C and 5% CO_2. On 4 DPI, the 96 well plates were frozen at -70C. The plates were thawed and 50µl of supernatant was removed for HA testing (Carbrey, Beard et al. 1974), an additional 20µl of supernatant was transferred onto fresh monolayers to serve as the inoculum for the second passage. The pattern described above was repeated to complete three passages.

Statistical Analysis: Statistical comparisons were made using GraphPad Prisma 5.0A (GraphPad Software, La Jolla, CA). Data was organized into contingency tables and analyzed using the Fisher exact test as well by the Cohen Kappa statistic. Results from ECEs VI and RRT-PCR detection were compared for significant differences in detection. The VI efficiency for each cell culture was also compared to ECE VI for significant differences in VI.

RESULTS

Real-Time Reverse Transcriptase Polymerase Chain Reaction: One hundred and ninety- six samples were collected from laughing gulls; 20.4% (n=40) of samples were positive for the AI virus matrix gene. One hundred and twelve samples collected from mallards were tested for AI virus Matrix gene and 31.2% (n=35) were positive for AI virus matrix gene.

Embryonating Chicken Eggs: One hundred and ninety-six samples collected from laughing gulls were inoculated into ECEs of which 24. 0 % (n=47) were positive by HA. Of the one hundred and twelve samples collected from mallard, 34.8% (n=39) were positive by HA.

Additional Embryo Inoculations: Comparison of the laughing gull RRT-PCR detection and ECEs VI results showed 16.8 % (n=33) of the total 196 samples resulted in conflicting results (i.e. RRT-PCR-/VI HA+ or RRT-PCR+/VI HA-) with the remaining 83.2% (n=163) showing consistent results (i.e. RRT-PCR+/VI HA+ or RRT-PCR-/VI HA-). A conflicting sample is defined as a sample that is positive by only one method of assay. Of the 33 conflicting samples, 61.6% (n=20) were RRT-PCR-/VI HA+ and the remaining 39.4% (n=13) were RRT-PCR +/VI HA-. All 33 conflicting samples were reinoculated into ECEs for two CAS VI attempts followed by one YS inoculation. 20 samples that were RRT-PCR-/ VI HA+ were also cultured for bacterial contamination and tested for AI virus using a commercial antigen capture ELISA (BinaxNow®; Binax, Inc., Scarborough, ME). The additional ECEs VI attempts resolved

36.4% (n=12) of the initial 33 conflicting samples. Overall 89.3 % (n=175) of samples had consistent results between RRT-PCR detection and ECEs VI. The majority of the disagreement (table 4.3), which consisted of 12 samples RRT-PCR +/ VI- and 9 samples RRT-PCR -/VI+, occurred in cloacal swabs collected 4, 7, and 10 DPI, and with a smaller number of conflicting results in the oropharyngeal samples on 10 DPI.

Comparison of the mallard RRT-PCR detection and ECEs VI results showed 19.6% (n=22) of the total 112 samples resulted in conflicting results (i.e. RRT-PCR-/VI HA+ or RRT-PCR+/VI HA-) with the remaining 80.4% (n=90) showing consistent results (i.e. RRT-PCR+/VI HA+ or RRT-PCR-/VI HA-). A conflicting sample is defined as a sample that is positive by only one method of assay. Of the 22 conflicting samples, 69.1% (n=13) were RRT-PCR -/VI HA+ and the remaining 40.9% (n=9) were RRT-PCR +/ VI HA-. The 22 conflicting samples were reinoculated into ECEs for two CAS VI attempts followed by one YS inoculation. 13 samples that were RRT-PCR- /VI HA+ were also cultured for bacterial contamination and tested for AI virus using a commercial antigen capture ELISA (BinaxNow®; Binax, Inc., Scarborough, ME). The additional VI attempts resulted in an additional 11 AI virus isolates. After the additional VI pathway, a total 90.2% (n=101) of the mallard samples had consistent results between RRT-PCR detection and ECE VI; i.e. RRT-PCR+/VI+ or RRT-PCR-/VI -. The majority of the disagreement (Table 4.3), which consisted of 7 samples RRT-PCR + /VI- and 4 samples RRT-PCR –/VI+, occurred between 7 and 10DPI cloacal samples.

In the laughing gull experiment, peak levels of respiratory shedding was detected on 1 and 2 DPI while intestinal shedding was not detected until 3DPI, with peak occurrence of intestinal shed on 4 and 7 DPI. In the mallard experiment, peak levels of respiratory shedding was detected on 2 and 4 DPI, which also accompanied by increased intestinal shedding on 3DPI.

The peak occurrence of intestinal shed was on 3, 4, and 7 DPI. Figures 4.1 and 4.2 illustrate the infection course for the laughing gull and the mallard experiments respectively, as represented by the number of positive samples.

Cell Culture: Forty laughing gull oropharyngeal and cloacal samples positive by ECEs were inoculated into five cell cultures for three VI attempts. The MDCK cell line supported replication of 80 % (n=32), the Mv1Lu cell line 42.5% (n=17), CEF cell culture 40 % (n=16), the QT-35 cell line 20% (n=8) and the HD11 cell line 5% (n=2). Thirty-eight mallard oropharyngeal and cloacal samples were also inoculated into five cell cultures for three passages. The MDCK cell line supported replication of 63.2 % (n=24), the HD11 cell line 42.1% (n=16), the CEF cell culture 23.7% (n=9), and the Mv1Lu and the QT-35 cell lines both resulted in 13.2 % (n=5) positive. The VI efficiency for each cell culture was averaged for the laughing gull and mallard samples. The MDCK cell VI had an overall VI efficiency of 71.6%, the Mv1Lu 55.7%, the CEF 26.6%, the HD11 23.7%, and the QT-35 had a VI efficiency of 16.5%.

Statistical Analysis: The Fisher Exact analysis from ECEs and RRT-PCR for the laughing gull sample set showed no significant differences in detection. The Cohen Kappa statistics for the laughing gulls was 0.66, which falls within the range of substantial amount of agreement between RRT-PCR and ECEs. The MDCK cell line produced VI results with no significant difference in AI virus detection compared to ECEs, however numerically the MDCK produced lower number of virus isolates. The remaining four cell cultures had a significantly lower rate of AI virus VI compared to ECEs VI. The Fisher Exact test indicated no significant difference in detection for ECEs and RRT-PCR for the mallard sample set. The Cohen Kappa statistics for the mallards was 0.77, which also falls within the range of substantial amount of agreement between RRT-PCR and ECEs. Results from the cell culture VI using the mallard

samples showed that all VI attempts using cell cultures produces significantly lower frequency of AI virus VI compared to ECEs VI.

DISCUSSIONS

When the results obtained using RRT-PCR for AI virus matrix gene were aligned with results from VI using ECEs for both the laughing gull and the mallard experiments, a number of samples with conflicting results required additional testing. The additional embryo inoculation pathway was used to amplify the RRT-PCR+ samples with LPAI virus below the detectable level of the HA test in initial VI assay. The VI performed in this study used only 100µl of sample material inoculated into each of three ECEs, which may have lowered the sensitivity of VI. After the use of additional embryo inoculations, which provided for the amplification of AI virus, the final results did show substantial levels of association between RRT-PCR and VI results.

The laughing gulls were obtained from the wild as nestlings and the mallard were reared in outdoor aviaries. As these are not SPF birds, the level and types of bacterial present in the oropharyngeal and cloacal swabs may not have been controlled with level of antibiotics added to the sample collection media, which may have caused some of the conflicting results, specifically those samples that were AI matrix gene negative by RRT-PCR but HA positive in ECEs could be from bacterial growth and not from the presence of AI virus (Spackman, Suarez et al. 2008). ECEs are known to support growth of viable AI virus as well as contaminating bacteria or other pathogens while molecular methods are directed to specific templates on the viral RNA regardless of the pathogen's viability (Suarez, Spackman et al. 2009). The majority of the remaining conflicting results between AI virus matrix gene RRT-PCR positive samples and ECEs virus isolation negative samples occurred mainly in the cloacal samples late in the infectious period, which may imply that viruses present in these samples could be below the

level of detection by ECEs and HA for VI, but still detectible by molecular methods. Other possibilities for RRT-PCR positive samples that fail to produce viable virus using embryos may be defective virus particles that are unable to replicate and even the degradation of samples cause by bacterial enzymes (Spackman, Suarez et al. 2008).

An interesting observation was the replication of LPAI virus and its recovery from the oropharyngeal samples. The collective dogma for LPAI virus replication in wild birds is limited to intestinal replication (Webster, Yakhna et al. 1978). In both the laughing gull and mallard studies, respiratory shedding was most frequent in the early stages of infection; i.e. 1-4 DPI. During the middle to the end of the experiments, intestinal shedding was more frequently observed in the gulls and mallards than was respiratory shedding. The four LPAI viruses used for inoculation of the laughing gulls are listed in Table 4.2. The H7N3 and H6N4 inoculation resulted in respiratory shed between 1 and 4 DPI with minimal amounts of virus detected on 7 and 10 DPI. Intestinal shed for the H7N3 occurred mainly between 4 and 10 DPI, while the H6N4 intestinal shed occurred mainly between 2 and 4 DPI. The H3N8 virus produced only respiratory shed on 1 and 2 DPI, while the H2N3 resulted in delayed intermittent respiratory shedding on 4 and 10 DPI. The two LPAI viruses used for inoculation of the mallards are also listed in Table 4.2. The H4N8 inoculation resulted in respiratory shed on 1, 2, 3, 4, 7, and 10 DPI with intestinal shed occurring minimally on 4 and 7 DPI. The H5N6 inoculation showed delayed detection with respiratory shed occurring on 2, 3, 4, and 7 DPI with intestinal shed occurring on 4, 7, and 10 DPI. However, the number of positive samples, determined by VI and HA using ECEs, for intestinal versus respiratory shedding were similar.

Costa *et al* found that mallards infected with LPAI viruses A/Mallard/Mn/199106/99 (H3N8) and A/Mallard/Mn/355779/00 (H5N2) also excreted virus via oropharynx and cloaca.

Respiratory and intestinal shed occurred mainly on 1-4 DIP with sporadic virus shed detected from 7-21 DPI (Costa, Brown et al. 2010). Kim *et al* infected chickens and magpies with a LPAI virus obtained from magpies during surveillance from 2003 to 2007. Virus was re-isolated from swabs collected from the experimentally infected chicken from 3 to 7 DPI from the oropharynx and from 3 to 7 DPI from the cloaca. From the experimentally infected magpies, virus was re-isolated from only the oropharynx from 1 to 5 DPI, with no virus detected from the cloaca (Kim, Jeong et al. 2010). Tumpey *et al* infected chickens and turkeys with a A/turkey/Virginia/15851/02 (H2N2) LPAI virus. Virus was recovered from the oropharynx of both species during the first 5 days of infection, with low to undetectable virus titers recovered from cloacal samples from both species. Their results indicated that the H7N2 replicated more efficiently in the respiratory tract (Tumpey, Kapczynski et al. 2004). The small differences noted in respiratory versus intestinal shed of all LPAI virus discussed may be a result of differences in particular virus strains or the frequency of virus detected in the oropharyngeal cavity may be a result of route of exposure; i.e. intranasal. Results shown in figures 4.1 and 4.2 indicate early oral shed (i.e. 1 - 4 DPI) with intestinal shed spanning a longer duration (i.e. 2 to 10 DPI). A combination of oropharyngeal and cloacal samples may be beneficial in wild bird sampling programs to maximize obtaining AI viruses (Bulaga, Garber et al. 2003), however when sampling occurs during the later stages of infection, cloacal sampling may prove to be more successful.

The use of cell culture for virus isolation from experimental samples was shown to have an overall lower sensitivity than that of either RRT-PCR methods or VI in ECEs. The MDCK cell line was superior to the other cultures tested producing substantially more positive results than the remaining four cultures and was the only group with VI results that were similar to

ECEs VI for laughing gull samples. As a kidney epithelial cell line, the MDCK cells produce endogenous proteases that aid in the cleavage of the AI virus precursor HA_0 protein into HA_1 and HA_2, which renders the virus infective and allows multiple round of replication (Bosch and Garten 1981; Spackman, Suarez et al. 2008). However, the MDCK cells failed to isolate the LPAI viruses as consistently as ECEs suggesting that differences may still exist in cell receptors to bind the viruses and produce replication. The use of cell culture as a virus isolation system does carry certain advantages such as limiting dependence on ECEs that require lengthy incubation periods (Spackman, Suarez et al. 2008). However, the limited sensitivity of cell culture may restrict its practical application.

REFERENCES

Asaoka, N., Y. Tanaka, T. Sakai, Y. Fujii, R. Ohuchi and M. Ohuchi 2006. Low growth ability of recent influenza clinical isolates in mdck cells is due to their low receptor binding affinities. Microbes and Infection 8(2): 511-519.

Bosch, F. X. and W. Garten 1981. Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between ha1 and ha2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. Virology 113(2): 725-735.

Brand, C. and P. Palese 1980. Sequential passage of influenza virus in embryonated eggs or tissue culture: emergence of mutants. Virology 107(2): 424-433.

Bulaga, L. L., L. P. Garber, D. A. Senne, T. J. Myers, R. Good, S. Wainwright, S. Trock and D. L. Suarez 2003. Epidemiologic and surveillance studies on avian influenza in live-bird markets in new york and new jersey, 2001. Avian Diseases 47: 996-1001.

Carbrey, E. A., C. W. Beard, R. J. Cooper, R. P. Hansen and B. S. Pomeroy 1974. Hemagglutination and hemagglutination-inhibition tests with newcastle disease virus--

microtiter technique. 17th Annual Proceedings of the American Association of Veterinary Laboratory Diagnosticians: 1-6.

Costa, T. P., J. D. Brown, E. W. Howerth and D. E. Stallknecht 2010. The effect of age on avian influenza viral shedding in mallards (*Anas platyrhynchos*). Avian Diseases 54: 581-585.

Ip, H., P. L. Flint, J. C. Franson, R. T. Dusek, D. V. Derksen, R. E. Gill, L. R. Ely, J. M. Pearce, R. B. Lanctat, S. M. Matsuoka, D. B. Irons, J. B. Fischer, R. M. Oates, M. R. Peterson, T. F. Fondell, D. A. Rocque, J. C. Pedersen and T. C. Rothe 2008. Prevalence of influenza a viruses in wild migratory birds in alaska: patterns of variation in detection at a crossroads of intercontinental flyways. Virology Journal 5: 71-81.

Kim, M. C., O. M. Jeong, H. M. Kang, M. R. Paek, J. S. Kwon, C. S. Song, Y. K. Kwon, J. G. Lee, J. H. Kwon and Y. J. Lee 2010. Pathogenicity and transmission studies of h7n7 avian influenza virus isolated from feces of magie origin in chickens and magpie. Veterinary Microbiology 141: 268-274.

Lee, C. W., K. Jung, S. J. Jadjao and D. L. Suarez 2009. Evaluation of chicken-origin (df-1) and quail-origin (qt-35) fibroblast cell lines for replication of avian influenza viruses. Journal of Virological Methods 153(1): 22-28.

Lira, J., K. A. Moresco, D. E. Stallknecht and D. E. Swayne 2009. Single and combination diagnostic test efficiency and cost analysis for detection and isolation of avian influenza virus from wild bird cloacal swabs. Avian Diseases 54(1S): 606-612.

Spackman, E., D. A. Senne, L. L. Bulaga, T. J. Myers, M. L. Perdue, L. P. Garber, K. Lohman, L. T. Daum and D. L. Suarez 2003. Development of real-time rt-pcr for the detection of avian influenza virus. Avian Diseases 47(3S): 1079-1082.

Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum and D. L. Suarez 2002. Development of a real-time reverse transcriptase pcr assay for type a influenza virus and the avian h5 and h7 hemagglutinin subtypes. Journal of Clinical Microbiology 40: 3256-3260.

Spackman, E., D. L. Suarez and D. A. Senne 2008. Avian influenza diagnostic and surveillance methods. Avian Influenza Ed. D.E. Swayne, Blackwell Publishing, Ames, IA: 299-308.

Stallknecht, D. E. and J. B. Brown 2008. Ecology of avian influenza in wild birds. Avian Influenza Ed. D.E. Swayne, Blackwell Publishing, Ames, IA: 43-58.

Suarez, D. L., E. Spackman, D. A. Senne, L. L. Bulaga, A. C. Welsch and K. Froberg 2009. The effect of various disinfectants on detection of avian influenza virus by real-time rt-pcr. Avian Diseases 47(3S): 1091-1095.

Sugimura, T., Y. Murakami and T. Ogawa 2000. The susceptibility of culture cells to avian influenza viruses. Journal of Veterinary Medical Sciences 62(6): 659-660.

Swayne, D. E., D. A. Senne and C. W. Beard 1998. Avian influenza, in: a laboratory manual for the isolation and identification of avian pathogens. 5th Edition. Ed. D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson, WlM. Reed. American Association of Avian Pathologists (Jacksonville, FL): 128-134.

Tumpey, T. M., D. R. Kapczynski and D. E. Swayne 2004. Comparative susceptibility of chickens and turkeys to avian influenza a h7n2 virus infection and protective efficacy of a commercial avian influenza h7n2 virus vaccine. Avian Diseases 48: 167-176.

Webster, R. G., M. Yakhna, V. S. Hinshaw, W. J. Bean and K. C. Murti 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology 84(2): 268-278.

Woolcock, P. R., M. D. McFarland, S. Lai and P. Chin 2001. Enhanced recovery of avian influenza virus isolates by a combination of chicken embryo inoculation methods. Avian Diseases 45: 1030-1035.

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TABLES AND FIGURES

Table 4.1. Avian influenza virus strains used in laughing gull and mallard experiments

Experiment	Virus Strain	Subtype	Isolate Source	Inoculum Titer (EID_{50}/ml)
Laughing gull	A/HerringGull/532/NJ/2005	H2N3	Dr. Stallknecht[A]	$10^{6.6}$/1.0ml
	A/LaughingGull/AI05-768/2006	H3N8	Dr. Stallknecht[A]	$10^{6.5}$/1.0ml
	A/LaughingGull/AI00-470/2000	H6N4	Dr. Stallknecht[A]	$10^{6.5}$/1.0ml
	A/LaughingGull/AI00-2455/2000	H7N3	Dr. Stallknecht[A]	$10^{7.6}$/1.0ml
Mallard	A/Mallard/OH/338/86	H4N8	Dr. Slemons[B]	$10^{6.7}$/1.0ml
	A/Mallard/WI/34/75	H5N6	Dr. Slemons[B]	$10^{6.5}$ /1.0ml

[A] Southeast Cooperative Wildlife Disease Study, Athens, GA

[B] Department of Veterinary Preventive Medicine, Columbus, OH

Table 4.2. Cell lines/culture used for virus isolation with laughing gull and mallard samples

Name	Designation	Origin	Cell Type
Chicken Embryo Fibroblast	CEF	Embryo	Fibroblast
[A] Chicken Myeloblast	HD11	Bone Marrow	Leukocyte
[B] Mink Lung Epithelial	Mv1Lu	Lung	Epithelial
[C] Japanese Quail Fibroblast	QT-35	Fibrosarcoma	Fibroblast
Madin-Darby Canine Kidney	MDCK	Kidney	Epithelial

[A] Dr. Kapczynski (SEPRL, Athens)

[B] Dr. Sellers (UGA, Athens)

[C] Dr. Stallknecht (UGA, Athens)

Table 4.3. Summary of remaining conflicting samples from laughing gull and mallard experiments

	Number of Samples	RRT-PCR	ECE	Additional Embryo Inoculation
Laughing gull	12	+	-	-
	9	-	+	+
Mallard	7	+	-	-
	4	-	+	+

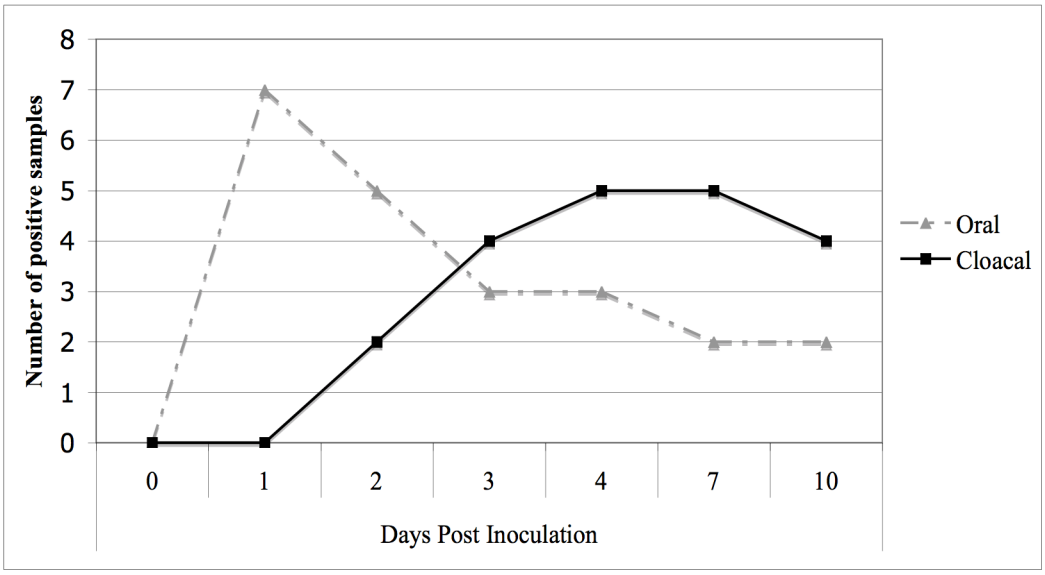

Figure 4.1 Respiratory and intestinal shed observed in laughing gulls

Number of positive samples was based on virus isolation in embryonating chicken eggs from oropharyngeal and cloacal swabs.

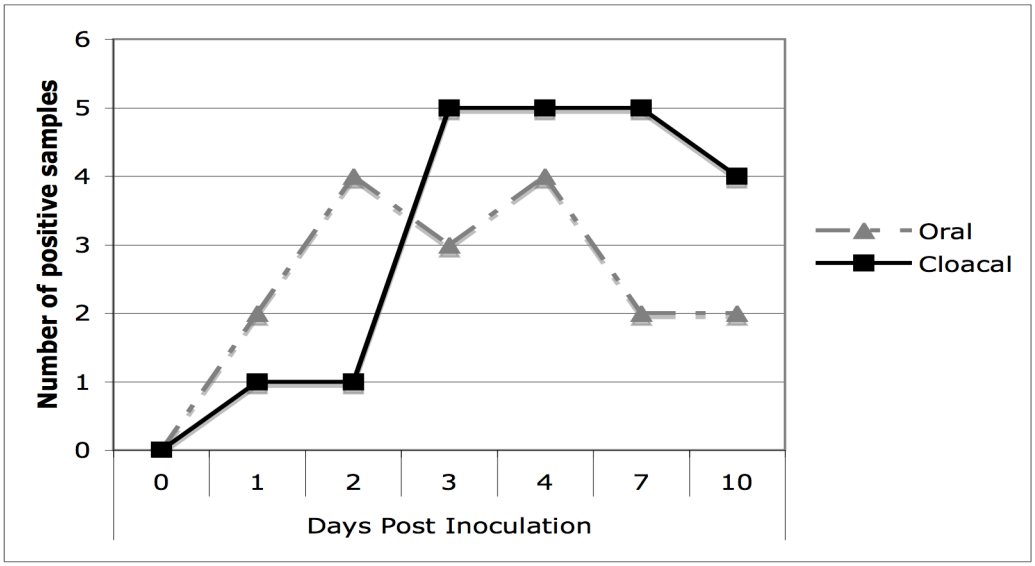

Figure 4.2. Respiratory and intestinal shed observed in mallards

Number of positive samples was based on virus isolation in embryonating chicken eggs from oropharyngeal and cloacal swabs.

CHAPTER 5

AVIAN EMBRYOS, REAL TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION, AND CELL CULTURE FOR DETECTION OF AVIAN INFLUENZA AND AVIAN PARAMYXOVIRUSES IN WILD BIRD SURVEILLANCE SAMPLES [3]

[3] Moresco, K.A., D.E. Stallknecht, D.E. Swayne. To be submitted to *Clinical and Vaccine Immunology.*

INTRODUCTION

Wild aquatic birds are the reservoir for avian influenza (AI) viruses and avian paramyxovirus type 1 (APMV-1). AI viruses are classified in the family *Orthomyxoviridae*, genus *Influenzavirus A*, and can be one of 16 different hemagglutination subtypes (H1-H16) in combination with one of nine different neuraminidase subtypes (N1-N9). The AI viruses genome contains single strand negative sense RNA with at least eight segments (Swayne and Halvorson 2008). At least two primary wild aquatic bird reservoirs for low pathogenicity avian influenza viruses (LPAI viruses) exist in nature; birds in the order *Anseriformes*, which are composed of ducks, geese, and swans, and birds in the order *Charadriiformes*, which are composed of gulls, terns, and waders (Stallknecht and Shane 1988; Hanson, Luttrell et al. 2008).

APMV-1 is classified in the family *Paramyxoviridae,* genus *Avulavirus* and can be one of nine serotypes (APMV1-9); Newcastle disease virus (NDV) is the virulent strains of APMV-1. The APMV-1 genome contains single stranded, non-segmented, negative sense RNA (Alexander 1995; Alexander 2008). APMV isolates have frequently been obtained from migratory waterfowl and aquatic birds and it has been reported that infections have been established in at least 241 species of birds (Alexander and Senne 2008). AI viruses and avian paramyxovirus -1 (APMV-1) have been transmitted to domestic poultry causing asymptomatic infections to mild or even severe disease.

The standard methods of virus isolation for AI viruses and APMV-1 include inoculation of sample material into the chorioallantoic sac (CAS) of embryonating chicken eggs (ECEs). AI viruses and APMV-1 both grow well in ECEs, as well as display the trait of hemagglutination of avian and mammalian erythrocytes (Alexander 2008; Swayne, Senne et al. 2008), but specific identification requires further testing such as standard hemagglutination inhibition assays to

confirm virus identity (Carbrey, Beard et al. 1974). Molecular based assays such as real-time reverse transcriptase polymerase chain reaction (RRT-PCR) are often used. Spackman *et al* developed a RRT-PCR assay directed to the matrix gene of AI viruses (Spackman, Senne et al. 2002; Spackman, Senne et al. 2003) and in 2008, Kim *et al* refined a RRT-PCR assay that was able to detect and distinguish between class I and class II APMV-1, with detection of APMV-1 matrix and polymerase genes (Wise, Suarez et al. 2004; Kim, Suarez et al. 2008).

Since the 1940s, ECEs have been the most successfully used VI method and are termed the "gold standard" (Spackman, Suarez et al. 2008). A large portion of AI virus surveillance studies have focused on the use of RRT-PCR followed by VI using ECEs. In this study, surveillance samples collected from wild birds from 2002 to 2008 were tested for AI viruses and APMV-1 by RRT-PCR. Virus isolation was performed on all RRT-PCR positive samples for AI viruses and APMV-1 using ECEs, embryonating duck eggs (EDEs), and embryonating turkey eggs (ETEs). Virus isolation was also performed using Madin-Darby canine kidney (MDCK) cells and African green monkey kidney (VERO) cells.

METHODS AND MATERIALS

Surveillance Samples. Two hundred samples collected from wild birds spanning 2003 to 2008 with 50% (n=100) obtained from Charadriiformes (Ruddy Turnstone); 48.5% (n=97) from Anseriformes consisting of mallard (n=58), blue-winged teal (n=28), green-winged teal (n=1), wood duck (n=4), gadwall (n=1), ring-neck duck (n=1), and ruddy duck (n=1); 1% (n=2) from Gruiformes (American coot); and 0.5% (n=1) from Galliformes (chicken). The wild bird samples were selected to represent the natural reservoirs of AI viruses in nature as well as species from which AI viruses are frequently isolated.

Real-time reverse transcriptase polymerase chain reaction. All samples were tested using RRT-PCR directed to the AI virus matrix gene and a duplex RRT-PCR test directed to APMV-1 matrix and polymerase genes. 100µl of each sample was added to 750µl of TRIzol LS Reagent® (Invitrogen, Carlsbad, CA) and 150µl of RNase-free water (Ambion®, Austin, TX). 400µl of the aqueous phase was removed and processed into high quality RNA using the Ambion RNA extraction kit® (Ambion, Austin, TX). All primers and probes were purchased from Integrated DNA Technologies (IDT, Skokie, Illinois) and include the following: AI virus forward primer (MA +25), the reverse primer (MA-124), and probe (MA +64) and APMV-1 forward primers (M+4100 and L+8738), reverse primers (M-4220 and L-8847), and probes (M+4169 and L+8762). The RRT-PCR was performed using the Qiagen One Step PCR Kit® (QIAGEN, Valencia, CA) in the manner previously described (Spackman, Senne et al. 2002; Spackman, Senne et al. 2003; Wise, Suarez et al. 2004; Kim, Suarez et al. 2008).

Avian Embryos: Samples positive for AI virus and APMV-1 by RRT-PCR were inoculated into 10 day of age specific pathogen free (SPF) embryonating chicken eggs (ECEs), and 12 day of age SPF embryonating turkey eggs (ETEs). High quality AI virus antibody negative embryonating duck eggs (EDEs) were obtained from a commercial source and were inoculated on 12 days of age. 100µl of sample material was injected into the chorioallantoic sac (CAS) of three eggs using standard methods (Swayne, Senne et al. 2008) and the embryos were incubated for 4 days at 37C. Chorioallantoic fluid (AAF) from all embryos was harvested for hemagglutination (HA) testing using 0.5% chickens erythrocytes (Carbrey, Beard et al. 1974). AAF from dead embryos was harvested separately; AAF from live embryos was harvested and pooled. If HA negative, 200µl of AAF was reinoculated into CAS for a second virus isolation (VI) attempt by the previously described method. For the third VI attempt, 200µl of AAF was

inoculated into the yolk sac (YS) of 6 day of age ECEs, 8 day of age ETEs and EDEs and allowed to incubate for 4 days at 37C. The yolk sac membrane and the chick were collected and forcefully passed through a syringe. Contents were centrifuged at 2500 rpm for 10 minutes; supernatant was collected and tested for HA (Woolcock, McFarland et al. 2001; Lira, Moresco et al. 2009).

Cell Culture: Samples positive for AI virus and APMV-1 by RRT-PCR were inoculated into MDCK and VERO cell culture for three isolation attempts. Cells were seeded into 96 well cell culture plates with 1X Dulbecco's Modified Eagle Medium (DMEM), 5% Fetal Bovine Serum (FBS), and antibiotic/antimycotic solution (10,000 U/ml Penicillin G, 10,000 μg/ml Streptomycin, 25 μg/ml Amphotercin B) and allowed to monolayer overnight at 37C and 5% CO_2. The monolayers were washed twice with 1X sterile phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA) and inoculated with 20μl of original sample into each of three wells and allowed to incubate for 45 minutes at 37C. After incubation, 150μl of media supplemented with antibiotic/antimycotic solution (10,000 U/ml Penicillin G, 10,000 μg/ml Streptomycin, 25 μg/ml Amphotercin B) was added. Infected cells were allowed to incubate for four days. On 4 DPI, cells were frozen at -80C and thawed. 50μl of supernatant was harvested for HA testing (Carbrey, Beard et al. 1974) and an additional 20μl of supernatant was used as inoculum for the second VI attempt. This method was repeated for a total of three VI attempts.

Any sample with positive HA was grown to a volume sufficient for differential diagnostic testing. The MDCK and VERO cells were seeded into 48 well cell culture plates with 1X DMEM, 5% FBS, and 1% antibiotic/antimycotic (10,000 U/ml Penicillin G, 10,000 μg/ml Streptomycin, 25 μg/ml Amphotercin B) solution and allowed to monolayer overnight. The monolayers were washed twice with 1X sterile phosphate buffered saline (PBS) (Invitrogen,

Carlsbad, CA) and 100µl of HA positive supernatant was inoculated into each of three wells and allowed to incubate for 45 minutes. 500µl of 1X DMEM supplemented with 1% antibiotic/antimycotic solution was added. Plates were incubated for 4 days. On 4 DPI, cells were frozen at -80C and thawed once. All supernatant was harvested, pooled, and HA tested (Carbrey, Beard et al. 1974).

Specific Virus Identification: Positive identification of AI virus was achieved using a commercial antigen capture ELISA (BinaxNow®; Binax, Inc., Scarborough, ME). Positive identification of APMV was determined by standard hemagglutination inhibition assay (Carbrey, Beard et al. 1974). APMV serotype was determined using reference antigen/antiserum combinations for APMV-1, 2, 3, 4, 6, and 7. The APMV-2, 4, 6, and 7 reference standards originated from wild bird isolates.

Statistical Analysis: Statistical comparisons were made using GraphPad Prism 5.0A (GraphPad Software, La Jolla, CA). Data was organized into contingency tables and analyzed for significant differences in detection using the Fisher exact test and confirmed using the Chi-Square test. Statistical analysis was performed for AI virus and APMV-1 in the same manner. Positive detection by RRT-PCR was compared to positive VI in ECEs, ETEs, EDEs, MDCK and VERO cell culture independently for a total of five combinations. Positive VI results for ECEs, ETEs, EDEs, MDCK and VERO cell culture were all compared to each other for a total of nine combinations.

RESULTS

Real-time reverse transcriptase polymerase chain reaction: 23% (n=46) of samples were positive for AI virus matrix gene; 54.3% (n=25) of the positives were obtained from Anseriformes and 45.6% (n=21) obtained from Charadriiformes. The cycle threshold (CT)

values ranged from 29.39 to 43.32. For the duplex APMV-1 RRT-PCR, 8.5% (n=17) were positive for Class I and II NDV; 47.2% (n=8) were from Anseriformes and the remaining 52.9% (n=9) from Charadriiformes. The CT values ranged from 28.19 to 39.85.

Virus Isolation in Embryonating Chicken Eggs: Viable AI viruses were recovered in 21.7% (n=10) of RRT-PCR positive samples; seven were recovered on pass 1, two on pass 2, and one on pass 3. The range of RRT-PCR CT values for positive virus isolation was 29.39 to 37.95; this group contained a total of 16 samples. The yield of positive VI for ECEs was 62.5% (n=10) among RRT-PCR positive with CT < 37.95 (n=16). The range of the remaining RRT-PCR CT values was 38 to 43.32 (n=30) and no AI viruses were isolated. In addition, there was no CT value, below which there was 100% predictability for isolation of AI viruses. ECEs supported growth of three AI virus isolates not recovered using ETEs and EDEs.

APMV-1 was recovered in 52.9% (n=9) of RRT-PCR positive samples and five APMV-1 isolates recovered from swabs that were RRT-PCR negative. There was one APMV-4 isolate recovered. In total for 14 APMV-1 isolates, eight, three, and three isolates were recovered in pass 1, 2, and 3, respectively. The APMV-1 RRT-PCR CT values ranged from 28.19 to 39.85 for positive virus isolation. The majority of the VI positives occurred in the RRT-PCR CT values of 36 or less (n=8). For these samples, ECEs had a VI rate of 75% (n=6). An occasional VI positive occurred in the RRT-PCR values 37 to 39.85 (n=9). For these samples, ECEs had a VI rate of 33% (n=3). There was no CT value, below which there was predictable isolation of APMV-1. ECEs supported the growth of one APMV-1 isolate not recovered using ETEs or EDEs. One isolate with a positive HA was unable to be identified as AI virus or APMV-1, 2, 3, 4, 6, 7.

Virus Isolation in Embryonating Turkey Egg: AI virus was recovered from 17.4% (n=8) of RRT-PCR positive samples (n=46); six in pass 1, and two in pass 2. The majority of the VI occurred in the RRT-PCR CT values of 37 or less (n=16). With RRT-PCR CT values of less than 37, ETEs had a VI rate of 50% (n=8). The remaining RRT-PCR CT values were 38 to 43.32 (n=30) and no AI viruses were isolated. There was no CT value, below which there was predictable isolation of AI virus. ETEs supported growth of one AI virus isolate not recovered using ECEs or EDEs.

APMV-1 was recovered in 58.8% (n=11) of RRT-PCR positive samples. There were six APMV-1 isolates recovered that were not detected by RRT-PCR in original samples. There was one APMV-4 isolate recovered. In total, ten, five, and two isolates were recovered in pass 1, 2, and 3, respectively. ETEs supported growth of three APMV-1 isolates not recovered using ECEs or EDEs. The APMV-1 RRT-PCR CT value ranged from 28.19 to 39.85. The majority of VI occurred in the RRT-PCR CT values of 36 or less (n=8) with a 100% (n=8) VI rate. The remaining RRT-PCR CT values were 36 to 39.85 (n=9) had VI rate of 33% (n=3). One sample with a positive HA on isolation attempt was not identified as AI virus or APMV-1, 2, 3, 4, 6, 7.

Virus Isolation in Embryonating Duck Eggs: AI virus was recovered in 17.4% (n=8) of AI matrix gene RRT-PCR positive samples; six in pass 1, and two in pass 2. The majority of the VI positives occurred in the RRT-PC CT values of 37 or less (n=16) with VI rate of 43.8% (n=7). The remaining RRT-PCR CT values were 38 to 43.32 (n=30) with VI rate of 3% (n=1) There was no CT value, below which there was predictable isolation of AI virus. EDEs supported growth of one AI virus isolate not recovered using ECEs and ETEs.

APMV-1 was recovered in 70.5% (n=12) of RRT-PCR positive samples. There were four APMV-1 viruses isolated from RRT-PCR negative samples. In total, twelve, four and zero

isolates were recovered in pass 1, 2, and 3, respectively. EDEs supported growth of one APMV-1 isolate not isolated in ECEs or ETEs. The samples with positive virus isolation had RRT-PCR CT values ranged from 28.19 to 39.84. The majority of the VI occurred in RRT-PCR CT values of 36 or less (n=8) for a VI rate of 100% (n=8). The remaining RRT-PCR CT value range was 37 to 39.85 (n=9) with a VI rate of 44% (n=4). One sample with a positive HA on virus isolation attempt was unable to be identified as AI virus or APMV-1, 2, 3, 4, 6, 7. There was one APMV-4 isolate recovered.

Virus Isolation in MDCK Cell Culture. AI viruses were recovered in 10.9% (n=5) of RRT-PCR positive samples (n=46), all on pass 1. The RRT-PCR CT values of virus isolates ranged for 28.39 to 36.83. There was no CT value, below which there was predictable isolation of AI virus. No APMV-1 was recovered in the MDCK cell culture VI.

Virus Isolation in VERO Cell Culture. AI virus was recovered in 10.9% (n=5) of RRT-PCR positive samples (n=46); four on pass 1 and one on pass 2. The RRT-PCR CT values samples with virus isolation ranged from 28.39 to 36.83. There was no CT value, below which there was predictable isolation of AI virus. APMV-1 was recovered in 29.4% (n=5) of RRT-PCR positive samples; four on pass 1 and one on pass 2. The RRT-PCR CT values for samples with virus isolation ranged from 28.19 to 39.42. There was no CT value, below which there was predictable isolation of APMV-1. One sample with positive HA activity on virus isolation attempt was not identified as AI virus or APMV-1, 2, 3, 4, 6, 7.

Statistical Analysis. The frequency of detection of AI virus by RRT-PCR was compared to VI in ECEs, ETEs, EDEs, MDCK and VERO cell culture by the Fisher Exact test and confirmed using the Chi Square test. Analysis revealed that all methods of VI resulted in

significantly lower rates of AI virus detection compared to RRT-PCR. There were no significant differences in frequency of detection between the five methods for AI virus isolation.

Detection of APMV-1 by RRT-PCR was compared to VI in ECEs, ETEs, EDEs, MDCK and VERO cell culture by the Fisher Exact test and confirmed using the Chi Square test. Frequency of VI was significantly lower compared to detection of AI virus by RRT-PCR. Frequency of VI in ECEs, ETEs, and EDEs were not significantly different. Also, frequency of VI in VERO cell culture was not significantly different compared to VI in ECEs, however numerically, there were less isolates recovered using VERO cells. There were significant differences in detection when VI using VERO cell culture was compared to VI using ETEs and EDEs individually, with an overall reduction in the number of isolates obtained using Vero cell culture. VI using MDCK cell culture was also compared to all VI methods, and analysis reveals significant differences in detection, with no positive VI for APMV-1.

DISCUSSIONS

The preferred method of virus isolation for AI viruses and APMV-1 is inoculation of nine-to-eleven day of age ECEs via the CAS (Swayne and Halvorson 2008). The YS route of inoculation is not a standard method of VI but has been used when CAS inoculation fails (Swayne and Halvorson 2008; Swayne, Senne et al. 2008). Several reports indicate an increased sensitivity and frequency of AI virus isolation when the YS route of inoculation was performed (Woolcock, McFarland et al. 2001; Suarez, Woolcock et al. 2002; Lira, Moresco et al. 2009). Lira *et al* described an increased frequency of AI virus isolation with YS inoculation, with an additional yield of 18% (n=9) after three YS VI attempts compared to three CAS inoculations (Lira, Moresco et al. 2009). However, in our study we only obtained a 2% (n=1) addition yield of AI virus isolates after one YS inoculation. Comparison of the two studies showed two factors

that may account for the different results. First, the number of YS and CAS isolation attempts differed between the two studies. Lira *et al* compared three CAS inoculations to three YS inoculations (Lira, Moresco et al. 2009), whereas this study uses a combination of the two inoculation methods in an attempt to maximize positive isolation with a minimal increase in cost and time. Second, the range of RRT-PCR CT values of the Lira *et al*. study were lower and ranged from 24.92 to 37.62 (Lira, Moresco et al. 2009), verses the AI virus CT values in this study ranged from 29.39 to 43.32, with the majority of CT values over 36. There may exist an inverse association between CT value and efficiency of virus recovery with the YS route of inoculation; i.e. low CT values may increase the chances of VI by YS route. While there is no definite predictive quality associated with CT values that pertains to positive VI, there may be a correlation between CT values of greater than 37 and reduced efficacy of YS inoculation to yield a viable virus isolate. If so, it is recommended for laboratories to evaluation the RRT-PCR CT value range obtain from samples and create a virus isolation pathway based on a maximum CT value that would provide the highest number of isolates using minimum materials. However further study and evaluation with a larger sample size would be necessary.

Infections by AI viruses are most frequent in domestic ducks, which are the same species as mallards, followed by domestic turkeys and least frequent in domestic chickens when comparing to the relative populations of each species. Because VI in ECEs yields fewer viruses than are detected by RRT-PCR, the possibility of increased isolation by using EDEs and ETEs was investigated. In our study, use of ECEs, EDEs, and ETEs showed no significant differences in VI, but ECEs yielded three additional isolates not recovered with ETEs or EDEs. In addition the VI efficiency was compared for the samples with CT values of less than or equal to 37 (n=16). ECEs VI efficiency was 62.5% (n=10) compared to the 50% (n=8) VI efficiency of

ETEs, and the 43.8% (n=7) VI efficiency of EDEs. Those factors, in combination with the possible difficulties in locating and maintaining an adequate supply of ETEs and EDEs favors the continued use of ECEs in surveillance studies as the primary substrate for AI virus isolation.

Similarly, VI for APMV-1 using ECEs, EDEs, and ETEs showed no significant difference in isolation frequency, but ETEs yielded three isolates not recovered with ECEs or EDEs. In addition, ETEs and EDEs yielded APMV-1 isolates on all samples with CT values of 37.81 or less. ETEs may be a reasonable alternative when ECEs fail to isolate APMV-1 virus on samples with CT values of 37.81 or less and could be used a secondary step for isolation.

There were a total of six APMV-1 isolates recovered using avian embryo VI that were not originally detected by the duplex RRT-PCR APMV-1 assay. The protocol described by Kim *et al* utilized 250µl of sample material and 750µl of TRIzol (Kim, Suarez et al. 2008). In this study, the volume of original sample material used for RRT-PCR detection was lowered to 100µl in an effort to conserve the original materials for testing using five VI substrates. The lower volume of sample material used for RRT-PCR detection may have contained a lower concentration of viral RNA, which may have led to failed detection.

The MDCK and VERO cell cultures showed no significant differences in VI frequency compared to ECEs, however such cultures on average produced five to eight less isolates. In Lira *et al.*, the MDCK cells used for VI from wild bird cloacal swabs yielded significantly fewer isolates than ECEs (Lira, Moresco et al. 2009). In addition, a cost analysis was performed which revealed that the addition of cell culture to virus detection assays and VI assays simply increase the cost of diagnostics without improving virus isolation frequency (Lira, Moresco et al. 2009). In laboratories where financial support and resources are not limited, the addition of MDCK or VERO cell culture provides no benefit or improvement to VI methods such as ECEs. However,

for laboratories with limited financial support and resources, where the use of molecular based assays and the embryos for VI are limited, the use of cell culture for VI may hold some advantage, with the understanding that the consistency and efficiency of the MDCK and VERO cell lines compared to VI using ECEs is markedly decreased.

REFERENCES

Alexander, D. J. 1995. The epidemiology and control of avian influenza and newcastle disease. Journal of Comparative Pathology 12(2): 105-126.

Alexander, D. J. 2008. Newcastle disease virus and other avian paramyxoviruses. In, A laboratory manual for the isolation and identification of avian pathogens. 4th Edition (Ed. D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson, W.M. Reed. American Association of Avian Pathologists (Jacksonville, FL): 156-163.

Alexander, D. J. and D. A. Senne 2008. Newcastle disease, other avian paramyxoviruses, and pheumovirus infections. Diseases of Poultry 12th Edition Ed. Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.E. Swayne, Blackwell Publishing (Ames, IA): 75-115.

Carbrey, E. A., C. W. Beard, R. J. Cooper, R. P. Hansen and B. S. Pomeroy 1974. Hemagglutination and hemagglutination-inhibition tests with newcastle disease virus--microtiter technique. 17th Annual Proceedings of the American Association of Veterinary Laboratory Diagnosticians: 1-6.

Hanson, B. A., M. P. Luttrell, V. H. Goekjian, L. Niles, D. E. Swayne, D. A. Senne and D. E. Stallknecht 2008. Is the occurrence of avian influenza virus in charadriiformes species and location dependent? Journal of Wildlife Diseases 44(2): 351-361.

Kim, M. L., D. L. Suarez and C. L. Afonso 2008. Detection of a broad range of class I and II newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. Journal of Veterinary Diagnostic Investigation 20: 414-425.

Lira, J., K. A. Moresco, D. E. Stallknecht and D. E. Swayne 2009. Single and combination diagnostic test efficiency and cost analysis for detection and isolation of avian influenza virus from wild bird cloacal swabs. Avian Diseases 54(1S): 606-612.

Spackman, E., D. A. Senne, L. L. Bulaga, T. J. Myers, M. L. Perdue, L. P. Garber, K. Lohman, L. T. Daum and D. L. Suarez 2003. Development of real-time rt-pcr for the detection of avian influenza virus. Avian Diseases 47(3S): 1079-1082.

Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum and D. L. Suarez 2002. Development of a real-time reverse transcriptase pcr assay for type a influenza virus and the avian h5 and h7 hemagglutinin subtypes. Journal of Clinical Microbiology 40: 3256-3260.

Spackman, E., D. L. Suarez and D. A. Senne 2008. Avian influenza diagnostic and surveillance methods. Avian Influenza Ed. D.E. Swayne, Blackwell Publishing, Ames, IA: 299-308.

Stallknecht, D. E. and S. M. Shane 1988. Host range of avian influenza virus in free-living birds. Veterinary Research Communications 12: 125-141.

Suarez, D. L., P. R. Woolcock, A. J. Bermudez and D. A. Senne 2002. Isolation from turkey breeder hens of a reassortant h1n2 influenza virus with swine, human, and avian lineage genes. Avian Diseases 46(1): 111-121.

Swayne, D. E. and D. Halvorson 2008. Influenza. Diseases of Poultry 12th Edition Ed. Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.E. Swayne, Blackwell Publishing (Ames, IA): 152-184.

Swayne, D. E., D. A. Senne and C. W. Beard 2008. Avian influenza, in: a laboratory manual for the isolation and identification of avian pathogens. 5th Edition. Ed. D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson, W.M. Reed. American Association of Avian Pathologists (Jacksonville, FL): 128-134.

Wise, M. G., D. L. Suarez, B. S. Seal, J. C. Pedersen, D. A. Senne, D. J. King, D. R. Kapczynski and E. Spackman 2004. Development of a real-time reverse-transcription pcr for detection of newcastle disease virus rna in clinical samples. Journal of Clinical Microbiology 42(1): 329-338.

Woolcock, P. R., M. D. McFarland, S. Lai and P. Chin 2001. Enhanced recovery of avian influenza virus isolates by a combination of chicken embryo inoculation methods. Avian Diseases 45: 1030-1035.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

The preliminary studies focused on growth parameters of low pathogenicity avian influenza (LPAI) viruses in various cell lines and cell cultures. The cell lines and cultures were selected to represent various animal species and cell types from various tissues. The majority of the culture and lines had previously been tested for permissiveness to a limited number of AI viruses, but not to all sixteen hemagglutinin subtypes of LPAI virus. Our studies determined the MDCK and CEK cell cultures were the most efficient for replication of LPAI virus with or without the addition of supplemental trypsin. The CEK and MDCK cells experienced less than 0.3 log increase with the addition of supplemental trypsin, however these two cultures produced mean tissue culture infectious dose ($TCID_{50}$) that most closely resembled the mean embryo infectious dose (EID_{50}). Since these kidney epithelial cells produce endogenous proteases, it may not be necessary to supplement these cell lines with trypsin or other proteases to facilitate cleavage of the HA_0 precursor protein (Bosch and Garten 1981).

The preliminary study also included growth parameters of LPAI viruses in three types of avian embryos: embryonating chicken eggs (ECEs), embryonating turkey eggs (ETEs), and embryonating duck eggs (EDEs). All embryos were inoculated with all sixteen hemagglutinin subtypes of LPAI virus and the EID_{50}s achieved by each embryo were compared. There was no difference in the growth titers of the LPAI viruses between the three types of embryos.

Laughing gulls (*Leucophaeus atricilla*) and mallards (*Anas platyrhynchos*), which belong to the orders Charadriiformes and Anseriformes, were inoculated with four and two subtypes of

LPAI viruses, respectively. Oropharyngeal and cloacal swabs were collected on 0, 1, 2, 3, 4, 7, and 10 DPI. All samples were assayed using a real-time reverse transcriptase polymerase chain reaction (RRT-PCR) directed to the matrix gene of AI virus. In addition, all samples were inoculated into ECEs for standard virus isolation. When results from RRT-PCR were compared to single passage in ECEs, there were samples with conflicting results, which were defined as positive by only one method such as RRT-PCR positive and ECEs negative or vice versa. The conflicting samples (i.e. RRT-PCR-/VI HA+ or RRT-PCR+/VI HA-) were inoculated into ECEs for two chorioallantoic sac (CAS) inoculations followed by one yolk sac (YS) inoculation. For RRT-PCR-/VI HA+ samples, allantoic fluid (AAF) was collected and checked for (1) sterility using thioglycolate broth and (2) the presence of AI virus was confirmed using a commercial antigen capture ELISA. The additional embryo inoculation pathway allowed agreement of 41.8% (n=23) of the original conflicting samples (n=55). Final results revealed an 89 to 90% agreement between RRT-PCR and ECEs. The RRT-PCR matrix assay was developed in 2003 by Spackman *et al* and when validated using samples collected from live bird markets, the test displayed a ninety percent agreement when RRT-PCR results were compared to VI using ECEs (Spackman, Senne et al. 2002; Spackman, Senne et al. 2003).

Four cell lines and one cell culture (MDCK, HD11, Mv1Lu, QT-35 and CEF) were selected based on the preliminary studies to perform VI on samples collected from the laughing gulls and mallards. All samples positive for AI virus by ECEs/HA were inoculated into the five cell cultures for three VI attempts. The only cell line to produce no significant differences in VI compared to VI using ECEs, was the MDCK cells inoculated with the ECEs/HA+ positive samples from the laughing gull study. However, the isolation results in MDCK cells were significantly different, with a significantly lower VI rate, compared to ECEs VI positive mallard

samples. While the MDCK cell line was far superior to any other culture tested, it produced less positive identifications and it seemed to lack the consistency achieved by ECEs for VI. All remaining lines and cultures produced significantly lower isolation rates than ECEs.

Two hundred surveillance samples collected from wild bird species consisting of near equal representation of the orders Charadriiformes and Anseriformes, with minimal numbers of sample originating from Guriformes and Galliformes, were used to determine VI efficiency of ECEs, ETEs, EDEs, MDCK and VERO cell culture compared to detection by RRT-PCR. All samples were tested by RRT-PCR for presence of AI virus and APMV-1. The RRT-PCR assay, used for AI virus detection, was developed by Spackman *et al* in 2003 with detection directed to the AI virus matrix gene (Spackman, Senne et al. 2002; Spackman, Senne et al. 2003). The RRT-PCR assay used for APMV-1 detection was developed by Kim *et al* in 2008 with detection directed to the matrix and polymerase genes, which is able to detect and distinguish between class I and II APMV-1 (Wise, Suarez et al. 2004; Kim, Suarez et al. 2008). VI was performed on all RRT-PCR positive samples for AI virus and APMV-1. CT values ranged from 29.29 to 43.32 for AI virus and 28.19 to 39.85 for APMV-1. The embryo inoculation pathway, which consisted of two CAS inoculations followed by one YS inoculation, showed similar AI virus and APMV-1VI results for ECEs, ETEs, and EDEs.

For AI virus, ECEs had a VI rate of 62.5%, ETEs 50%, and EDEs 43.8% for samples with RRT-PCR CT values less then or equal to 37 (n=16). The remaining samples (n=30) had CT values of greater then or equal to 38 and there was a rare occurrence of VI positive for these samples; i.e. ECEs and ETEs had a VI rate of 0% and EDEs had a VI rate of 3%. For APMV-1 VI, the ECEs had a VI rate of 75% while ETEs and EDEs had a VI rate of 100% for samples with CT values less then or equal to 36 (n=8). The remaining samples (n=9) had CT values of

greater then or equal to 37 and there was an occasional VI positive for these samples; i.e. ECEs and ETEs had a VI rate of 33% while EDEs had a VI rate of 44%. The two cell lines, MDCK and VERO, were able to support growth of a minimal number of isolates which was on average between five to twelve less isolates than obtain from avian embryos. Lira *et al* described a cost analysis of VI and RRT-PCR detection methods. She determined that the addition of cell culture VI to other methods of detection, such as RRT-PCR or ECEs, increased the cost of diagnostics without increasing yield when the samples had high frequency of positive results (Lira, Moresco et al. 2009).

Lira *et al* also described the increased VI rate with the use of YS inoculation. The study compared VI rate using three CAS inoculations to three YS inoculations and obtained an 18% increase in AI virus isolation using three YS inoculations. By RRT-PCR directed to the matrix gene of AI viruses, the CT value range was between 24.92 and 37.62 (Lira, Moresco et al. 2009). In comparison the embryo inoculation pathway in our study consisted of two CAS inoculations followed by one YS inoculation. Using this pathway there was only a 2% increase in AI virus isolation. However, the CT value range in this study determined by RRT-PCR, also directed to the matrix gene, was between 29.39 and 43.42 with the majority of the CT values were greater than 36. While there is no definite predictive quality associated with CT values that pertains to positive VI, there is an inverse association between CT values of greater than 37 and reduced efficacy of YS inoculation to yield a viable virus isolate.

REFERENCES

Bosch, F. X. and W. Garten 1981. Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between ha1 and ha2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. Virology 113(2): 725-735.

Kim, M. L., D. L. Suarez and C. L. Afonso 2008. Detection of a broad range of class I and II newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. Journal of Veterinary Diagnostic Investigation 20: 414-425.

Lira, J., K. A. Moresco, D. E. Stallknecht and D. E. Swayne 2009. Single and combination diagnostic test efficiency and cost analysis for detection and isolation of avian influenza virus from wild bird cloacal swabs. Avian Diseases 54(1S): 606-612.

Spackman, E., D. A. Senne, L. L. Bulaga, T. J. Myers, M. L. Perdue, L. P. Garber, K. Lohman, L. T. Daum and D. L. Suarez 2003. Development of real-time rt-pcr for the detection of avian influenza virus. Avian Diseases 47(3S): 1079-1082.

Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum and D. L. Suarez 2002. Development of a real-time reverse transcriptase pcr assay for type a influenza virus and the avian h5 and h7 hemagglutinin subtypes. Journal of Clinical Microbiology 40: 3256-3260.

Wise, M. G., D. L. Suarez, B. S. Seal, J. C. Pedersen, D. A. Senne, D. J. King, D. R. Kapczynski and E. Spackman 2004. Development of a real-time reverse-transcription pcr for detection of newcastle disease virus rna in clinical samples. Journal of Clinical Microbiology 42(1): 329-338.