DEVELOPMENT OF METHODS FOR THE DETECTION AND CONTROL OF LOW-PATHOGENIC AVIAN INFLUENZA VIRUS

by

LAUREN MARY APPLEBY

(Under the Direction of Egbert Mundt)

ABSTRACT

The control of avian influenza is most effectively approached from multiple angles. In this research, two aspects of the management of this disease were examined. The first objective was to establish a species-independent competitive ELISA for the detection of antibodies against hemagglutinin 6 (H6). Characterization of the monoclonal antibodies (mAbs) developed toward this purpose revealed that all four mAbs were specific for linear epitopes within the esterase domain of H6. One of the mAbs was also found to be neutralizing. Another aim of the research was to evaluate the efficacy of metam-sodium as a disinfectant of poultry litter. The removal of pathogens which persist in litter is required for its safe disposal, yet few agents are able to effectively penetrate this porous material. It was found that the manufacturer's recommended concentration was able to completely eliminate infectious avian influenza virus and infectious bursal disease virus from contaminated poultry litter.

INDEX WORDS: avian influenza virus, hemagglutinin, H6, monoclonal antibody, competitive ELISA, metam-sodium, infectious bursal disease virus, disinfectant, litter

DEVELOPMENT OF METHODS FOR THE DETECTION AND CONTROL OF LOW-PATHOGENIC AVIAN INFLUENZA VIRUS

by

LAUREN MARY APPLEBY

B. S., University of Georgia, 2007

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2010

DEVELOPMENT OF METHODS FOR THE DETECTION AND CONTROL OF LOW-PATHOGENIC AVIAN INFLUENZA VIRUS

by

LAUREN MARY APPLEBY

Major Professor:	Egbert Mundt
Committee:	Maricarmen García Holly Sellers

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2010

DEDICATION

I would like to dedicate my thesis to my parents, who instilled in me their work ethic and always encouraged me to take on new challenges. Additionally, this work would not have been possible without the support and advice of my husband.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my major professor, Dr. Egbert Mundt, for taking me on first as a laboratory technician and then as a graduate student. He has always been extremely patient with me and from him I have learned a great deal.

I would also like to acknowledge the two other members of my graduate committee, Dr. Maricarmen García and Dr. Holly Sellers, for taking the time to monitor my progress and review my work.

Additionally, I appreciate the assistance and instruction which I have received on frequent occasions from other members of Dr. Mundt's group, in particular Dr. Ashwini Warke, Dr. Ruth Stricker, and Mr. Alan Icard.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Influenza A and B cause a disease with a broad spectrum of severity, from a mild disease which poses a mere annoyance to most of the millions it infects each year, to one of the deadliest scourges of all time. This seeming paradox arises as a result of the high mutability of the influenza virus. Not only does the virus experience a high mutation rate as a result of its error-prone replication process, but it also possesses the ability to transfer genetic material from one strain to another, also known as reassortment. These traits are advantageous to the virus because they result in a high level of adaptability in the host. In other words, the rapid rate of genetic change increases the odds that, within a given population of the virus, there will be individual virions equipped, for example, to recognize the cellular receptors of a new host species should the opportunity arise. Truly, the unique characteristics of a given strain of influenza virus are the result of a complex synthesis of myriad genetic factors. Such factors can align to give rise to both killer pandemics and minor seasonal infections.

The family *Orthomyxoviridae* contains the genera *Influenza A*, *Influenza B,* and *Influenza C* as well as *Thogotovirus* and *Isavirus*. These viruses are characterized by their segmented, single-stranded RNA genomes of negative polarity. The influenza A and B viruses each have genomes consisting of eight segments while the genome of influenza C has seven. The three influenza virus groups are distinguished on the basis of two of their internal proteins, matrix protein 1 (M1) and nucleoprotein (NP) (83). Influenza A and B are widespread in humans, while influenza C is much more mild and is thus an often undiagnosed disease. The natural hosts of influenza A virus are wild ducks and shorebirds [avian influenza virus (AIV)]; however, AIV also infects domestic poultry species and mammals, including swine and humans (reviewed in 153). Influenza A viruses are further categorized into subtypes based on hemagglutinin (HA) and neuraminidase (NA), two surface glycoproteins (152). So far, sixteen HA (H1-H16) and nine NA (N1-N9) subtypes have been described (43). As a whole, influenza viruses are a group of extremely important human and animal pathogens. Human-adapted AIV is largely responsible for the seasonal influenza which sweeps through the population each year. The rate of change,induced by the host's protective immune response, of hemagglutinin (HA) is so great that infection or vaccination with the strain from one year offers minimal protection against infection with the strains arising in subsequent years. Seasonal influenza kills approximately 36,000 people in the United States annually (16). Its victims are principally those with less robust immune systems, such as the very young and the very old.

While the annual death toll may seem surprisingly high, every so often an atypical influenza virus emerges and spreads rapidly through the naïve population, resulting in higher case fatality rates and frequently affecting age groups unaccustomed to influenza complications. These influenza strains, which are of global distribution and significance, are designated pandemic strains. Influenza has been implicated in four such pandemics in recent history. The outbreak of "Spanish flu" from 1918-1919 culminated in an estimated 50 million deaths world-wide (72). In the years 1957-1958 and 1968-1969 influenza virus again asserted itself. Most recently, in 2009, a novel swine-origin influenza virus began its spread around the globe (158). Each of these pandemics was preceded by the introduction of genes from the vast avian influenza A reservoir into viruses capable of infecting humans. The pandemic strains from 1957 and 1968 each received polymerase genes from an avian source (75). Reassortment of avian, swine, and human strains followed by further circulation in swine gave rise to the 2009 virus (5). Conversely, the 1918 strain is thought to be a fully avian virus that underwent adaptation in swine before transferring to humans (142). Although not capable of human to human spread, and therefore of limited distribution, the highly pathogenic H5N1 virus which emerged in the live bird markets of Hong Kong in 1997 had the ability to directly infect humans without prior adaptation (24). A total of 498 infections with this virus to date have resulted in 294 deaths (159). These outbreaks carry with them a recurring theme: the gene pool of AIVs present in avian species represents a virtually endless supply of genetic material, which, if transferred to humans, can have disastrous consequences.

Given the situation, there is undeniable prudence in conducting influenza virus surveillance on a grand scale. There are many tests for the detection of influenza, each with its own merits; however, in monitoring operations of a broad scope there are several important considerations. First, any test used must be sensitive, accurate, and able to produce relatively quick results. It must also be possible to process a large number of samples, including samples from several different species. The competitive enzyme-linked immunosorbent assay (cELISA) has all of these advantages. It is a rapid test which can be automated and its competitive design enables the detection of virus subtype-specific antibodies in serum from any species. One goal of this research was to develop a species-independent cELISA against subtype 6 hemagglutinin (H6).

Another factor in the control of influenza is environmental risk reduction. Although this term encompasses many measures which can be taken, perhaps the most basic and widespread of these is the application of chemical disinfectants. Out of the many compounds with antiviral properties, the ideal substance should be safe for humans, animals, and the environment and fast-acting on both porous and non-porous surfaces. Metam-sodium is a commonly used soil fumigant which is approved by the United States Environmental Protection Agency for this purpose (38). The second aim of this research was to examine the efficacy of metam-sodium as a disinfectant for poultry litter.

CHAPTER 2

REVIEW OF LITERATURE

General Biology of Avian Influenza Viruses

Influenza A viruses belong to the family *Orthomyxoviridae* within the order *Mononegavirales*. They are categorized based on the subtypes of two of the three surface proteins, hemagglutinin (HA) and neuraminidase (NA) (152). At this point in time, a total of sixteen HA and nine NA subtypes have been documented (43). Wild aquatic waterfowl, such as ducks and shorebirds, are the natural hosts of influenza A viruses [avian influenza virus (AIV)], but this does not mean that AIVs are limited in distribution to these hosts. Avian influenza viruses are known to infect humans and other mammals from time to time as well as domestic poultry (reviewed in 153). As a whole, influenza viruses are a group of extremely important human and animal pathogens. Apart from seasonal influenza, which results in an annual average of approximately 36,000 fatalities in the United States (16), particularly lethal and widespread pandemic strains are known to emerge on occasion. The 1918-1919 outbreak of “Spanish flu” was responsible for an estimated 50 million deaths worldwide (72). Since the identification of the influenza virus as the etiological agent of influenza in the 1930s, three other pandemics have occurred. One emerged during the years 1957-1958 (H2N2), the second in 1968-1969 (H3N2), and, most recently, the novel H1N1 in 2009 (157). All of these pandemic viruses have contained genes of avian origin (75, 142).

Genome Organization

One of the most salient aspects of influenza viruses is their ability to reassort, giving rise to novel virus strains. These reassortment events, in which genes are exchanged between different viruses, are what can occasionally give rise to pandemic strains (75) and are the result of the segmented nature of the influenza genome. The avian influenza virus genome is composed of eight segments of negative-sense, single-stranded RNA (reviewed in 152). The genome encodes a total of eleven viral proteins (19).

Viral Proteins

Protein Basic 2 (PB2)

Segment one of the avian influenza virus genome encodes PB2, which forms part of the heterotrimeric polymerase complex (31, 143). PB2 is known to be responsible for binding 5' cap groups of cellular mRNAs, a function which is crucial to the initiation of viral mRNA synthesis. UV cross-linking assays first demonstrated that PB2 was the sole protein responsible for cap-binding by showing that only PB2 co-migrated with labeled cap groups after cross-linking (13, 128, 144). Once bound by PB2, the cellular mRNA is cleaved 10-14 nucleotides downstream from the cap structure by the viral protein PA (see below) which contains endonuclease activity (30, 40, 54, 165). The liberated cap group is then used to prime the synthesis of viral mRNA. The cap-binding activity of PB2 has been mapped to three separate regions of the primary amino acid sequence. Not only do these regions show sequence homology to other known cap-binding proteins, but antibodies directed against these areas or site-directed mutagenesis within them have both been shown to inhibit cap-binding and transcription (39, 59).

Apart from its role in cap-binding and transcription initiation, it has been demonstrated that PB2 is required for viral replication (60, 62, 110). Site-directed mutagenesis of conserved residues in the N-terminal region of the protein was found to inhibit the accumulation of both positive-sense copy RNA (cRNA) and negative-sense viral RNA (vRNA) (48). PB2 is known to interact with PB1 (139), the viral RNA-dependent RNA polymerase, and also the nucleoprotein (NP) as described by Biswas et al. (12). The PB1- and NP-binding regions share significant overlap and it has been shown that these proteins compete with one another for binding to PB2. This suggests that PB2 may play a role in regulating the switch of the polymerase complex activity from transcription to replication by interacting with either one protein or the other (114).

Protein Basic 1 (PB1)

PB1 is encoded by the second segment of the AIV genome. It is the viral RNA-dependent RNA polymerase and is responsible for both elongation of viral mRNA from the 5' cap and unprimed synthesis of viral complementary RNA (cRNA) and viral genomic RNA (vRNA) (98). PB1 shares conserved amino acid sequence motifs with other known nucleotide polymerases and mutation of these residues abolishes polymerase activity (11). PB1 is known to interact with both PB2 and PA (111) and is thought to form the backbone of the polymerase complex (25, 31, 51).

PB1-F2

A second open reading frame resides within segment two of the AIV genome, and this encodes PB1-F2. Unrelated in function to PB1, PB1-F2 is a pro-apoptotic protein. PB1-F2 is primarily expressed when the virus infects monocytic cells (19). Once the protein is expressed it induces the formation of pores in the inner and outer mitochondrial membranes, causing the dissipation of the membrane potential and initiating the signaling cascade which leads to apoptosis. This activity has been mapped to an amphipathic helix near the C-terminus of the protein (49).

Protein Acidic (PA)

The third genome segment encodes PA, which forms part of the polymerase complex along with PB1 and PB2. Most reports indicate that there is a direct interaction between PA and PB1 while no such interaction exists between PA and PB2 (51, 143). This was challenged, however, by a recent study that was able to detect binding of these two subunits through a bimolecular fluorescence complementation assay (55). The biological significance of this potentially transient interaction is unknown. Although its role is still imperfectly understood, PA has been shown to be essential to viral replication (98). It was once thought that the endonuclease activity associated with the viral polymerase resided with the PB1 subunit; however mounting evidence indicates that PA is responsible for endonucleolytic cleavage of cellular mRNA (30, 40, 54, 165).

Hemagglutinin (HA)

Hemagglutinin is a trimeric type I membrane protein encoded by segment four of the AIV genome. It binds to sialic acid receptors on the cell surface, initiating cell-mediated endocytosis (reviewed in 131). HA is a pivotal protein in the determination of host range and tissue tropism. HAs of AIV show a preference for receptors with α2, 3-linked sialic acids, which are prevalent in the upper avian respiratory tract. Conversely, mammals have a higher prevalence of α2, 6-linked sialyl receptors in the upper respiratory tract (101). HAs from mammal-adapted strains show a higher affinity for α2, 6-linkages than avian strains while maintaining the ability to bind α2, 3-linked sialyl receptors (101, 118). Despite these differences, the human cases of H5N1 demonstrate that it is possible for AIV to infect humans without acquiring human influenza genes or passing through an intermediate host (24). Clearly then, HA, although an important determinant of host range, is not the only contributing factor. HA is, however, a significant determinant of pathogenicity for AIVs in chickens and the marker which is used to distinguish between high pathogenic and low pathogenic avian influenza viruses (HPAI and LPAI, respectively). Infection with HPAI results in severe disease in chickens, with mortality approaching 100% in most cases. HPAI is defined by having several basic amino acid residues at the hemagglutinin cleavage site (3). Full-length HA, called HA0, is posttranslationally cleaved to yield two peptides, HA1 and HA2, which remain connected by disulfide bonds. Cleavage and subsequent conformational change as a result of the acidification of the cellular endosome result in the exposure of the hydrophobic N-terminus of HA2. This fusion peptide, as it is called, embeds in the endosomal membrane, causing the membrane and the viral envelope to fuse and permitting the release of the viral ribonucleoprotein complex into the cytoplasm (131, 135). The cleavage of hemagglutinin is essential to viral infection and the additional basic residues allow the hemagglutinin protein to be cleaved by furin-like proteases, which are ubiquitous, rather than solely by trypsin-like proteases, which are limited in distribution to the respiratory and digestive tracts (61, 137). As a result of this trait, HPAI can display altered tissue tropism and lead to systemic viral infection. So far, every strain of HPAI isolated has been of either the H5 or H7 subtype (3). Tests which detect antibodies to different HA subtypes are widely used for influenza surveillance.

Nucleoprotein (NP)

Segment five encodes NP, which associates with viral RNA and the polymerase complex to form the ribonucleoprotein complex (RNP). Multiple subunits of NP interact with one another and with RNA to form a helical structure (71, 76, 121). NP is also known to interact with M1 and the polymerase complex proteins PB1 and PB2 (12, 163) and it is essential for the production of full-length cRNAs and vRNAs (94, 125). While bound to the viral RNA, NP is imported into the nuclei of infected cells (91, 106). At least two separate regions within the primary sequence are responsible for this targeting (27, 102). Additionally, NP is a phosphoprotein (77) and its phosphorylation may also play a role in nuclear import (102).

Neuraminidase (NA)

Encoded by segment six, NA, like HA, is an antigenically diverse surface glycoprotein. It can be found as a homotetramer in the viral envelope. As a class II membrane protein, its transmembrane domain is located near the amino-terminus. A stalk region connects the transmembrane domain to the catalytic center located in the globular head (146). The enzymatic function of NA is to cleave terminal sialyl residues from cell surface glycoconjugates (1). This is essentially the opposite function of HA, which binds these residues, and a delicate balance between HA and NA activity is required for efficient viral infection (64, reviewed in 147). Through its receptor-destroying activity, NA prevents HA from binding mucus proteins rather than the targeted epithelial cells, permits the release of progeny virions from infected cells, and makes it so virus particles do not adhere to one another (109). NA inhibitors are a major class of anti-influenza A drugs, although it appears that influenza A viruses are able to rapidly evolve resistance to these compounds (97).

Matrix 1 (M1)

Segment seven encodes two proteins, one of which is M1. M1 undergoes homo-oligomerization to form a meshwork on the internal face of the viral envelope, lending shape and support to virus particles (104). Apart from associating with the viral envelope and elements of the cytoskeleton (166), M1 is also known to interact with NP (52, 104), and through this contact it plays a role in the nuclear import and export of RNPs. Bui et al. (14) established that RNPs were unable to enter the nucleus when bound to M1. Additionally, they demonstrated that RNPs could undergo normal nuclear import when this interaction was disrupted by a transient decrease in pH (14). Since the viral protein M2 (discussed below) increases the acidity of the virion interior, and the M2 targeting anti-influenza drugs amantadine and rimantadine have been shown to block the nuclear import of RNPs (91), the current understanding is that M1 undergoes a conformational change upon the acidification of the interior of the virus particle, releasing its bound RNPs and thereby permitting them to enter the nucleus. During the later stages of infection, M1 enters the nucleus (92) and permits the export of RNPs for packaging into progeny virions (91). M1 is also an important factor in virus budding. When expressed in cell culture in the absence of other viral proteins, it can assemble into virus-like particles which are released from cells (50).

Matrix 2 (M2)

Splicing of segment seven mRNA gives rise to a second mRNA which encodes the M2 protein (80). It is an integral membrane protein (82) which embeds in the viral envelope as a homotetramer (58) and functions as an ion channel (112). M2 is activated in response to a lowered external pH, a condition which occurs within the late virus particlecontaining endosome prior to viral uncoating. It selectively permits protons to enter the inner virion and thereby leads to the acidification of the interior of the virus particle (22). It has been shown that the cytoplasmic tail of the M2 protein is important in several ways. M2 mutants lacking the cytoplasmic tail region located at the carboxy-terminus of the protein not only had an unusual filamentous morphology, but they were found to be inefficient in the incorporation of RNPs into progeny virions (68). The importance of M2 in RNP packaging is thought to result from its interaction with M1 (17). As integral membrane proteins, M2 and HA translocate to the plasma membrane via the trans-Golgi network. When M2 is specifically blocked by the antiviral drug amantadine, the low pH conformation of HA is expressed on the surface of infected cells (140). HA adopts this conformation as a result of the acidity of trans-Golgi vesicles. Functional M2 allows protons to exit the vesicle, thereby raising the pH and permitting HA to adopt its proper conformation (23).

Non-Structural 1 (NS1)

The co-linear mRNA of segment eight encodes NS1 (79), which serves in multiple capacities during viral infection. It has been shown to bind to the poly-(A) tails of both viral and cellular mRNAs, inhibiting their export from the nucleus (116). This is thought to be conducive to viral transcription by making sure that there are cellular mRNAs available to the polymerase complex, which must use the 5’-cap structure of cellular mRNAs to prime viral mRNA synthesis. Indeed, NS1 appears to be highly involved in various aspects of the regulation of viral gene expression. It has been shown to inhibit the splicing of pre-mRNAs, with the exception of NS1 mRNA (88). It also stimulates the transcription of the M1, NP, and NA mRNA (37) and interacts directly with viral transcription-replication complexes in the cell nucleus, possibly influencing late viral gene expression (90). Additionally, the role of NS1 in modulating the host immune response has been well established. It has been demonstrated that an influenza A virus lacking the NS1 gene could replicate in the absence of interferon but was severely attenuated in systems where interferon was present (47). The NS1 protein from a lethal H5N1 isolate was shown to be responsible for the resistance of the isolate to the effects of interferons and tumor necrosis factor α (122). Li et al. (85) demonstrated that NS1 from a highly pathogenic H5N1 virus was crucial in determining the replication potential of the virus in chickens. Specifically, a single amino acid in NS1 was found to be important to the viral inhibition of alpha and beta interferon in this host.

Nuclear Export Protein (NEP)

Splicing of the segment eight RNA transcripts gives rise to an mRNA encoding the nuclear export protein (NEP) (79). As its name suggests, it is involved in the export of RNP complexes from the nucleus (107). It has also been shown to drastically inhibit the synthesis of cRNA, vRNA, and mRNA from a model template RNA in cells expressing the polymerase proteins and NP, suggesting that NEP may play a regulatory role in the cycle of infection (15).

Replication Cycle

Attachment and Entry

The influenza A cycle of infection, as with any virus, begins with attachment to the host cell. This initial stage is mediated by HA, which binds to terminal sialic acid residues of glycoproteins or glycolipids on the cell surface (157). Several specific amino acid residues in HA have been identified as important in determining which type of sialic acid residue is preferentially bound (7, 26, 119). While some HAs have a preference for α2, 6-linked sialyl residues, others are more inclined to bind residues with α2, 3-linkages (118). This variation in binding affinity plays a significant role in the determination of host range. This is because the upper avian airways contain more α2, 3-linked sialyl residues while in humans and other mammals there is a higher incidence of α2, 6-linkages (101). Swine possess a more or less even distribution of the two linkage types, making them uniquely susceptible to both avian-adapted and mammalian-adapted influenza A viruses (131). While the HA receptor-binding domain represents a significant host range determinant, it is not the only factor at work. Indeed, the H5N1 virus which emerged during 1997 in Hong Kong was a fully avian virus which was transmitted to humans without prior adaptation (24).

Once bound to the cell, virions are internalized via endocytosis (93). While the majority of influenza A virus particles enter cells through a clathrin-mediated pathway, a clathrin- and caveolin-independent entry mechanism is also utilized by the virus (120, 130). In the case of clathrin-dependent entry, the *de novo* formation of clathrin-coated pits at the sites of viral attachment appears to be a process which is somehow induced by the virus itself, although the mechanism is unknown (120). Studies employing real-time fluorescence microscopy have elucidated the movements of individual virus particles within the cell prior to fusion. The endocytic vesicle containing the virus first fuses with the early endosome. This compartment then undergoes dynein-directed transport to the perinuclear region, eventually maturing into a late endosome with an approximate pH of 5, at which point fusion of the viral envelope with the endosomal membrane occurs (78).

Uncoating

Uncoating is the process by which the viral RNPs is released into the cell from the virus particle. The fusion and the events immediately prior to it are highly contingent upon the low pH (approx. 5.0) environment of the late endosome (154, 164). In demonstration of this, the introduction of compounds which raise the pH of this cellular compartment effectively blocks influenza uncoating (93, 164). The main viral protein mediators of fusion and release of the RNPs into the cytoplasm are M2, M1, and HA.

The low pH of the late endosome triggers the activation of the ion channel made by the tetrameric M2, which permits protons to enter the interior of the virion (22). The subsequent acidification of the interior of the virus particle induces a conformational change in M1. This change causes M1 to release its bound RNPs so that they can enter the cytoplasm once fusion of the viral envelope with the endosomal membrane occurs (14).

The actual fusion step is brought about through the action of HA. In order to be fully functional, HA must be post-translationally cleaved by extracellular proteases (89, 154, 155). This cleaves the precursor HA0 into its component peptides HA1 and HA2. The two remain connected via disulfide bonds, but the cleavage permits the later exposure of the hydrophobic N-terminus of HA2, which is essential for membrane fusion (33). While in the endosome, hemagglutinin undergoes a conformational change in response to the increased acidity in the endosomal compartment. This change fully exposes the N-terminus of HA2, which then embeds in the membrane of the late endosome. The result is to draw the membrane and the viral envelope into close proximity, permitting them to fuse (136).

Transcription and Replication

After they are released into the cytoplasm of the cell, the RNPs are actively transported to the nucleus and enter through the nuclear pore complexes (91). Since all of the constituent proteins of each RNP contain their own nuclear localization signal (NLS) (73, 91, 100, 103), it was initially unclear whether the RNP components were transported to the nucleus individually or as partial or whole complexes. While the individual proteins are capable of entering the nucleus in the absence of other viral proteins, it has been demonstrated that not all of the RNP constituent proteins are able to efficiently accumulate in the nucleus when expressed alone (41, 103). This suggested that at least some interaction between the proteins occurs during nuclear transport. The current understanding is that PB1 and PA form a heterodimer in the cytoplasm before entering the nucleus while PB2 is transported independently of the other two polymerase proteins (29, 63). Additionally, it appears that complex formation between PB1-PA and PB2 is the event which results in the retention of PB1-PA in the nucleus (63).

Once inside the nucleus, the RNPs can begin the task of synthesizing mRNAs from the viral genome. First, PB2 binds the 5’-cap group of a cellular mRNA (13, 144). The mRNA is cleaved 10-13 nucleotides downstream of the cap by PA (30, 40, 54, 113, 165). While the cellular mRNA which is released will eventually be degraded, its stolen 5’ cap will be used to prime the transcription of a viral mRNA. PB1 is responsible for the elongation of the mRNA transcript from the primer (98). A 3' poly-A tail is added to mRNAs through reiterative copying of a short stretch of U residues near the 5' end of the vRNA. Polyadenylated viral mRNAs are transported from the nucleus to the cytoplasm where they are translated into the viral proteins (20).

The ribonucleoprotein complexes produce three different species of RNA. mRNAs are primarily synthesized early during viral infection so that viral proteins can be translated. At later time points post-infection, the RNPs switch to the synthesis of positive-sense cRNA and, using this cRNA as a template, transcribe negative-sense vRNA (20). It is thought that NP plays an important role in mediating this change in function; however the exact mechanism is unknown (12). The synthesis of cRNA and vRNA, unlike that of mRNA, does not require a primer (81).

Packaging, Budding and Release

In the final stage of an infection, the next generation of virus particles is assembled and released from the cell. The progeny virions must contain the genetic information copied from the parent virus and certain structural proteins which will enable them to infect new cells. Studies with defective interfering RNAs (DIs), which are viral RNAs with internal deletions, have established that influenza vRNAs are not packaged randomly (34, 46). Instead, there is a selective process by which one copy of each vRNA segment is contained within a budding virion (99). DIs have also helped to elucidate which parts of vRNAs are necessary for their incorporation into virus particles. Each vRNA has 5' and 3' untranslated regions containing sequences conserved among all eight segments as well as segment-specific sequences. Although it was originally thought that these untranslated regions were all that was required for vRNA packaging (65), it has now been established that at least part of the coding sequences of vRNAs are also needed in order for the segments to be incorporated into virions (35, 44, 46) and that length of the coding regions, rather than sequence, may be important (108).

vRNAs exit the nucleus and approach the plasma membrane in the context of RNPs. M1 and NEP are thought to be the major mediators of this process. Both of these proteins enter the nucleus (91, 162). In the absence of M1, RNPs fail to be exported from the nucleus. Nuclear export is restored upon the addition of exogenous M1 protein (92). Although M1 is known to be able to bind to NP (52), it is unclear whether it orchestrates the export of RNPs through direct contact or by some indirect mechanism. It has been shown that nuclear export can occur even if M1 remains in the nucleus, although virions are prevented from assembling (156). It is known that NEP binds to both M1 (161) and Crm1, a protein which is part of the cellular nuclear export pathway (2), and interacts with nuclear pore complexes (107). Inhibition of Crm1 with leptomycin B also inhibits the nuclear export of RNPs (36, 150). Although it is presumed that the export of viral RNPs from the nucleus is controlled through the interactions between Crm1, NEP, M1, and NP, the process has yet to be fully elucidated. Once outside the nucleus, M1 associates with the cytosolic face of the cell membrane (166), docking the bound RNPs in place at the site of budding. The remaining structural proteins of the influenza virus, HA, NA, and M2, are transmembrane proteins which become embedded in the plasma membrane during synthesis (157). Several studies have shown that the cytoplasmic tails of these proteins are necessary for their efficient packaging into virions (17, 95). Furthermore, M1 is known to be capable of interacting with the cytoplasmic domain of M2 (17), indicating a possible mechanism by which the viral integral membrane proteins are positioned at the site of budding.

Beneath the plasma membrane, M1 self-associates to form a meshwork (104). In so doing, it is thought to generate the necessary membrane curvature for the virion to bud from the cell (166). Indeed, in a system in which only the M1 protein is expressed, virus-like particles develop and are released from cells (50). After budding from the cell surface, the newly-formed virus particles, which, at this stage, are in close proximity to one another, would tend to form aggregates were it not for the action of NA (109). NA cleaves the terminal sialic acid residues from the glycoproteins of neighboring virus particles, thereby disrupting the sialic acid binding mediated by HA and freeing the virions to disperse and subsequently infect new cells.

Prevalence, Distribution, and Significance of H6-subtype Viruses

Avian influenza viruses of the H6 subtype are among those most commonly isolated from wild waterfowl in North America (53, 74, 126, 127, 134). They are also detected at high frequencies in both wild ducks and domestic poultry in parts of Asia (69, 84, 129). Although frequently detected in ducks (order Anseriformes), H6-subtype viruses are rarely isolated from shorebirds and gulls (order Charadriiformes) (74). Nevertheless, it is apparent that these viruses experience occasional intercontinental transfer between Asia and North America. This is most clearly evidenced by the large-scale replacement of H6 genes of the North American lineage with those of the Asian lineage, such that the North American genes have all but disappeared over the past 20 years (105).

Although all viruses of the H6 subtype recorded to date have been grouped to the LPAI (3), these viruses are implicated in increased flock mortality, respiratory distress, decreased egg production, and nephritis in infected chicken flocks (84) and are therefore of economic concern to the poultry industry. During 2000 and 2001, nine isolates of H6N2 were recovered from chickens in California over a span of 20 months. The results of phylogenetic analysis of the viruses suggested that they might be capable of sustained circulation in chickens and may even undergo reassortment while replicating in this host (151).

Although they do not normally infect mammals (45), H6-subtype viruses can replicate in experimentally infected human volunteers (10). Furthermore, one of the most notable features of the influenza viruses is their ability to acquire new characteristics, such as a different host range, through reassortment events. Indeed, a reassortant H6 virus was isolated from a green-winged teal during the 1997 outbreak of highly pathogenic H5N1. This virus, called W312, shared seven of its eight genome segments with the circulating HPAI- only the segment encoding hemagglutinin had been exchanged (57). W312 can still be isolated from wild birds in southern China (21), meaning that, although HK/97 has been eradicated, the majority of its genes are still in circulation and need only a single reassortment event in order to reemerge as a major animal and human health threat.

Overall, the influenza outbreaks within the last decade have served to underline the importance of conducting large-scale surveillance of circulating viruses. At-risk areas can be identified in this way before outbreaks begin. For example, if a subtype known to have a propensity for infecting domestic poultry is identified in a wild bird population near an area of poultry production, extra precautions, such as vaccination against that subtype, can be taken. By keeping domestic animals free of influenza viruses, the risk of zoonotic human infection should be greatly lessened (42).

Avian Influenza Control

Surveillance

Surveillance of circulating avian influenza viruses has become a high priority in recent years due to the increasing number of outbreaks of highly pathogenic avian influenza (3, 42, 123). For surveillance it is possible to either look for the virus itself in swab or tissue samples or to test serum samples for the presence of antibodies raised against the virus. Virus isolation is the most common and sensitive method for the direct detection of AIV. In this test, 9- to 14-day-old embryonated chicken eggs are inoculated with 100-200μl of a given sample and then further incubated for 2 to 3 days. Allantoic fluid is harvested from the eggs and tested for the ability to agglutinate erythrocytes in a Hemagglutination Assay (HA) (141). While sensitive, this method becomes time-consuming and expensive when large numbers of samples are involved. Additionally, avian paramyxoviruses also agglutinate red blood cells (149) and so the possibility that samples contain viruses from this group, rather than AIV, cannot be eliminated by an HA. A more rapid and specific test for the detection of avian influenza virus is the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (132). RNA extracted from the samples to be tested is first reverse-transcribed into a complementary DNA copy (cDNA) before undergoing multiple cycles of amplification with AIV-specific primers. Only those samples which contain the AIV genome will yield an RT-PCR product. Depending on the primers used, it is also possible to detect specific HA and NA subtypes. Both infectious and inactivated virus will be detected by this method (138). Since influenza viruses are typically cleared from infected individuals in a matter of days, there is only a small window in which to collect samples which are potentially positive for virus (87). For this reason, the detection of antibodies produced by a host in response to AIV infection, which can be long-lasting, is the preferred method for large-scale surveillance projects (32).

There are many antibody detection tests available. In the Agar Gel Precipitation Test (AGPT), serum samples are placed into wells in a sheet of agar adjacent to wells containing antigen. The formalin-inactivated antigen is prepared from the chorioallantoic membranes of embryonated chicken eggs infected with a type A influenza virus (8). If the serum samples contain antibodies to the influenza A nucleoprotein which is contained in the antigen, a visible precipitate of antigen-antibody complexes will form in the agar gel between the wells. This test can identify serum samples which are positive for antibodies to any type A influenza virus in avian and mammalian sera (8, 9). Unfortunately, relatively large quantities of both antigen and serum are required and the test takes at least 24 hours to develop (167). Another method is the Hemagglutination Inhibition (HI) test. A serum sample is determined to have antibodies to a particular reference strain if it is able to inhibit the agglutination of erythrocytes which is caused by this strain. Only the HA subtype against which the antibodies are raised can be determined in this test (160). Additionally, numerous AIV reference strains are required in order to cover all subtypes. One issue with the HI assay is that it typically requires the use of infectious virus, which necessitates that it be conducted using certain safety precautions in specialized facilities (123, 167).

The Enzyme-Linked Immunosorbent Assay (ELISA) is one which can be carried out on the bench top as it usually need not utilize infectious virus. In this method, a 96-well plate is coated with a viral protein antigen. The level of specificity of the test can be altered depending on which antigen is used. For example, there are commercially available ELISA kits which use NP (IDEXX FlockChek™ AI MultS-Screen Ab Test Kit, IDEXX Laboratories, Westbrook, ME), which is conserved among all influenza A viruses (160). These kits can identify serum samples which are positive for AIV (66). Conversely, by using HA or NA as the viral protein antigen, it is possible to determine whether or not a serum sample contains antibodies to the particular subtype used. In a typical indirect ELISA, the coated plate is first incubated with the experimental serum samples followed by incubation with an enzyme-tagged conjugate which specifically binds antibodies of the species from which the sera were taken (160). Upon the addition of a substrate, a colorimetric change is observed in those wells which contain the conjugate bound to serum antibodies, which are in turn bound to the viral antigen.

One drawback to this assay is that it is species-specific. A variation on the ELISA which sidesteps this problem is the competitive ELISA (cELISA). This test typically utilizes a monoclonal antibody specific for the viral protein antigen. Rather than measuring the amount of serum antibody, if any, which binds the antigen, the extent to which the antibodies in the serum compete for binding of the antigen with the monoclonal antibody is measured. In this way, the conjugate need not be specific for the serum species of origin, only the monoclonal antibody species of origin, the mouse. The cELISA enables all domestic and wild avian species to be covered by one test (32, 167). In addition to its broad application, the cELISA can be carried out rapidly, with a minimum of specialized equipment, and, if desired, it can be fully automated.

Disinfection of Poultry Facilities

Although disinfection need not be performed through chemical means, the application of antimicrobial compounds is perhaps the most widespread and economical practice. Such substances are utilized as both a precautionary or preventative measure as well as for the disinfection of facilities and equipment after a disease outbreak. The spread of AIV from the initial site of outbreak has often been attributed to the movement of contaminated equipment (28), and thus thorough disinfection of any potentially contaminated materials is an essential component of any AI control program (124). Among viruses, influenza A is grouped into the category of those most readily inactivated (115). Nevertheless, depending on the particular strain and the environment, it can persist for long periods of time. During the 1983 outbreak of HPAI H5N2 in Pennsylvania, influenza virus could be isolated from poultry house manure more than 30 days after infected birds were removed from the facility (56). In the experimental infection of specific pathogen free (SPF) chickens with LPAI H7N2, Lu et al. (87) reported that virus could be isolated from manure up to two weeks after the birds had stopped shedding virus. An aqueous environment provides even more favorable conditions for AIV persistence. At 17°C and pH 8.2, for a LPAI [A/mottled duck/LA/38M/87 (H6N2)] was shown to remain infectious in fresh water for up to 100 days (133). The isolation of infectious AIV from unconcentrated lake water has also been reported (67).

Although potentially highly persistent in fresh water, AIV is readily inactivated upon the addition of chlorine (117). This makes it possible to quickly and safely treat drinking water which is destined for commercial poultry. Inactivation of AIV in other types of environments poses a different set of challenges. Heat treatment is usually quite effective at eliminating AIV in egg products when temperatures of at least 56°C are used and in thigh and breast meat at a temperature of 70°C (reviewed in 28). Unfortunately, heat inactivation is not feasible for the disinfection of poultry facilities or larger equipment. In this type of situation, chemical disinfectants are preferable.

In order to be classified as effective at eliminating a particular infectious agent, the United States Environmental Protection Agency (EPA) requires that a virucidal compound be able to completely inactivate the virus while at least four logs of virus particles per milliliter are recovered from untreated controls (145). There are many classes of chemical disinfectants, each with its own benefits and drawbacks. Soaps and detergents are particularly effective at washing away organic matter. They also are capable of disrupting the lipid envelopes of influenza virions (6). However, it is recommended that they are used with other disinfectants (28), as they may not be able to completely inactivate AIV on their own (4).

AIV is sensitive to acid treatment due to the conformational change in HA which is induced at low pH (96). As a result, many acidic compounds can inactivate AIV. Among those described are acetic acid (1-5%), citric acid (1-3%), and hydrochloric acid (2-5%). All are capable of inactivating the virus on hard, non-porous surfaces, although hydrochloric acid is highly corrosive to metals (4, 86).

Alkaline compounds which have shown the ability to inactivate AIV include sodium hydroxide (2-5%), calcium hydroxide (1-3%), and sodium carbonate (10%). These are most effective above room temperature. Sodium hydroxide is a corrisive substance, particulary on aluminum (4).

Chlorine compounds, including calcium hypochlorite (2-3%) and sodium hypochlorite (household bleach, 2-3%), are also effective on hard, non-porous surfaces, although their efficacy is greatly reduced in the presence of organic matter and at alkaline pH (86). Other substances which have been shown to inactivate AIV are aldehydes, such as formalin (8%) and glutaraldehyde (1-2%) (148); oxidizing agents, including the product Virkon® and hydrogen peroxide; phenol compounds; quaternary ammonium compounds (138, 148); and alcohols, such as ethanol (70%) (87). Despite this wide array of virucidal compounds, few of them are able to effectively penetrate organic material, such as poultry litter, in order to inactivate AIV which is present below the surface (28).

References

1. Air, G. M. and W. G. Laver. The neuraminidase of influenza-virus. Proteins – Structure, Function, and Genetics. 6:341-356. 1989.

2. Akarsu, H., W. P. Burmeister, C. Petosa, I. Petit, C. W. Müller, R. W. H. Ruigrok, and F. Baudin. Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2). The EMBO Journal. 22:4646-4655. 2003.

3. Alexander, D. J. An overview of the epidemiology of avian influenza. Vaccine. 25:5637-5644. 2007.

4. Alphin, R. L., K. J. Johnson, B. S. Ladman, and E. R. Benson. Inactivation of avian influenza virus using four common chemicals and one detergent. Poultry Science. 88:1181-1185. 2009.

5. Arias, C. F., M. Escalera-Zamudio, M. de los Dolores Soto-Del Río, A. G. Cobián-Güemes, P. Isa, and S. López. Molecular anatomy of 2009 influenza virus A (H1N1). Archives of Medical Research. 40:643-654. 2009.

6. Ausvetplan, 2005: Australian Veterinary Emergency Manual Plan Avian Influenza - Updated Interim Draft (1,891), 3rd ed., Version 3.1, 2005. (http://www.animalhealthaustralia.com.au/aahc)

7. Aytay, S. and I. T. Schulze. Single amino acid substitutions in the hemagglutinin can alter the host range and receptor binding properties of H1 strains of influenza virus. Journal of Virology. 65:3022-3028. 1991.

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

9. Beard, C. W. Avian influenza antibody detection by immunodiffusion. Avian Diseases. 14:337-341. 1970.

10. Beare, A. S. and R. G. Webster. Replication of avian influenza viruses in humans. Archives of Virology. 119:37-42. 1991.

11. Biswas, S. K. and D. P. Nayak. Mutational analysis of the conserved motifs of influenza A virus polymerase basic protein 1. Journal of Virology. 68:1819-1826. 1994.

12. Biswas, S. K., P. L. Boutz, and D. P. Nayak. Influenza virus nucleoprotein interacts with influenza virus polymerase proteins. Journal of Virology. 72:5493-5501. 1998.
13. Blaas, D., E. Patzelt, and E. Kuechler. Identification of the cap binding protein of influenza virus. Nucleic Acids Research. 10:4803-4812. 1982.

14. Bui, M., G. Whittaker, and A. Helenius. Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. Journal of Virology. 70:8391-8401. 1996.

15. Bullido, R., P. Gómez-Puertas, M. J. Saiz, and A. Portela. Influenza A virus NEP (NS2 protein) downregulates RNA synthesis of model template RNAs. Journal of Virology. 75:4912-4917. 2001.

16. Centers for Disease Control and Prevention (CDC). *Influenza: The Disease*. CDC, 2009. Available at http://www.cdc.gov/flu/about/disease/index.htm. [Access date: March 31, 2010]

17. Chen, B. J., G. P. Leser, D. Jackson, and R. A. Lamb. The influenza virus M2 protein cytoplasmic tail interacts with the M1 protein and influences virus assembly at the site of virus budding. Journal of Virology. 82:10059-10070. 2008.

18. Chen, H., R. A. Bright, K. Subbarao, C. Smith, N. J. Cox, J. M. Katz, and Y. Matsuoka. Polygenic virulence factors involved in pathogenesis of 1997 Hong Kong H5N1 influenza viruses in mice. Virus Research. 128:159-163. 2007.

19. Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J. R. Bennink, and J. W. Yewdell. A novel influenza A virus mitochondrial protein that induces cell death. Nature Medicine. 7:1306-1312. 2001.

20. Cheung, T. K. W. and L. L. M. Poon. Biology of influenza A virus. Annals of the New York Academy of Sciences. 1102:1-25. 2007.

21. Chin, P. S., E. Hoffmann, R. Webby, R. G. Webster, Y. Guan, M. Peiris, and K. F. Shortridge. Molecular evolution of H6 influenza viruses from poultry in Southeastern China: prevalence of H6N1 influenza viruses possessing seven A/HongKong/156/97 (H5N1)-like genes in poultry. Journal of Virology. 76:507-516. 2002.

22. Chizhmakov, I. V., F. M. Geraghty, D. C. Ogden, A. Hayhurst, M. Antoniou, and A. J. Hay. Selective proton permeability and pH regulatino of the influenza virus M2 channel expressed in mouse erythroleukemia cells. Journal of Physiology. 494:329-336. 1996.

23. Ciampor, F., C. A. Thompson, S. Grambas, and A. J. Hay. Regulation of pH by the M2 protein of influenza A viruses. Virus Research. 22:247-258. 1992.

24. Claas, E. C. J., A. D. M. E. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet. 351:472-477. 1998.

25. Coloma, R., J. M. Valpuesta, R. Arranz, J. L. Carrascosa, J. Ortín, and J. Martín-Benito. The structure of a biologically active influenza virus ribonucleoprotein complex. PLoS Pathogens. 5:1-10. 2009.

26. Connor, R. J., Y. Kawaoka, R. G. Webster, and J. C. Paulson. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology. 205:17-23. 1994.

27. Davey, J., N. J. Dimmock, and A. Colman. Identification of the sequence responsible for the nuclear accumulation of the influenza virus nucleoprotein in *Xenopus* oocytes. Cell. 40:667-675. 1985.

28. De Benedictis, P., M. S. Beato, and I. Capua. Inactivation of avian influenza viruses by chemical agents and physical conditions: a review. Zoonoses and Public Health. 54:51-68. 2007.

29. Deng, T., J. Sharps, E. Fodor, and G. G. Brownlee. In vitro assembly of PB2 with a PB1-PA dimer supports a new model of assembly of influenza A virus polymerase subunits into a functional trimeric complex. Journal of Virology. 79:8669-8674. 2005.

30. Dias, A., D. Bouvier, T. Crépin, A. A. McCarthy, D. J. Hart, F. Baudin, S. Cusack, and R. W. H. Ruigrok. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature. 458:914-918. 2009.

31. Digard, P. V. C. Blok, and S. C. Inglis. Complex formation between influenza virus polymerase proteins expressed in *Xenopus* oocytes. Virology. 171:162-169. 1989.

32. Dlugolenski, D., R. Hauck, R. J. Hogan, F. Michel, and E. Mundt. Production of H5-specific monoclonal antibodies and the development of a competitive enzyme-linked immunosorbent assay for detection of H5 antibodies in multiple species. Avian Diseases. 53:644-649. 2009.

33. Doms, R. W., A. Helenius, and J. White. Membrane fusion activity of the influenza virus hemagglutinin- the low pH-inducced conformational change. The Journal of Biological Chemistry. 260:2973-2981. 1985.

34. Duhaut, S. D. and J. W. McCauley. Defective RNAs inhibit the assembly of the influenza virus genome segments in a segment-specific manner. Virology. 216:326-337. 1996.

35. Duhaut, S. and N. J. Dimmock. Approximately 150 nucleotides from the 5' end of an influenza A segment 1 defective virion RNA are needed for genome stability during passage of defective virus in infected cells. Virology. 275:278-285. 2000.

36. Elton, D., M. Simpson-Holley, K. Archer, L. Medcalf, R. Hallam, J. McCauley, and P. Digard. Interaction of the influenza virus nucleoprotein with the cellular CRM1-mediated nuclear export pathway. Journal of Virology. 75:408-419. 2001.

37. Enami, K., T. A. Sato, S. Nakada, and M. Enami. Influenza virus NS1 protein stimulates translation of the M1 protein. Journal of Virology. 68:1432-1437. 1994.

38. Environmental Protection Agency (EPA). 2010. *Methyldithiocarbamate salts (metam sodium/potassium).* Available at: http://www.epa.gov/oppsrrd1/reregistration/metamsodium/. [Access date: May 18 2010]

39. Fechter, P., L. Mingay, J. Sharps, A. Chambers, E. Fodor, and G. G. Brownlee. Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding. Journal of Biological Chemistry. 278:20381-20388. 2003.

40. Fodor, E., M. Crow, L. J. Mingay, T. Deng, J. Sharps, P. Fechter, and G. G. Brownlee. A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. Journal of Virology. 76:8989-9001. 2002.

41. Fodor, E. and M. Smith. The PA subunit is required for efficient nuclear accumulation of the PB1 subunit of the influenza A virus RNA polymerase complex. Journal of Virology. 78:9144-9153. 2004.

42. Fouchier, R. A. M., A. D. M. E. Osterhaus, and I. H. Brown. Animal influenza virus surveillance. Vaccine. 21:1754-1757. 2003.

43. Fouchier, R. A. M., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. M. E. Osterhaus. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. Journal of Virology. 79:2814-2822. 2005.

44. Fujii, K., Y. Fujii, T. Noda, Y. Muramoto, T. Watanabe, A. Takada, H. Goto, T. Horimoto, and Y. Kawaoka. Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. Journal of Virology. 79:3766-3774. 2005.

45. Fujii, K., C. Kakumoto, M. Kobayashi, S. Saito, T. Kariya, Y. Watanabe, Y. Sakoda, H. Kida, and M. Suzuki. Serological evidence of influenza A virus infection in Kuril Harbor Seals (Phoca vitulina stejnegeri) of Hokkaido, Japan. Journal of Veterinary Medical Science. 69:259-263. 2007.

46. Fujii, Y., H. Goto, T. Watanabe, T. Yoshida, and Y. Kawaoka. Selective incorporation of influenza virus RNA segments into virions. Proceedings of the National Academy of Sciences. 100:2002-2007. 2003.

47. García-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology. 252:324-330. 1998.

48. Gastaminza, P., B. Perales, A. M. Falcón, and J. Ortín. Mutations in the N-terminal region of influenza virus PB2 protein affect virus RNA replication but not transcription. Journal of Virology. 77:5098-5108. 2003.

49. Gibbs, J. S., D. Malide, F. Hornung, J. R. Bennink, and J. W. Yewdell. The influenza A virus PB2-F2 protein targets the inner mitochondrial membrane via a predicted basic amphipathic helix that disrupts mitochondrial function. Journal of Virology. 77:7214-7224. 2003.

50. Gómez-Puertas, P., C. Albo, E. Pérez-Pastrana, A. Vivo, and A. Portela. Influenza virus matrix protein is the major driving force in virus budding. Journal of Virology. 74:11538-11547. 2000.

51. González, S., T. Zürcher, and J. Ortín. Identification of two separate domains in the influenza virus PB1 protein involved in the interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure. Nucleic Acids Research. 24:4456-4463. 1996.

52. Hankins, R. W., K. Nagata, A. Kato, and A. Ishihama. Mechanism of influenza virus transcription inhibition by matrix (M1) protein. Research in Virology. 141:305-314. 1990.

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

54. Hara, K., F. I. Schmidt, M. Crow, and G. G. Brownlee. Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. Journal of Virology. 80:7789-7798. 2006.

55. Hemerka, J. N., D. Wang, Y. Weng, W. Lu, R. S. Kaushik, J. Jin, A. F. Harmon, and F. Li. Detection and characterization of influenza A virus PA-PB2 interaction through bimolecular fluorescence complementation assay. Journal of Virology. 83:3944-3955. 2009.

56. Henzler, D. J., D. C. Kradel, S. Davison, A. F. Ziegler, D. Singletary, P. DeBok, A. E. Castro, H. Lu, R. Eckroade, D. Swayne, W. Lagoda, B. Schmucker, and A. Nesselrodt. Epidemiology, production losses, and control measures associated with an outbreak of avian influenza subtype H7N2 in Pennsylvania (1996-98). Avian Diseases. 47:1022-1036. 2003.

57. Hoffmann, E., J. Stech, I. Leneva, S. Krauss, C. Scholtissek, P. S. Chin, M. Peiris, K. F. Shortridge, and R. G. Webster. Characterization of the influenza A virus gene pool in avian species in Southern China: was H6N1 a derivative or a precursor of H5N1? Journal of Virology. 74:6309-6315. 2000.

58. Holsinger, L. J. and R. A. Lamb. Influenza virus-M2 integral membrane-protein is a homotetramer stabilized by formation of disulfide bonds. Virology. 183:32-43. 1991.

59. Honda, A., K. Mizumoto, and A. Ishihama. Two separate sequences of PB2 subunit constitute the RNA cap-binding site of influenza virus RNA polymerase. Genes to Cells. 4:475-485. 1999.

60. Honda, A., K. Mizumoto, and A. Ishihama. Minimum molecular architectures for transcription and replication of the influenza virus. Proceedings of the National Academy of Sciences. 99:13166-13171. 2002.

61. Horimoto, T. and Y. Kawaoka. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. Journal of Virology. 68:3120-3128. 1994.

62. Huang, T. S., P. Palese, and M. Krystal. Determination of influenza virus proteins required for genome replication. Journal of Virology. 64:5669-5673. 1990.

63. Huet, S., S. V. Avilov, L. Ferbitz, N. Daigle, S. Cusack, and J. Ellenberg. Nuclear import and assembly of influenza A virus RNA polymerase studies in live cells by fluorescence cross-correlation spectroscopy. Journal of Virology. 84:1254-1264. 2010.

64. Hughes, M. T., M. Matrosovich, M. E. Rodgers, M. McGregor, and Y. Kawaoka. Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice. Journal of Virology. 74:5206-5212. 2000.

65. Hutchinson, E. C., J. C. von Kirchbach, J. R. Gog, and P. Digard. Genome packaging in influenza A virus. Journal of General Virology. 91:313-328. 2010.

66. IDEXX. FlockChek AI MultiS-Screen Ab Test Kit Information Sheet. 2010. Available at: http://www.idexx.com/view/xhtml/en_us/livestock-poultry/poultry/avian-influenza.jsf?conversationId=128053. [Access date: June 3, 2010]

67. Ito, T., K. Okazaki, Y. Kawaoka, A. Takada, R. G. Webster, and H. Kida. Perpetuation of influenza-A viruses in Alaskan waterfowl reservoirs. Archives of Virology. 140:1163-1172. 1995.

68. Iwatsuki-Horimoto, K., T. Horimoto, T. Noda, M. Kiso, J. Maeda, S. Watanabe, Y. Muramoto, K. Fujii, and Y. Kawaoka. The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. Journal of Virology. 80:5233-5240. 2006.

69. Jahangir, A., Y. Watanabe, O. Chinen, S. Yamazaki, K. Sakai, M. Okamura, M. Nakamura, and K. Takehara. Surveillance of avian influenza viruses in Northern Pintails (*Anas acuta*) in Tohoku District, Japan. Avian Diseases. 52:49-53. 2008.

70. Jennings, P. A., J. T. Finch, G. Winter, and J. S. Robertson. Does the higher order structure of the influenza virus ribonucleoprotein guide sequence rearrangements in influenza viral RNA? Cell. 34:619-627. 1983.

71. Johnson, N. P. A. S. and J. Mueller. Updating the accounts: global mortality of the 1918-1920 “Spanish” influenza pandemic. Bulletin of the History of Medicine. 76:105-115. 2002.

72. Jones, I. M., P. A. Reay, and K. L. Philpott. Nuclear location of all three influenza polymerase proteins and a nuclear signal in polymerase PB2. The EMBO Journal. 5:2371-2376. 1986.

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

74. Kawaoka, Y., S. Krauss, and R. G. Webster. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. Journal of Virology. 63:4603-4608. 1989.

75. Kingsbury, D. W. and R. G. Webster. Some properties of influenza virus nucleocapsids. Journal of Virology. 4:219-225. 1969.

76. Kistner, O., K. Müller, and C. Scholtissek. Differential phosphorylation of the nucleoprotein of influenza A viruses. Journal of General Virology. 70:2421-2431. 1989.

77. Lakadamyali, M., M. J. Rust, H. P. Babcock, and X. Zhuang. Visualizing infection of individual influenza viruses. PNAS. 100:9280-9285. 2003.

78. Lamb, R. A. and P. W. Choppin. Segment 8 of the influenza virus genome is unique in coding for two polypeptides. Proceedings of the National Academy of Sciences. 76:4908-4912. 1979.

79. Lamb, R. A., C.-J. Lai, and P. W. Choppin. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. Proceedings of the National Academy of Sciences. 78:4170-4174. 1981.

80. Lamb, R. A. and P. W. Choppin. The gene structure and replication of influenza virus. Annual Reviews in Biochemistry. 52:467-506. 1983.

81. Lamb, R. A., S. L. Zebedee, and C. D. Richardson. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell. 40:627-633. 1985.

82. Lamb, R. A. and R. M. Krug. Orthomyxoviridae: the viruses and their replication. In: Fields Virology 4th ed. D. M. Knipe and P. M. Howley, eds. Lippincott, Williams, and Wilkins, Philadelphia. pp 1487-1531. 2001.

83. Lee, M. S., P. C. Chang, J. H. Shien, M. C. Cheng, C. L. Chen, H. K. Shieh. Genetic and pathogenic characterization of H6N1 avian influenza viruses isolated in Taiwan between 1972 and 2005. Avian Diseases. 50:561-571. 2006.

84. Li, Z., Y. Jiang, P. Jiao, A. Wang, F. Zhao, G. Tian, X. Wang, K. Yu, Z. Bu, and H. Chen. The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. Journal of Virology. 80:11115-11123. 2006.

85. Lombardi, M. E., B. S. Ladman, R. L. Alphin, and E. R. Benson. Inactivation of avian influenza virus using common detergents and chemicals. Avian Diseases. 52:118-123. 2008.

86. Lu, H., A. E. Castro, K. Pennick, J. Liu, Q. Yang, P. Dunn, D. Weinstock, and D. Henzler. Survival of avian influenza virus H7N2 in SPF chickens and their environments. Avian Diseases. 47:1015-1021. 2003.

87. Lu, Y., X.-Y. Qian, and R. M. Krug. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. Genes and Development. 8:1817-1828. 1994.

88. Maeda, T., K. Kawasaki, and S.-I. Ohnishi. Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2. Proceedings of the National Academy of Sciences. 78:4133-4137. 1981.

89. Marión, R. M., T. Zürcher, S. de la Luna, and J. Ortín. Influenza virus NS1 protein interacts with viral transcription-replication complexes *in vivo*. Journal of General Virology. 78:2447-2451. 1997.

90. Martin, K. and A. Helenius. Transport of incoming influenza virus nucleocapsids into the nucleus. Journal of Virology. 65:232-234. 1991.

91. Martin, K. and A. Helenius. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell. 67:117-130. 1991.

92. Matlin, K. S., H. Reggio, A. Helenius, and K. Simons. Infectious entry pathway of influenza virus in a canine kidney cell line. The Journal of Cell Biology. 91:601-613. 1981.

93. Medcalf, L., E. Poole, D. Elton, and P. Digard. Temperature-sensitive lesions in two influenza A viruses defective for replicative transcription disrupt RNA binding by the nucleoprotein. Journal of Virology. 73:7349-7356. 1999.

94. Mitnaul, L. J., M. R. Castrucci, K. G. Murti, and Y. Kawaoka. The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. Journal of Virology. 70:873-879. 1996.

95. Mittal, A., T. Shangguan, and J. Bentz. Measuring p*K*a of activation and p*K*i of inactivation for hemagglutinin from kinetics of membrane fusion of virions and of HA expressing cells. Biophysics Journal. 83:2652-2666. 2002.

96. Monto, A. S., J. L. McKimm-Breschkin, C. Macken, A. W. Hampson, A. Hay, A. Klimov, M. Tashiro, R. G. Webster, M. Aymard, R. G. Hayden, and M. Zambon. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrobial Agents and Chemotherapy. 50:2395-2402. 2006.

97. Nakagawa, Y., K. Oda, and S. Nakada. The PB2 subunit alone can catalyze cRNA synthesis, and the PA subunit in addition to the PB1 subunit is required for viral RNA synthesis in replication of the influenza virus genome. Journal of Virology. 70:6390-6394. 1996.

98. Nakajima, K. and A. Sugiura. Three-factor cross of influenza virus. Virology. 81:486-489. 1977.

99. Nath, S. T. and D. P. Nayak. Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1N1). Molecular and Cellular Biology. 10:4139-4145. 1990.

100. Nelson, J., S. S. Couceiro, J. C. Paulson, and L. G. Baum. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in hemagglutinin receptor specificity. Virus Research. 29:155-165. 1993.

101. Neumann, G., M. R. Castrucci and Y. Kawaoka. Nuclear import and export of influenza virus nucleoprotein. Journal of Virology. 71:9690-9700. 1997.

102. Nieto, A., S. de la Luna, J. Bárcena, A. Portela, J. Valcárcel, J. Melero, and J. Ortín. Nuclear transport of influenza virus polymerase PA protein. Virus Research. 24:65-75. 1992.

103. Noton, S. L., E. Medcalf, D. Fisher, A. E. Mullin, D. Elton, and P. Digard. Identification of the domains of the influenza A virus M1 matrix protein required for NP binding, oligomerization and incorporation into virions. Journal of General Virology. 88:2280-2290. 2007.

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

105. O'Neill, R. E., R. Jaskunas, G. Blobel, P. Palese, and J. Moroianu. 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. 1995.

106. O'Neill, R. E., J. Talon, and P. Palese. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. The EMBO Journal. 17:288-296. 1998.

107. Ozawa, M., J. Maeda, K. Iwatsuki-Horimoto, S. Watanabe, H. Goto, T. Horimoto, and Y. Kawaoka. Nucleotide sequence requirements at the 5' end of the influenza A virus M RNA segment for efficient virus replication. Journal of Virology. 83:3384-3388. 2009.

108. Palese, P., K. Tobita, and M. Ueda. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology. 61:397-410. 1974.

109. Perales, B. and J. Ortín. The influenza A virus PB2 polymerase subunit is required for the replication of viral RNA. Journal of Virology. 71:1381-1385. 1997.

110. Pérez, D. R. and R. O. Donis. A 48-amino-acid region of influenza A virus PB1 protein is sufficient for complex formation with PA. Journal of Virology. 69:6932-6939. 1995.

111. Pinto, L. H., L. J. Holsinger, and R. A. Lamb. Influenza virus M2 protein has ion channel activity. Cell. 69:517-528. 1992.

112. Plotch, S. J., M. Bouloy, I. Ulmanen, and R. Krug. A unique cap(m7Gpppxm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral-RNA transcription. Cell. 23:847-858. 1981.

113. Poole, E., D. Elton, L. Medcalf, and P. Digard. Functional domains of the influenza A virus PB2 protein: identification of NP- and PB1-binding sites. Virology. 321:120-133. 2004.

114. Prince, H. N. and D. L. Prince. Principals of viral control and transmission. In: Disinfection, sterilization, and preservation, 5th ed. S. S. Block, ed. Williams and Wilkins, Philadelphia, PA. pp. 543-571. 2001.

115. Qiu, Y. and R. M. Krug. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). Journal of Virology. 68:2425-2432. 1994.

116. Rice, E. W., N. J. Adcock, M. Sivaganesan, J. D. Brown, D. E. Stallknecht, and D. E. Swayne. Chlorine inactivation of highly pathogenic avian influenza virus (H5N1). Emerging Infectious Diseases. 13:1568-1570. 2007.

117. Rogers, G. N. and J. C. Paulson. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology. 127:361-373. 1983.

118. Rogers, G. N., J. C. Paulson, R. S. Daniels, J. J. Skehel, I. A. Wilson, and D. C. Wiley. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature. 304:76-78. 1983.

119. Rust, M. J., M. Lakadamyali, F. Zhang, and X. Zhuang. Assembly of endocytic machinery around individual influenza viruses during viral entry. Nature Structural and Molecular Biology. 11:567-573. 2004.

120. Scholtissek, C. and H. Becht. Binding of ribonucleic acids to the RNP-antigen protein of influenza viruses. Journal of General Virology. 10:11-16. 1971.

121. Seo, S. H., E. Hoffmann, and R. G. Webster. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nature Medicine. 8:950-954. 2002.

122. Shafer, A. L., J. B. Katz, and K. A. Eernisse. Development and validation of a competitive enzyme-linked immunosorbent assay for detection of type A influenza antibodies in avian sera. Avian Diseases. 42:28-34. 1998.

123. Shahid, M. A., M. Abubakar, S. Hameed, and S. Hassan. Avian influenza virus (H5N1); effects of physico-chemical factors on its survival. Virology Journal. 6:38. 2009.

124. Shapiro, G. I., and R. M. Krug. Influenza virus RNA replication in vitro: sythesis of viral template RNAs and virion RNAs in the absence of an added primer. Journal of Virology. 62:2285-2290. 1988.

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

126. Sharp, G. B., Y. Kawaoka, D. J. Jones, W. J. Bean, S. P. Pryor, V. Hinshaw, and R. G. Webster. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. Journal of Virology. 71:6128-6135. 1997.

127. Shi, L., J. M. Galarza, and D. F. Summers. Recombinant-baculovirus-expressed PB2 subunit of the influenza A virus RNA polymerase binds cap groups as an isolated subunit. Virus Research. 42:1-9. 1996.

128. Shortridge, K. F. Avian influenza-A viruses of Southern China and Hong-Kong – ecological aspects and implications for man. Bulletin of the World Health Organization. 60:129-135. 1982.

129. Sieczkarski, S. and G. R. Whittaker. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. Journal of Virology. 76:10455-10464. 2002.

130. Skehel, J. J. and D. C. Wiley. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual Review of Biochemistry. 69:531-569. 2000.

131. 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. 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. 2002.

132. Stallknecht, D. E., M. T. Kearney, S. M. Shane, and P. J. Zwank. Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. Avian Diseases. 34:412-418. 1990.

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

134. Stegmann, T., J. M. White, and A. Helenius. Intermediates in influenza induced membrane fusion. The EMBO Journal. 9:4231-4241. 1990.

135. Stegmann, T., J. M. Delfino, F. M. Richards, and A. Helenius. The HA2 subunit of influenza hemagglutinin inserts into the target membrane prior to fusion. The Journal of Biological Chemistry. 266:18404-18410. 1991.

136. Steineke-Gröber, 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.

137. Suarez, D. L., E. Spackman, D. A. Senne, L. Bulaga, A. C. Welsch, and K. Froberg. The effect of various disinfectants on detection of avian influenza virus be real time RT-PCR. Avian Diseases. 47:1091-1095. 2003.

138. Sugiyama, K., E. Obayashi, A. Kawaguchi, Y. Suzuki, J. R. H. Tame, K. Nagata, and S.-Y. Park. Structural insight into the essential PB1-PB2 subunit contact of the influenza virus RNA polymerase. The EMBO Journal. 28:1803-1811. 2009.

139. Sugrue, R. J., G. Bahadur, M. C. Zambon, M. Hall-Smith, A. R. Douglas, and A. J. Hay. Specific structural alteration of the influenza hemagglutinin by amantadine. The EMBO Journal. 9:3469-3476. 1990.

140. Swayne, D. E., D. A. Senne, and C. W. Beard. Avian Influenza. In: Isolation and identification of avian pathogens, 4th ed. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed eds. Kennett Square, PA. pp. 150-155. 1998.

141. Taubenberger, J. K., A. H. Reid, R. M. Lourens, R. Wang, G. Jin, and T. G. Fanning. Characterization of the 1918 influenza virus polymerase genes. Nature. 437:889-893. 2005.

142. Toyoda, T., D. M. Adyshev, M. Kobayashi, A. Iwata, and A. Ishihama. Molecular assembly of the influenza virus RNA polymerase: determination of the subunit-subunit contact sites. Journal of General Virology. 77:2149-2157. 1996.

143. Ulmanen, I., B. A. Broni, and R. M. Krug. Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m^7GpppNm) on RNAs and in initiating viral RNA transcription. Proceedings of the National Academy of Sciences. 78:7355-7359. 1981.

144. US EPA. Efficacy data requirements: virucides. Registration Division, Office of Pesticide Programs. DIS/TSS-7. US EPA/National Service Center for Environmental Publications (NSCEP), Cincinnati, OH. 1981.

145. Varghese, J. N., W. G. Laver, and P. M. Colman. Structure of the influenza-virus glycoprotein antigen neuraminidase at 2.9-A-resolution. Nature. 303:35-40. 1983.

146. Wagner, R., M. Matrosovich, and H.-D. Klenk. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Reviews in Medical Virology. 12:159-166. 2002.

147. Wanaratana, S., R. Tantilertcharoen, J. Sasipreeyajan, and S. Pakpinyo. The inactivation of avian influenza virus suptype H5N1 isolated from chickens in Thailand by chemical and physical treatments. Veterinary Microbiology. 140:43-48. 2010.

148. Warke, A., L. Appleby, and E. Mundt. Prevalence of antibodies to different avian paramyxoviruses in commercial poultry in the United States. Avian Diseases. 52:694-697. 2008.

149. Watanabe, K., N. Takizawa, M. Katoh, K. Hoshida, N. Kobayashi, and K. Nagata. Inhibition of nuclear export of ribonucleoprotein complexes of influenza virus by leptomycin B. Virus Research. 77:31-42. 2001.

150. Webby, R. J., P. R. Woolcock, S. L. Krauss, D. B. Walker, P. S. Chin, K. F. Shortridge, and R. G. Webster. Multiple genotypes of nonpathogenic H6N2 influenza viruses isolated from chickens in California. Avian Diseases. 47:905-910. 2003.

151. Webster, R. G. and W. J. Bean. Genetics of influenza virus. Annual Review of Genetics. 12:415-431. 1978.

152. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. Evolution and ecology of influenza A viruses. Microbiological Reviews. 56:152-179. 1992.

153. White, J., K. Matlin, and A. Helenius. Cell fusion by Semliki forest, influenza, and vesicular stomatitis virus. The Journal of Cell Biology. 89:674-679. 1981.

154. White, J., J. Kartenbeck, and A. Helenius. Membrane fusion activity of influenza virus. The EMBO Journal. 1:217-222. 1982.

155. Whittaker, G., M. Bui, and A. Helenius. Nuclear trafficking of influenza virus ribonucleoproteins in heterokaryons. Journal of Virology. 70:2743-2756. 1996.

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

157. World Health Organization (WHO). *DG Statement Following the Meeting of the Emergency Committee*. Geneva: WHOI. 2009. Available at http://www.who.int/csr/disease/swineflu/4th_meeting_ihr/en/index.html. [Access date: March 31, 2010]

158. World Health Organization (WHO). *Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO*. 2010. Available at http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_05_06/en/index.html. [Access date: May 18, 2010]

159. Wu, R., S. Hu, Y. Xiao, Z. Li, D. Shi, and D. Bi. Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus. Veterinary Research Communications. 31:631-641. 2007.

160. Yasuda, J., S. Nakada, A. Kato, T. Toyoda, and A. Ishihama. Molecular assembly of influenza virus: association of the NS2 protein with virion matrix. Virology. 196:249-255. 1993.

161. Ye, Z., D. Robinson, and R. A. Wagner. Nucleus-targeting domain of the matrix protein (M1) of influenza virus. Journal of Virology. 69:1964-1970. 1995.

162. Ye, Z. T., Liu, D. P. Offringa, J. McInnis, and R. A. Levandowski. Association of influenza virus matrix protein with ribonucleoproteins. Journal of Virology. 73:7467-7473. 1999.

163. Yoshimura, A. and S.-I. Ohnishi. Uncoating of influenza virus in endosomes. Journal of Virology. 51:497-504. 1984.

164. Yuan, P., M. Bartlam, Z. Lou, S. Chen, J. Zhou, X. He, Z. Lv, R. Ge, X. Li, T. Deng, E. Fodor, Z. Rao, and Y. Liu. Crystal structure of an avian influenza polymerase PA_N reveals an endonuclease active site. Nature. 458:909-913. 2009.

165. Zhang, J. and R. A. Lamb. Characterization of the membrane association of the influenza virus matrix protein in living cells. Virology. 225:255-266. 1996.

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

CHAPTER 3

TESTING OF A NEW DISINFECTANT PROCESS FOR POULTRY VIRUSES[1]

---

[1] Appleby, L. and E. Mundt. Testing of a new disinfectant process for poultry viruses. Avian Diseases. 54 :763-767. 2010. Reprinted here with permission of publisher.

Abstract

The chemical compound metam-sodium was tested at three concentrations for the ability to inactivate the infectivity of low pathogenic avian influenza virus (LPAIV) and infectious bursal disease virus (IBDV) using virus contaminated chicken litter as the substrate. LPAIV was inactivated within one hour after the addition of metam-sodium independent of the concentration which was used. IBDV was not inactivated with the lowest amount of metam-sodium, but at higher concentrations the virus was inactivated within one hour after application. The results show that metam-sodium is able to penetrate chicken litter and inactivate enveloped as well as non-enveloped viruses due to its ability to form the active compound methyl isothiocyanate which acts as a fumigant.

Introduction and Literature Review

Outbreaks of infectious diseases usually start with an index case to which most subsequent outbreaks can be traced. Thus the control of the index case which includes a fast diagnostic and an immediate response is of importance. This may result in the depopulation of a particular animal setting. After the animals are removed facilities, equipment, vehicles and litter need to be treated with the aim of inactivating the particular infectious agent. This must be an essential part of control program for any animal disease. Disinfection can be achieved by several methods, such as physical methods, heat treatment, and inactivation with chemical compounds. The method of disinfection after an outbreak is a very critical tool for the control of the disease. The objective of disinfection measures is to make it such the disease can not spread to other facilities and thus controlling the outbreak. Since secondary spread occurs mainly due to human-related behavior and is connected with movement of people and equipment (3, 9) the control measures at the index case, if known, and also at places of the subsequent occurrence of the disease is of utmost importance.

Avian influenza virus (AIV) is a member of the family *Orthomyxoviridae* and belongs to the large group of enveloped viruses. The virus contains eight segments of a single-stranded RNA genome of negative polarity which encodes for 11 different proteins. Within AIV two subgroups have been described for gallinaceous birds, low pathogenicity (LP) and high pathogenicity (HP) AIV. LPAIV cause mainly a mild respiratory disease which is sometime accompanied with low mortality rates, and a drop in egg production in chickens and turkeys. This is not associated with specific HA or NA subtypes. In contrast, HPAIV can cause 100% mortality in susceptible gallinaceous birds and this has been associated in the past exclusively with AIV encoding either H5 or H7. Since every H5 and H7 encoding AIV has the capacity to obtain this phenotype these viruses belonging now to the category of notifiable avian influenza (NAI) regardless of whether they are LPAIV or HPAIV (OIE, 2008). Usually once an AIV of subtype H5 or H7 has been detected the premise will be subjected to control measures which can include depopulation. This can be performed by gassing (8, 11, 18) or foam (1). After depopulation the virus needs to be inactivated. One method which has been successfully used is composting of infected birds (23, 25, 29). The disinfection of surfaces also plays a role in controlling the spread of disease to other facilities. Mostly liquid disinfectants have been used for this purpose. Lombardi et al. (13) used several chemical compounds and compound mixtures (acetic acid, citric acid, calcium hypochlorite, sodium hypochlorite, laundry detergent with peroxygen, commercial iodine/acid disinfectant) to disinfect LPAIV. In another study HPAIV was inactivated by treatment with chlorine (20). HPAIV in chicken meat has been successfully inactivated upon exposure to temperatures in excess of 70°C (27).

Infectious bursal disease virus (IBDV) is a non-enveloped virus and belongs to the family *Birnaviridae*. It is endemic in poultry worldwide and can be controlled by vaccination. The viral genome consists of two segments of double-stranded RNA which encode**s** five viral proteins. IBDV is difficult to inactivate. Benton et al. (2) found that treatment with 0.5% phenol (5 h incubation) and 0.0125% merthiolate (1 h incubation) had no effect on the virus infectivity, but an incubation for 6h with 0.5 % formalin resulted in a marked reduction of the infectivity of the virus. Other compounds (staphene, hyamine) showed no effect under the conditions in which they were used (2). Incubation at a pH of 12 for 1 h at 30°C inactivated the virus whereas under the same conditions at pH 2 no inactivation was observed (2). Furthermore, a heat treatment at 56°C for 5 h did not significantly affect the infectivity of IBDV (2). Shirai et al (23) used invert soap in combination with 0.05% sodium hydroxide and found a strong inactivating effect on the infectivity of the virus. The effect of heat treatment was investigated by Mandeville et al. (15). The greatest reduction in virus titer was observed by incubation for 1 min at temperatures above 65°C. The highest level of inactivation, which was still incomplete, was observed at 100°C for 1 min. Landgraf et al. (12) showed that IBDV was inactivated after a 30 min exposure at 70°C. These observations demonstrate that the only way to control IBDV is vaccination in combination with measures to reduce the virus load in the chicken house.

The disinfection with heat and disinfectants either alone or in combination is widely used where infectious material needs to be inactivated. In this study we evaluated the compound metam-sodium for its ability to inactivate viral infectivity using IBDV and LPAIV as models for non-enveloped and enveloped viruses, respectively. Inactivation of viral infectivity on porous surfaces (here, chicken litter) might become a problem likely due to the lack of accessibility of the compound to the virus. To overcome this problem experiments were performed using metam-sodium, which degrades to a gas in presence of moisture (see below), and thus probably has a higher rate of penetration on porous surfaces. The compound metam-sodium was first registered in the United States in 1975. Metam-sodium is one of the most widely used agricultural pesticides in the United States and is presently registered for use on a wide variety of food and feed crops. During 1999, over 7.7 million kg of metam-sodium were used in the production of agricultural crops in California (28). Metam-sodium is also registered for a variety of antimicrobial and industrial uses and is under consideration as a replacement for methyl bromide which is on the list of substances that deplete the stratospheric ozone layer. The use of methyl bromide as fumigant in the crop industry for control of soil diseases will phase out by 2015 in developing countries, except for quarantine and pre-shipment uses and critical emergencies (4, 5, 6). Metam-sodium degrades to methyl isothiocyanate (MITC) in presence of moisture (24). The resultant MITC, as a gas, can effectively penetrate materials for thorough fumigation. MITC reacts directly with amino acids (21) and the mechanism by which it is thought to inactivate certain infectious agents is through an alteration of protein structure.

Materials and Methods

Virus and cells

The H5N2 influenza virus Ck/PA/13609/93 was kindly provided by Dr Suarez (USDA-ARS, SEPRL, Athens, GA, USA). The virus was propagated in the allantoic fluid of 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs (SPAFAS, Franklin, CN, USA). The EID_{50} was determined by 10-fold dilutions of harvested allantoic fluid in 9-day-old embryonated SPF eggs. Four days after inoculation the allantoic fluid was harvested and tested for the presence of hemagglutinating activity using 1% chicken red blood cells (RBCs) diluted in

PBS. The EID_{50} was calculated following the method of Reed and Muench (19). The cell culture-adapted IBDV strain D78 was provided by Ruud Hein (Intervet/Schering-Plough, Millsboro, DE, USA). The virus was propagated in DF1 cells grown in DMEM/High glucose (HyClone, South Logan, Utah, USA) containing 10% fetal bovine serum (Manassas, VA, USA). The $TCID_{50}$ of the propagated IBDV strain D78 was determined using 10-fold dilutions following the formula of Reed and Muench (19).

Contamination of chicken litter

Chicken litter from a commercial broiler farm was kindly provided by John Smith (Fieldale Farms Corporation, Baldwin, GA, USA). The chicken litter was packed in quantities of 300 g in autoclaved bags and incubated at 121°C for 60 min. To determine the moisture content of the chicken litter, 100 g were weighed before and after drying. 300 g of sterile chicken litter were poured under sterile conditions into sterile plastic boxes with lids. The chicken litter was contaminated with 4 ml of allantoic fluid of LPAIV H5N2 diluted in 11 ml of sterile DMEM which resulted in a titer of $10^{8.35}$ EID_{50}/300 g chicken litter. For contamination of the chicken litter with IBDV, 4 ml (10^7 $TCID_{50}$/ml) of IBDV strain D78 was added to 11 ml of sterile cell culture medium. For the controls 15 ml of medium without virus was used. The virus suspension and the control were evenly added to the chicken litter with a pipette and the lid was closed. The contents in the boxes were manually mixed by rigorous shaking.

Treatment with Metam-Sodium

The initial treatment was performed using 5 ml of metam-sodium (Trade name RID-A-VEC, Evergreen Organics Inc., Carson City, NE, USA) which is the approximate equivalent of

16.8 l per 1000 kg of chicken litter. The solution was added to half of the boxes of contaminated chicken litter and half of the controls. The remaining boxes were left untreated with the compound. In a second study, 1 ml, 2 ml, and 5 ml of metam-sodium were used along with the same untreated controls. Once the metam-sodium solution was added, the lid was closed and the chicken litter was manually mixed by rigorous shaking to equally distribute the compound in the litter. The boxes were incubated at room temperature (22°C). Three samples of approximately 1 g each were obtained from three different randomly selected places in each box at 1 h, 12 h, 24 h, 36 h, and 48 h (study 1) and 1 h, 12 h, 24 h, and 48 h (study 2) post-contamination by dipping a 50 ml reaction test tube into the litter under sterile conditions in a class 2 biosafety cabinet. The sample was resuspendend in 5 ml of virus transport medium (1X minimal essential medium, 7.5% sodium bicarbonate, 15 mM HEPES, 1% fetal bovine serum, 4,000 U/ml penicillin, 400 µg/ml gentamycin, 8 µg/ml amphotericin B, 4,000 µg/ml streptomycin, 1000 µg/ml kanamycin sulfate). The resuspended material was vortexed for 30 s and centrifuged at 4000 rpm for 20 min at 4°C. The supernatant was aliquotted in three samples (1 ml each) and stored at -70 °C until analysis.

Analysis of the treatment

To investigate whether or not infectious influenza virus was present in the samples, 9-day-old embryonated SPF eggs were used. Eggs were inoculated into the allantoic cavity with 100 µl of the obtained samples. The inoculated eggs were incubated at 37.0°C for 4 days and were candled daily. After the incubation period, the eggs were chilled at 4 °C for 24 h. Now the allantoic fluid was then removed and analyzed for the presence of AIV by hemagglutination test using 1% of chicken RBCs diluted in PBS. Samples which showed agglutination of the RBCs were counted as positive. Positive samples were titrated in eggs to investigate the EID_{50} of the virus using 4 embryonated eggs per dilution.

For the detection of IBDV DF1 cells were cultivated in 48-well plates until confluence. The medium was removed, and 0.2 ml of serum free medium was added followed by 100 µl of the sample. The cells were then incubated for 60 min at 37°C. After this time the supernatant was removed and fresh cell culture medium (DMEM-1% fetal calf serum) containing penicillin (100 IU/ml)/ streptomycin (100 µg/ml) was added. The cells were incubated for three days. The presence of infectious virus was determined by immunofluorescence using a rabbit polyclonal antiserum raised against IBDV as described before (16). The samples were counted positive when immunofluorescence positive cells were detected. Immunoflorescence was chosen since the presence of minor amounts of virus will be detected by this method if only single cells were infected. This procedure was necessary since due to the presence of metam-sodium a possible cytopathic effect could be misinterpreted. Those samples where fluorescence was observed were titrated in DF1 cells to determine the $TCID_{50}$ per 100µl of sample following standard procedures.

Results, Discussion, and Conclusion

The inactivation of the LPAIV strain Ck/PA/13609/93 (H5N2) was investigated using virus contaminated chicken litter as a substrate. The virus was used at a concentration of $10^{8.75}$ EID_{50} per 300 g of chicken litter. This resulted in a theoretical contamination of $10^{5.9}$ EID_{50}/g of chicken litter. Under the conditions which were used, where 1 g of litter was taken and diluted in 5 ml of virus transport medium, the viral titer would theoretically be 10^3 EID_{50}/100 µl medium at the time point after contamination (0 h). In the first experiment 5 ml metam-sodium was used. MITC is lighter than air and thus mixing ensured an equal distribution to the litter. One hour after the addition of metam-sodium no infectious virus was detected (Figure 1.1) as measured by the inoculation of the litter sample supernatants into 9 day-old embryonated SPF eggs. This indicated that LPAIV was inactivated within 60 min after application of the disinfectant. It has to be mentioned that at no time point after the inoculation of eggs with samples of the noninfected/nontreated and noninfected/metam-sodium treated controls embryonic lethality was observed. This indicated that neither the amount of metam-sodium/MITC nor the soluble compounds from the chicken litter did induce embryonic lethality in these eggs. In contrast, 10^1/100µl EID_{50} of virus was detected at 1 h after contamination in the control, where no metam-sodium was added. Interestingly, the observed titer was consistent between all three samples which support the assumption that the virus was evenly distributed in the chicken litter. But the dynamic of the decrease of the virus titer during the first hour without treatment was not investigated. At 12 h after contamination, no H5N2 virus was detected in any of the samples as measured by the presence of HA using the allantoic fluid of the inoculated eggs. This result shows that H5N2 virus was inactivated 1 h after the addition of metam-sodium. Also in the non-treated and treated controls no virus able to induce hemagglutination was detected. The next experiments were performed to investigate if it was possible to use a lower amount of metam-sodium for the complete inactivation of LPAIV (Figure 1.2). To this end a similar set up of the experiments was performed. The only difference was that the time point 36 h after exposure to metam-sodium was not measured. The obtained EID_{50} one hour after exposure in the mock treated control was again 10^1/100 µl. Again there was no measurable difference between the three samples which were taken, indicating an even distribution of infectious virus within the chicken litter. The addition of 5 ml metam-sodium as in the previous experiment inactivated the virus 1 h after incubation. Interestingly, reduction of metam-sodium to 60% (3 ml metam-sodium) and 20% (1 ml metam-sodium) also completely inactivated LPAIV strain H5N2 within one hour of exposure to the fumigant. On the other hand, the virus was also inactivated after 12 h without the addition of fumigant. This indicates that the ambient conditions in the chicken litter were sufficient to eliminate the infectivity of the virus. This result was surprising. A longer period of time was expected where the LPAIV was infectious since previous published reports indicated that avian influenza virus was infectious up to three days on several surfaces (26). It has been described that mixing of AIV with field chicken manure inactivated the virus five to ten times faster than under unmixed conditions (14). These data and our data indicate that components in the chicken litter have a detrimental effect on the infectivity of LPAIV. The inactivation of an enveloped virus is a process with components which bring lipids into solution, such as alcohol-containing disinfectants, due to the nature of the envelope. The use of chlorine inactivated HPAIV at a level of >3 orders of magnitude but did not inactivate the virus completely (20). Several chemical compounds reduced the virus titer significantly, but were not able to entirely inactivate the virus (13). One reason for the reduction but not 100% inactivation might be explained with the fact that these compounds are used in a liquid form and they interact with the surface of the virus containing-matter and denature it. But under the surface some infectious virus is still present which cannot be reached by the chemical compound. In contrast, when metam-sodium comes into contact with moisture it forms a gas-like compound (MITC) which is able to penetrate organic matter and, as a result of this characteristic, is able to reach deeper within the contaminated material to inactivate the virus. Furthermore, the used volumes (1 ml versus 5 ml) of metam-sodium had likely not an effect of the inactivation of the virus since the impact is based on the MITC gas-like compound. This property of MITC has been used for the control of *Paratrichodorus allius* and corky ringspot disease in potatoes (10) and for the control of root-knot nematodes in greenhouses (7) by injecting metam-sodium into the soil.

The inactivation of the non-enveloped virus, IBDV, showed very similar kinetics to LPAIV when 5 ml of metam-sodium were used. One hour after the addition of this amount of metam-sodium, no infectious virus was observed by the combination of infection of cell culture with the supernatants from the litter samples and detection of infectious virus by immunofluorescence (Figure 1.1). Again 60 min was sufficient to inactivate this virus. It needs to be mentioned that the investigated samples did induce a slight, but transient CPE in DF1 cells from which the cells recovered during the 12 h after incubation. As expected, the virus infectivity persisted over the time investigated in the chicken litter without treatment. The first 12 h the virus titer did not change but between 12 h and 24 h the titer declined and stayed at a low level which might also have been contributed to the inactivation of IBDV. It was also observed that IBDV was uniformly distributed throughout the contaminated litter, as indicated by lack of deviation about the mean titer. The treatment with different amounts of metam-sodium showed that 1 ml of metam-sodium was not sufficient to provide a complete inactivation of IBDV (Figure 1.2). At 1 h post treatment 2 out 3 samples contained infectious virus whereas at 12 h, 24 h, and 36 h post treatment one out of three samples was still infectious (data not shown). The treatment of the chicken litter with 3 ml or 5 ml of metam-sodium resulted in a complete inactivation of the virus only 1 h after the addition of the compound. The kinetics of IBDV infectivity during the second study resembled the results from the first study where infectious virus was still detected 48 h after infection. In addition, the results show that there is a dose-dependent inactivation of IBDV. The inactivation of IBDV in such a short period of time has not been described before. In former inactivation studies of IBDV, only after incubation with 0.5% formalin for a period of 6 h was there a marked reduction, although still not complete inactivation, of the virus (2). Other treatments were either unable or able only to a very limited extent to inactivate the virus. In this study we observed a 100% inactivation of one enveloped virus and one non-enveloped virus due to the treatment with metam-sodium. As already mentioned above, the propensity of metam-sodium to form a gas might be the key to the effectiveness of this compound. The use of metam-sodium in the crop industry to control plant pests is widely applied. With this study it was shown for the first time that metam-sodium can also be used to inactivate viruses (enveloped and non-enveloped) in an efficient and definite application. This compound might broaden the viral disease-fighting arsenal by preventing the spread of viruses.

Acknowledgements

We would like to thank Ron Richardson (Evergreen Organics Inc., Carson City, NE, USA) for the provision of Rid-A-Vec for our experiments.

References

1. Benson, E., G. W. Malone, R. L. Alphin, M. D. Dawson, C. R. Pope, and G. L. Van Wicklen. Foam-based mass emergency depopulation of floor-reared meat-type poultry operations. Poultry Science. 86:219-224. 2007.

2. Benton, W. J., M. S. Cover, J. K. Rosenberger, and R. S. Lake. Physicochemical properties of the infectious bursal agent (IBA). Avian Diseases. 11:438-445. 1967.

3. De Benedictis, P., M. S. Beato, and I. Capua. Inactivation of avian influenza viruses by chemical agents and physical conditions: a review. Zoonoses and Public Health. 54:51-68. 2007.

4. De Cal, A. , A. Martinez-Treceño, J. M. Lopez-Aranda, and P. Melgarejo. Chemical alternatives to methyl bromide in Spanish strawberry nurseries. Plant Disease. 88:210-214. 2004.

5. Díaz-Pérez, M., F. Camacho-Ferre, F. Diánez-Martínez, M. De Cara-García, and J. C. Tello-Marquina. Evaluation of alternatives to methyl bromide in melon crops in Guatemala. Microbial Ecology. 57:379–383. 2009.

6. El Hadiri, N., M. Ammati, M. Chgoura, and K. Mounir. Behavior of 1, 3-dichloropropene and methyl isothiocyanate in undisturbed soil columns. Chemosphere. 52:893-899. 2003.

7. Giannakou, I. O., A. Sidiropoulos, and D. Prophetou-Athanasiadou. Chemical alternatives to methyl bromide for the control of root-knot nematodes in greenhouses. Pest Management Science. 58:290-296. 2002

8. Gerritzen, M. A., E. Lambooij, S. J. W. Hillebrand, J. A. C. Lankhaar, and C. Pieterse. Behavioral responses of broilers to different gaseous atmospheres. Poultry Science. 79:928–933. 2000.

9. Henzler, D. J., D. C. Kradel, S. Davison, A. F. Ziegler, D. Singletary, P. DeBok, A. E. Castro, H. Lu, R. Eckroade, D. Swayne, W. Lagoda, B. Schmucker, and A. Nesselrodt. Epidemiology, production losses, and control measures associated with an outbreak of avian influenza subtype H7N2 in Pennsylvania (1996–1998). Avian Diseases. 47:1022–1036. 2003.

10. Ingham, R. E., P. B. Hamm, M. Baune, and K. J. Merrifield. Control of *Paratrichodorus allius* and corky ringspot disease in potato with shank-injected metam sodium. Journal of Nematology. 39:258-262. 2007.

11. Lambooij, E., M. A. Gerritzen, B. Engel, S. J. W. Hillebrand, J. Lankhaar, and C. Pieterse. Behavioral responses during exposure of broiler chickens to different gas mixtures. Applied Animal Behavioral Science. 62:255–265. 1999.

12. Landgraf, H., E. Vielitz, and R. Kirsch. Occurrence of an infectious disease affecting the bursa of Fabricius (Gumboro disease). Dtsch. Tieraerztl. Wochenschr. 74:6–10. 1967.

13. Lombardi, M. E., B. S. Ladman, R. L. Alphin, and E. R. Benson. Inactivation of avian influenza virus using common detergents and chemicals. Avian Diseases. 52:118-123. 2008.

14. Lu, H., A. E. Castro, K. Pennick, J. Liu, Q. Yang, P. Dunn, D. Weinstock, and D. Henzler. Survival of avian influenza virus H7N2 in SPF chickens and their environments. Avian Diseases. 47(Suppl.):1015-1021. 2003.

15. Mandeville, W. F., Cook, F. K., and D. J. Jackwood. Heat lability of five strains of infectious bursal disease virus. Poultry Science. 79:838-842. 2000.

16. Mundt, E., Beyer, J., and H. Müller. Identification of a novel viral protein in infectious bursal disease virus-infected cells. Journal of General Virology. 76: 437-443. 1995.

17. OIE (2008). http://www.oie.int/eng/normes/mcode/en_chapitre_1.10.4.htm

18. Poole, G. H., and D. L. Fletcher. Comparison of a modified atmosphere stunning-killing system to conventional electrical stunning and killing on selected broiler breast muscle rigor development and meat quality attributes. Poultry Science. 77:342–347. 1998.

19. Reed, L. J. and H. Muench. A simple method of estimating fifty per cent endpoints. American Journal of Epidemiology. 27:493-497. 1938.

20. Rice, E. W., Adcock, N. J., Sivaganesan, M., Brown, J. D., Stallknecht, D. E., and D. E. Swayne. Chlorine inactivation of highly pathogenic avian influenza virus (H5N1). Emerging Infectious Diseases. 13:1568-1570. 2007.

21. Rowel, H.M. and J. Kroll. Some aspects of reaction of benzyl isothiocyanate with bovine sarcoplasmatic protein. Nahrung. 39:465–474. 1995.

22. Senne, D. A., B. Panigrahy, and R. L. Morgan. Effect of composting poultry carcasses on survival of exotic avian viruses: highly pathogenic avian influenza (HPAI) virus and adenovirus of egg drop syndrome-76. Avian Diseases. 38:733–737. 1994.

23. Shirai, J., R. Seki, R. Kamimura, and S. Mitsubayashi. Effects of invert soap with 0.05% sodium hydroxide on infectious bursal disease virus. Avian Diseases. 38:240-243. 1994.

24. Smelt, J. H. and M. Leistra. Conversion of metham-sodium to methyl isothiocyanate and basic data on the behavior of methyl isothiocyanate in soil. Pesticide Science. 5:401–407. 1974.

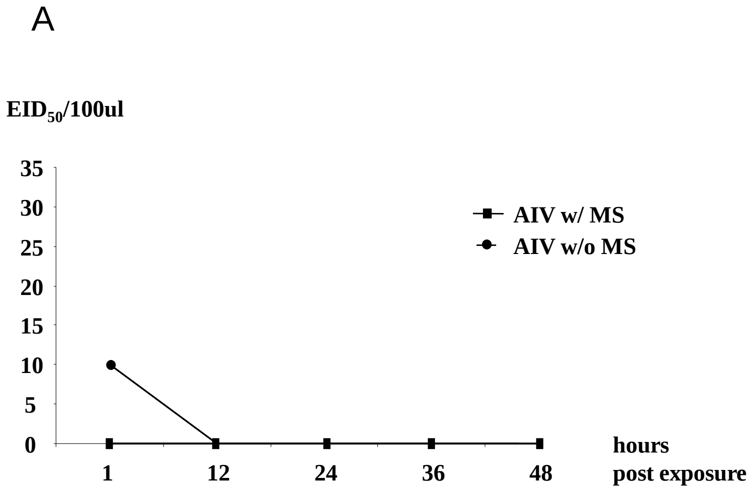
25. Spencer, L. AI – an overview of the Canadian experience, British Columbia 2004. Proceedings of the 13th Australian Poultry Convention. Gold Coast, Australia. Tour Hosts Pty. Ltd., Sydney, Australia. 2005.

26. Tiwari, A., D. P. Patnayak, Y. Chander, M. Parsad, and D. E. Goyal. Survival of two avian respiratory viruses on porous and nonporous surfaces. Avian Diseases. 50:284-287. 2006.

27. Thomas, C., D. J. King, and D. E. Swayne. Thermal inactivation of avian influenza and Newcastle disease viruses in chicken meat. Journal of Food Protection. 71:1214-1222. 2008.

28. Trout, T. California regulatory impacts on adoption of methyl bromide alternative fumigants. Methyl Bromide Alternatives. 7:4–7. 2001.

29. Wilkinson, K. G. The biosecurity of on-farm mortality composting. Journal of Applied Microbiology. 102:609–618. 2007.


A
EID$_{50}$/100ul
35
30
25
20
15
10
5
0
AIV w/ MS
AIV w/o MS
1
12
24
36
48
hours
post exposure

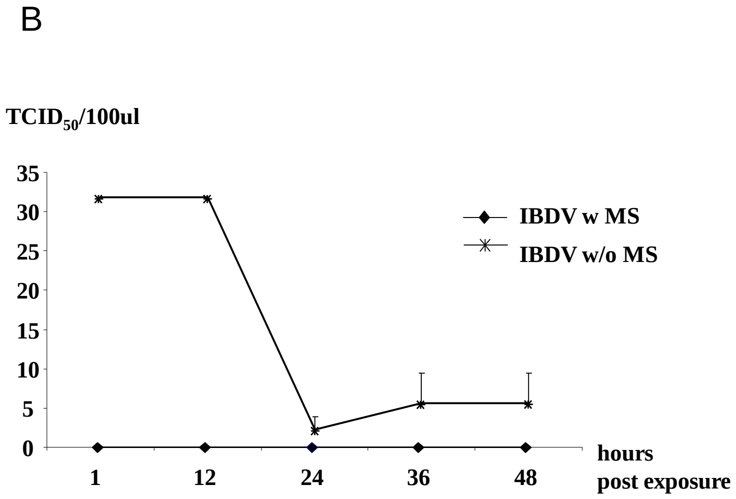
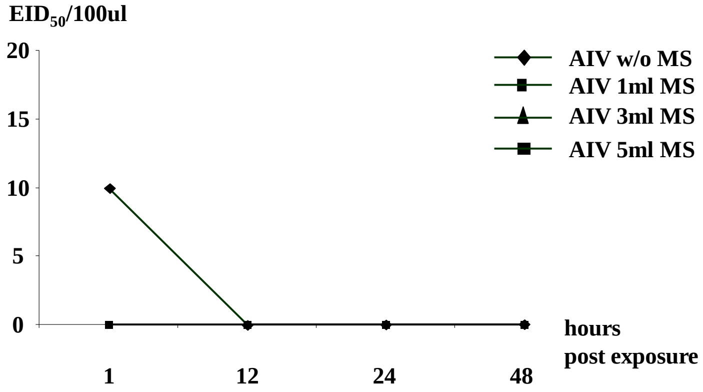

B
TCID$_{50}$/100ul
35
30
25
20
15
10
5
0
IBDV w MS
IBDV w/o MS
1
12
24
36
48
hours
post exposure

Figure 1.1: Inactivation of LPAIV and IBDV with 5ml of metam-sodium. 300g heat inactivated chicken litter (121°C 60min) placed in a plastic container with a lid was contaminated with (A) $10^{8.35}$ EID_{50} of LPAIV Ck/PA/13609/93 (H5N2) or (B) 4 x 10^{7} $TCID_{50}$/ml of IBDV strain D78. The content of the box was rigorously mixed, 5ml of metam-sodium (MS) was added and the content was mixed again (w/ MS). One box was not treated (w/o MS). At the time points indicated (hours post-exposure) samples were taken and analyzed for the presence of virus. Positive samples were titrated either in embryonated eggs (LPAIV) or in cell culture (IBDV) to determine the EID_{50} or $TCID_{50,}$ respectively. The calculated standard deviation is shown by error bars.

A


EID$_{50}$/100ul
20
15
10
5
0
1
12
24
48
hours
post exposure
AIV w/o MS
AIV 1ml MS
AIV 3ml MS
AIV 5ml MS

B

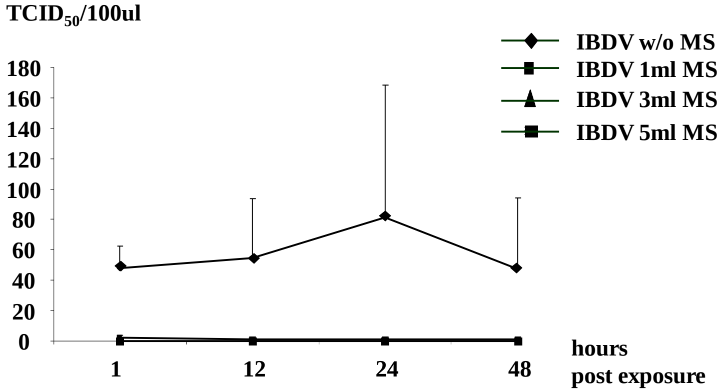

TCID$_{50}$/100ul
180
160
140
120
100
80
60
40
20
0
1
12
24
48
hours
post exposure
IBDV w/o MS
IBDV 1ml MS
IBDV 3ml MS
IBDV 5ml MS

Figure 1.2: Inactivation of IBDV with metam-sodium is dose-dependent. Plastic containers with lids were filled with 300g of heat inactivated chicken litter (121°C 60min). The litter was contaminated with (A) $10^{8.35}$ EID_{50} of LPAIV Ck/PA/13609/93 (H5N2) or (B) 4 x 10^7 $TCID_{50}$/ml of IBDV strain D78. The contaminated chicken litter was thoroughly mixed and treated with 1ml, 3ml, or 5ml of metam sodium (w/ MS). One box was not treated (w/o MS). The contents of the containers were mixed again. At the indicated time points (hours post-exposure) samples were taken and analyzed for the presence of virus. Positive samples were titrated either in embryonated eggs (LPAIV) or in cell culture (IBDV) to determine the EID_{50} or $TCID_{50,}$ respectively. The calculated standard deviation is shown by error bars.

CHAPTER 4

ESTABLISHMENT OF H6-SPECIFIC MONOCLONAL ANTIBODIES AND EXPERIMENTS TOWARD DEVELOPMENT OF A DIAGNOSTIC TEST[2]

---

[2] Appleby, L., R. Hogan, F. Michel, and E. Mundt. Establishment of H6-specific monoclonal antibodies and experiments toward development of a diagnostic test. To be submitted to Avian Diseases.

Abstract

The differentiation between HA subtypes of avian influenza virus is not possible with currently available commercial enzyme-linked immunosorbent assays (ELISAs). For the development of a H6-subtype specific ELISA the HA protein of the low pathogenic avian influenza virus (LPAI) A/duck/PA/486/1969 (H6N1) was expressed in a baculovirus system. The recombinant protein was purified by affinity chromatography and used for the immunization of mice. A panel of monoclonal antibodies (mAbs) to the recombinant protein was established and characterized. These mAbs were tested and found to be specific for H6 LPAI. The binding domain of all mAbs was mapped to an esterase domain of the HA1 subunit of the H6 protein. One mAb was able to specifically inhibit the infection of A/duck/PA/486/1969 (H6N1) in MDCK cells. The monoclonal antibodies were tested for their ability to be used in a competitive ELISA (cELISA). One mAb was selected and the cut-off value was calculated to be 39% inhibition from 80 AIV-negative sera. Using antisera specific for a variety of poultry pathogens and sera specific for H1-H13 AIV antigen resulted in no false positive reactions. The sensitivity was determined to be 39.94% whereas the specificity was 97%.

Introduction and Literature Review

Influenza A viruses belong to the virus family *Orthomyxoviridae*. According to their hemagglutinin (HA) and neuraminidase (N) surface glycoproteins, they are classified into subtypes. Currently 16 HA and 9 N subtypes are described (12, 33). Influenza A viruses are able to infect and cause disease in a great variety of mammalian an avian species. The natural reservoir for influenza A viruses are wild birds, thus they are named avian influenza viruses (AIV). Usually AIV infections in wild birds result in no clinical signs, allowing the virus to circulate in the environment without notice (43, 51). Most of the AIV belong to the low pathogenic avian influenza (LPAI) type whereas some LPAI, in particular those belonging to the HA5 (H5) and HA7 (H7) subtypes, can become highly pathogenic AIV (HPAI). Avian influenza viruses of the H6 subtype are among those most commonly isolated from wild waterfowl in North America (21, 44, 39, 40, 15). They are also detected at high frequencies in both wild ducks and domestic poultry in parts of Asia (19, Shortridge 1992, 25). Although frequently detected in ducks (order Anseriformes), H6-subtype viruses are rarely isolated from shorebirds and gulls (order Charadriiformes) (21). Nevertheless, it is apparent that these viruses experience occasional intercontinental transfer between Asia and North America. This is most clearly evidenced by the large-scale replacement of H6 genes of the North American lineage with those of the Asian lineage, such that the North American genes have all but disappeared over the past 20 years (32).

Although all viruses of the H6 subtype recorded to date have been grouped to the LPAI (2), these viruses are implicated in increased flock mortality, respiratory distress, decreased egg production, and nephritis in infected chicken flocks (25) and are therefore of economic concern to the poultry industry. During 2000 and 2001, nine isolates of H6N2 were recovered from chickens in California over a span of 20 months. The results of phylogenetic analysis of the viruses suggested that they might be capable of sustained circulation in chickens and may even undergo reassortment while replicating in this host (48). Although they do not normally infect mammals (13), H6-subtype viruses can replicate in experimentally infected human volunteers (4).

The key tool in identification and control of the threat caused by influenza A viruses is a constant and global AIV surveillance (11) and is mainly is performed by three methods. First, virus isolation in embryonated eggs, second, detection of viral RNA by RT-PCR, and third, analysis of serum samples for the presence of antibodies against AIV proteins (1). Replicating virus in the infected animal is present for a comparably short period of time but the presence of AIV antibodies lasts longer. By detection of the antibodies in the host serum the epidemiology, and thus the ecology, of the virus can be investigated without being dependent on the presence of replicating virus or viral RNA.

For an initial screening the sera are investigated for the presence of antibodies against the viral ribonucleoprotein (NP). NP is a group-specific antigen for all influenza A viruses. This can be performed by the agar gel precipitation test (AGPT) or enzyme-linked immunosorbent assay (ELISA) systems (1, 38, 45, 50, 52). After that, positive serum samples are investigated for antibodies specific for HA subtypes by hemagglutination inhibition (HI) assays (1). The HI assay is a highly sensitive method and is considered the serological standard method. The HI test is necessary for sub-typing of HA subtype-specific antibodies. Serum neutralization tests (SNT) also primarily detect antibodies against HA (49). Both tests are highly specific and sensitive but are laborious, not well suited for automation, and require the handling of infectious virus. The latter has an inherent risk of accidental release of the virus into the environment. The advantage of both tests is that they can be used in a species-independent approach. ELISA systems have been developed using a broad variety of antigens for the rapid screening of large numbers of serum samples. ELISAs can be readily automated, but depend on the availability of secondary antibodies against the immunoglobulin of the investigated species. Indirect ELISA systems for the detection of HA –specific antibodies have been found to have a low specificity (46). For the detection of antibodies in a species independent approach, the use of a competitive system is the method of choice (cELISA). Several species-independent HA-specific cELISAs have been described for H5 (10, 9, 35, 51) and H7 (9, 41). Just recently an H6-specific cELISA was reported (5).

Here we describe the establishment of a H6-specific cELISA system using recombinant antigen. The specificity and sensitivity has been determined using serum samples from chickens.

Material and Methods

Cells

Cells of the chicken fibroblast cell line DF-1 (16) and MDCK cells (kindly provided by Dr H Sellers, PDRC, University of Georgia, Athens, GA, USA) were grown in Dulbecco's Modified Eagles's Medium with 4.5 g/l glucose (DMEM-4.5, Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Mediatech, Manassas, VA, USA). Cells were cultivated in a humidified atmosphere at 37°C with 5% CO_2. For generation of recombinant baculovirus and its propagation the insect cell lines of *Spodoptera frugiperda* (*Sf*9, Invitrogen, Carlsbad, CA, USA), were cultivated in serum free SFX-Insect medium (Thermo Scientific, Waltham, MA, USA) at 28°C.

Generation of recombinant baculovirus

The H6N1 virus [A/duck/PA/486/1969 (H6N1), kindly provided by Dr. Dennis Senne (National Veterinary Service Laboratory, Ames, IA, USA)] was propagated in the allantoic cavity of nine-day-old specific pathogen free eggs (Sunrise Farms, Catskill, NY, USA) for 3 d at 37°C. The allantoic fluid was harvested and the viral RNA was extracted using the High Pure RNA Isolation Kit (Roche, Applied Science, Mannheim, Germany). The obtained viral RNA was reverse transcribed into viral cDNA using oligonucleotides Bm-HA-1 and Bm-NS-890R applying the conditions as previously described (19). The obtained PCR product was gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). After cloning into the plasmid pCR2.1-TOPO using the Topo TA cloning kit (Invitrogen, Carlsbad, CA) the sequence of isolated recombinant plasmids was determined (pCR2.1-HA6). Based on the sequence two oligonucleotides (H6BAC-FP and H6Bac-RP, see Table 2.1) were designed for PCR amplification of the open reading frame (ORF) encoding the HA6 protein. H6Bac-RP contained a sequence encoding for an Arg-Gly-Ser-6xHis tag (RGS-His) amino acid sequence located at the C-terminus of the recombinant protein. The gel purified PCR product was cleaved with *Rsr* II and *Spe* I, again gel purified and ligated into the appropriately cleaved baculo-transfer vector pFastBacDual (Invitrogen, Carlsbad, CA). After selection of plasmids containing an insert of the appropriate size sequencing was performed and a plasmid with a sequence 100% identical to pCR2.1-HA6 in respect to the HA6-ORF was chosen (pFAST-H6-RGSHis). The recombinant plasmid pFAST-H6-RGSHis was used to generate a recombinant baculovirus using the Bac-to-Bac system (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The obtained recombinant baculovirus (H6-Bac) was propagated in Sf9 cells and the supernatant of the second passage was used as viral stock. The $TCID_{50}$ for the viral stocks were determined by titration of the virus in 96-well tissue culture plates. To this end the virus stock was ten-fold diluted and 100 µl of the diluted virus was added to four wells per dilution step. To each well 100 µl of Sf9 cells (10^5 cells) were added and the plate was incubated for three days at 28ºC. In the next step the cells were fixed with ice-cold ethanol for 10 min. The dried cells were processed for indirect immunofluorescence using a baculovirus specific mAb (Anti-AcV5, Sigma-Aldrich) and FITC-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Inc. West Grove, PA, USA). The presence of mAb binding was visualized with fluorescein isothiocyanate (FITC) -labeled goat anti-mouse IgG (Jackson ImmunoResearch) and an inverted microscope (Axiovert 40 CFL; Carl Zeiss Ltd Scientific, Jena, Germany). The titer was calculated following the method of Reed and Muench (37).

Generation of plasmids encoding a truncated HA1 protein

Based on pCR2.1-HA6 the HA1 part of the HA6-ORF was truncated by using a set of oligonucleotides (see Table 2.1). The reverse oligonucleotide for each cDNA fragment encoded a FLAG-tag amino acid (aa) sequence (DYKDDDDK) resulting in a tag-sequence localized at the C-terminus of each protein. Four DNA fragments were generated using four primer pairs which coded either for the complete HA1 subunit (H6-1BsmBI-FP1/H6-1BsmBI-FLAG-RP1) or for cDNA fragments which were consecutively truncated (Δ) from the C-terminus of the HA1 protein in steps of 50 amino acids [Δ50 aa (H6-1BsmBI-FP1/H6-1BsmBI-FLAG-RP2), Δ100 aa (H6-1BsmBI-FP1/H6-1BsmBI-FLAG-RP3, Δ150 aa (H6-1BsmBI-FP1/H6-1BsmBI-FLAG-RP3)]. The PCR fragments were gel eluted, incubated with the restriction enzyme *BsmB* I and ligated into the vector pHW2000 (17). Recombinant plasmids (pH-H6-1FLAG, pH-H6-1FLAGΔ50, pH-H6-1FLAGΔ100, pH-H6-1FLAGΔ150) were sequenced to verify the identity of the sequence to the HA6-portion of pFAST-H6-RGSHis.

Detection and purification of recombinant proteins

The detection and purification of the recombinant protein has been described previously (10). Briefly, protein samples were analyzed for the presence of recombinant proteins either by protein stain using Imperial Protein Stain (Thermo Scientific) or by Western blot after separation on a sodiumdodecylsulphate-12% polyacrylamide gel by gel electrophoresis (SDS-PAGE).

Western blot was performed using standard conditions. For Western blot analysis several monoclonal antibodies (mAbs) were used: HA6-1, HA6-2, HA6-3, HA6-4 (specific for HA6, described in this paper), anti-RGS6xHis mAb (Qiagen, Valencia, CA, USA), anti-FLAG mAb **(**ANTI-FLAG® M2 mAb, Sigma-Aldrich**,** St. Louis, MO, USA). The binding of the mAb during Western blot analysis was visualized using a peroxidase-labeled goat anti-mouse IgG (Sigma-Aldrich) in combination with the chemiluminescent substrate Immobilon Western (Millipore, Billerca, MA, USA) and Gel Logic 2200 documentation system (Carestream Health, New Haven, CT, USA). For the detection of antibodies by immunofluorescence goat anti-mouse (FITC or Cy3 labeled) or goat anti-rabbit FITC labeled conjugates were used (Jackson ImmunoResearch, West Grove, PA, USA).

Transfection of cell cultures

For immunofluorescence studies 24-well tissue culture plates were seeded with DF1 cells. After the cells were grown to 80% confluence the medium was removed and the cells were transfected with 200 ng of the appropriate plasmid and 1 µl of Lipofectamine^{TM} 2000 Reagent (Invitrogen) as recommended by the manufacturer. Twenty-four hours after transfection the cells were fixed with ice-cold ethanol and processed for immunofluorescence following standard procedures. For Western blot analysis DF1 cells grown in six-well tissue culture plates were transfected with 3 ul of Lipofectamine^{TM} 2000 Reagent and 2 µg of plasmid DNA. Three hours after transfection the medium was removed and medium containing 1x penicillin/streptomycin (Sigma Aldrich) was added. For each experiment mock-transfected cells served as a negative control. Twenty-four hours later transfected cells were trypzinized, sedimented at 700x g and resuspended in 100 µl PBS. After addition of 100 µl of 2x sample buffer (23) the sample was heated at 95°C for 2 min, sonicated with Branson Sonifier (Branson, Danbury, CT, USA) and centrifuged for 5 min at 13000x g and the resulting lysate was used for Western blot analysis.

Source of serum samples

Several hyperimmune sera which had been prepared in goats or chickens directed against H1, H2, H3, H4, H5, H7, H8, H10, and H11 were obtained from American Type Culture Collection (Manassas, VA, USA). Furthermore, sera from chickens with antibodies against H1, H3, H9, H10, H11, H13, and H15 were kindly provided by Dr. D. Suarez (SEPRL, Athens, GA, USA). Sera from chickens infected with three different H5-subtype AIVs [A/Ck/PA/13609/93 (H5N2), A/Ck/TX/167280-4/02 (H5N3), A/Mute Swan/MI/ 451072/06 (H5N1)] were obtained from animal experiments (30). Serum samples from chickens infected with A/duck/PA/486/1969 (H6N1) were obtained during experiments where three-week-old SPF chickens (Merial Select, Gainesville, GA, USA) were infected with 10^6 EID via the oro-nasal route. At 7, 14, and 21 d. p. i. serum samples were taken in order to obtain serum samples with different HI titers. Another set of serum samples from chickens was obtained from Dr. Garcia and Dr. Jackwood (PDRC, University of Georgia, Athens, GA, USA). These chickens were infected with LPAI of the H6N2 subtype [A/Ck/Ca/139/01-(H6N2), A/Ck/CA/431/00 (H6N2)]. Dr. J. Gelb (University of Delaware) provided sera from chickens infected with an isolate from waterfowl [A/waterfowl/415/DE/2005 (H6N2)]. All serum samples were tested for the presence of HI antibodies using A/duck/PA/486/1969 (H6N1) as antigen. The test was performed following the OIE recommendations (31). Only the sera which tested HI positive were used for the ELISA. Furthermore, 80 sera from chicken flocks from the field known to be free of antibodies against influenza as tested by agar gel precipitation test were provided by Dr. S. Thayer (PDRC, Athens, GA, USA).

cELISA

The establishment of the cELISA followed essentially the protocol as described by (10). Briefly, using the protein detector ELISA kit (KPL, Gaithersburg, MD, USA) the antigen was diluted in coating buffer to a final concentration of 500 ng/ml and 50 µl were added to each well of a 96-well flat bottom plate (FisherBrand, Santa Clara, CA, USA). After incubation and blocking the plate was incubated with the appropriate sera and the competing H6 mAb diluted appropriately, as determined by checkerboard titration, in dilution buffer (Synbiotics, San Diego, CA, USA). All incubation steps were performed at 37°C. After addition of the stop solution the optical density (OD) values were measured at 405 nm using an ELISA reader (ELx 808, BioTek, Winooski, VT, USA). The unimpaired binding (100% binding value) of the H6 mAb was determined by adding buffer without serum to four wells. The inhibition of the mAb binding for each serum was calculated according to the formula $100 - OD_{serum}/OD_{mAb}\ 100 = \%$ inhibition of the mAb binding. One positive standard control serum was included in each 96-well plate. Over the course of the study a serum pool obtained from SPF chickens infected with A/duck/PA/486/1969 (H6N1) was used as the positive control serum (E. Mundt, unpublished results).

Generation of H6-specific monoclonal antibodies

The generation of monoclonal antibodies was performed essentially as previously described with some differences (10). For the immunization of three female BALB C mice

(Harlan Laboratories, Inc. Indianapolis, IN, USA) 200 µg purified HA6-Bac antigen was used for each injection. The immunization protocol was as described before (10). The presence of H6-specific antibodies were initially screened by an indirect ELISA using goat anti-mouse IgG specific HRP conjugate (Thermo Fisher Scientific, Rockford, IL, USA). The ELISA plates were coated with the purified recombinant antigen. Hybridoma supernatants tested positive in the ELISA were subsequently tested for reactivity using H6N1-infected DF1 cells.

Testing for specificity

DF1 cells were seed into 96-well tissue culture plates. Twelve hours later the cells were infected with LPAI strains encoding different HA-subtypes: A/AGWT/LA/213GW/1987 (H1N1), A/AGWT/NC/6423-165/2006 (H2N7), A/MALL/MN/AI07-4724/2007 (H3N8), A/MALL/MN/AI07-4714/2007 (H4N6), A/MUSW/MI/451072/2006 (H5N1), A/MALL/MN/AI07-3174/2007 (H6N1), A/duck/PA/486/1969 (H6N1), A/MALL/MN/AI07-3174/2007 (H6N4), A/Ty/OR/1971 (H7N3), A/NSHO/MN/A08-24-50/2008 (H8N4), A/turkey/Wisconsin/1/1966 (H9N2), A/RUTU/NJ/ AI00-1185/2000 (H10N7), A/ABDU/NC/674-1066/2006 (H11N9), A/RUTU/NJ/AI07-677/2007 (H12N5), and A/LAGU/NJ/AI08-1460/2008 (H13N9). All viruses were provided by Dr. D. Stallknecht (University of Georgia, Athens, USA) except for the H9N2 and H7N3 viruses which were provided by Dr. D. Senne (National Veterinary Service Laboratory, Ames, IO, USA). Twelve hours after infection the cells were fixed with ice-cold ethanol and incubated either with the H6-specific mAbs or a rabbit NP-specific antiserum (22). The binding of the antibodies were detected by using species-specific FITC-conjugated antibodies as described above.

Plaque assay and virus neutralization assay

Plaque assay and virus neutralization (VN) tests were performed using confluent MDCK cells grown in a 6-well tissue culture plate. Allantoic fluid from A/duck/PA/486/1969 (H6N1)-infected 9-day-old SPF eggs was used in a series of 10-fold dilutions in serum-free DMEM. The cell culture supernatant was removed and the cells were rinsed once with serum-free DMEM before 1 ml of serum-free DMEM was added. This was followed by the addition of 100 µl of the appropriate virus dilution. The plate was incubated for 1 h at 37°C to allow the virus to infect the cells. Next, the medium was removed from the cells, the cells were rinsed again with serum-free medium, and finally overlaid with a semi-liquid medium [1.2% Avicel RC-581 (FMC BioPolymer, Philadelphia, PA, USA), 1x minimal essential medium (Invitrogen), 20 mM HEPES, 10 mM L glutamine, 0.75% NaHCo3, 1x penicillin/streptomycin solution (Hyclone, Rockford, IL, USA), 1 µg/ml TPCK-treated trypsin (Worthington Biochemical Corp. Lakewood, NJ, USA)]. The cells were incubated for 2 d at 37°C. The overlay was removed, the cells were rinsed with phosphate buffered solution (PBS), and fixed with ice-cold acetone/methanol (40%/60% vol/vol) for 10 min. The fixation solution was removed and the cells were air dried and stained with a 1:1 mixture of PBS and crystal violet solution [5% methanol, 11.1 % formaldehyde, 0.13 % crystal violet (w/vol)] for 10 min. Finally, the solution was removed, the cells were rinsed once with tap water, and then air dried. For the VN test a virus dilution containing approximately 100 plaque forming units (PFU) in 100 µl DMEM was mixed with either 100 µl of hybridoma cell culture supernatant or 100 µl of an H6N1 chicken serum diluted 1:10. As negative controls, SPF chicken serum and no serum were used. The mixture was incubated for 60 min at 37°C and subsequently added to the MDCK cells. The remaining procedure followed the protocol as described for the plaque assay.

Results

Generation of monoclonal antibodies

For the generation of monoclonal antibodies a recombinant baculovirus (H6His-Bac) was constructed using the recombinant plasmid pFAST-H6-RGSHis and the Bac-to-Bac system as recommended by the manufacturer. Cellular lysates of H6His-Bac infected cells were purified by affinity chromatography and analyzed by both protein stain and Western blot (Figure 2.1). After purification of the recombinant H6-His protein, two single bands with a molecular mass of approximately 62 kD were detected in the protein-stained gel (Fig. 2.1A). The presence of an RGSHis-tag sequence was confirmed by using an appropriate monoclonal antibody. To further characterize the antigen, chicken serum obtained 21 d after infection with A/duck/PA/486/1969 (H6N1) was used and showed a reaction with the appropriate band at 62 kDa. The identity of the H6-Bac protein was finally confirmed by protein sequencing using MALDI-TOF MS/MS (University of Georgia, Chemical and Biological Sciences Mass Spectrometry Facility). After repeated immunizations of female BALB C mice with the recombinant H6-His, seven different mAbs were selected (mAb HA6-1 to HA6-7) which were positive in an indirect ELISA using H6-His protein. The specificity was tested by indirect immunofluorescence using A/duck/PA/486/1969 (H6N1) infected DF1 cells. Only 4 supernatants (mAb HA6-1, HA6-2, HA6-4, HA6-5) reacted with infected cells. This was confirmed by indirect double-fluorescence using the supernatants and goat anti-rabbit FITC-and goat anti-mouse Cy3-conjugated antibodies (data not shown). For further characterization and validation of the specificity of the selected mAbs, indirect immunofluorescence was performed using LPAI encoding H1-H13. The results showed that the selected mAb reacted only with H6-encoding viruses. No reaction with other

LPAI was observed. The successful infection of the cells was monitored by using a rabbit anti-NP specific serum (Table 2.2).

One H6-specific mAb shows virus-neutralizing properties

In further assay the mAbs were tested for their ability to inhibit hemagglutination and neutralize the virus. The HI test using the wild type virus showed no HI activity with any of the monoclonal antibodies. The VN test showed that H6-1 was able to neutralize the virus from 73 plaques (no serum) to 18 plaques. The incubation with the remaining monoclonal antibodies did not significantly reduce the virus titer (H6-2: 61 plaques, H6-3: 46 plaques, H6-5: 50 plaques). The H6N1-specific chicken serum neutralized the virus completely as evidenced by the absence of any plaques.

Characterization of the binding region of the H6-specifc mAbs

For the determination of the binding region of the anti-H6 mAbs it was tested whether the antibodies reacted with either the HA1 or the HA2 subunit of the HA6 protein. Allantoic fluid of A/duck/PA/486/1969 (H6N1)-infected SPF eggs was used for Western blot analysis. The serum of the H6-Bac vaccinated mouse used for the fusion was used as a positive control. It was observed that the mouse serum reacted with the HA0, HA1, and HA2 proteins. Surprisingly, all four monoclonal antibodies reacted with the HA1 subunit of the HA protein. In the next experiments the binding region for the HA6 mAbs was determined by both immunofluorescence and Western blot analysis using the plasmids pH-H6-1FLAG, pH-H6-1FLAGΔ50, pH-H6-1FLAGΔ100, and pH-H6-1FLAGΔ150. For the generation of the plasmids the pHW2000 vector was used since we were not able for unknown reasons to observe expression after cloning the

ORF into the eukaryotic expression vector pcDNA3 (Invitrogen). After transfection immunofluorescence was observed in all wells transfected with the recombinant plasmids when the anti-FLAG mAb was used (data not shown). Surprisingly, a fluorescent signal was observed with all four H6-mAbs only in cells transfected with pH-H6-1FLAG and pH-H6-1FLAGΔ50. Western blot experiments using cellular lysates of transfected DF1 cells confirmed the observation made during immunofluorescence investigations (Figure 2.3). Only transfection with pH-H6-1FLAG and pH-H6-1FLAGΔ50 resulted in protein bands of the appropriate size when the H6-specific mAbs were used. Expression of each recombinant protein was indicated by the presence of an appropriate band when the FLAG-mAb was used. The results indicate that all four mAbs bind to a non-conformational epitope located between amino acids 244 and 294 of the HA1 subunit of the H6 protein. This domain in the HA1 protein has been characterized as esterase domain (26).

Experiments towards the development of an H6-specific cELISA

In the next assays the binding of the four monoclonal antibodies in an indirect ELISA was investigated. During the first experiments the binding profiles of the mAbs were compared. The mAb HA6-5 showed the lowest OD value and was not further considered. The remaining three mAbs were further tested in checkerboard titrations using different amounts of antigen and different dilutions of several H6-specific chicken sera. The combination of a dilution of 1:300 of mAb HA6-2 and a serum dilution of 1:10 resulted in the highest value of inhibition of the binding of the mAb when several chicken sera from birds which had been infected with A/duck/PA/486/1969 (H6N1) were used. Next, 80 AGPT-negative chicken sera were used to determine the cut-off value for the cELISA. We found that a serum dilution of 1:10 and a 1:300

antibody dilution of mAb HA6-2 resulted in the best ratio for the determination of the cut off value. The inhibition was calculated as described before (10). The average inhibition was determined to be 16.95% with a minimum of 1.6 % and maximum of 32.34%. The standard deviation was determined to be 7.66%. Next the necessary n-fold standard deviation was determined where all 80 serum samples would be recognized as truly negative. Only at three standard deviations above the mean was a specificity of 100% accomplished. This resulted in a cut-off value of 39.93%. For the determination of the specificity to H6, several antisera were used which contained antibodies against H1-H13. None of these sera were recognized as positive by the cELISA. In additional experiments sera were used which were AIV antibody negative but contained antibodies against other poultry pathogens [reticuloendotheliosis virus, chicken anemia virus, avian encephalomyelitis virus, infectious bronchitis virus (subtypes DE-072, Connecticut, Arkansas, Massachusetts), Newcastle disease virus, infectious laryngotracheitis virus, avian reovirus 1133, avian reovirus 41560, avian reovirus 40963, avian rotavirus, avian adenovirus (serotype 1 - serotype 12), mycoplasma gallisepticum, mycoplasma synovia, infectious bursal disease virus serotype 1 (classic and variant) and serotype 2]. These sera were obtained from Charles River SPAFAS (N Franklin CT, USA) and provided by Dr. Sellers (PDRC, University of Georgia, Athens, GA, USA). None of these sera showed an inhibition of the binding of mAb H6-2 above the determined cut-off value. The inhibition of the mAb binding ranged between 0% and 24.75% with an average of 5.12% (standard deviation 6.22%). These results show that the cELISA was highly specific in regard to other poultry pathogens and HA antigens other than HA6.

Finally, experiments were performed with serum samples from experimentally infected chickens. Only serum samples from chickens which had a positive HI titer were used for the experiment. The sensitivities of the test at 1, 2, and 3 standard deviations above the mean were 62.5%, 43.75% and 39.94%, respectively. Since at one and two standard deviations a number of LPAI-negative sera would be recognized as false positives, the sensitivity of the test was 39.94%.

Discussion and Conclusion

The surveillance of wild bird populations for the presence or absence of LPAI is an important tool to prevent the introduction of LPAI in commercial poultry. Two methods for surveillance can be used, the direct detection of the infectious agent by means of detection of nucleic acid through RT-PCR (35, 34, 24), real time RT-PCR (6, 7, 42), or DNA micro array (14) and the isolation of the virus in embryonated eggs from AIV-negative chickens (31). Another diagnostic approach is the detection of antibodies resulting from an infection. To this end, the HI assay is widely used and it is considered the gold standard for the subtyping of LPAI antibodies. The assay is time consuming, laborious, and depends on the handling of infectious virus, but it is highly specific and can be used on serum from any species. On the other hand, increased surveillance can be expected to generate a larger number of samples and other serological assays need to be implemented which can be used for preliminary screening. For this purpose, ELISA tests have been developed (8, 20, 45) and several commercial tests are on the market. These ELISA tests are either indirect or based on a competitive approach using antibodies directed against the nucleoprotein of influenza A virus. The latter approach can also be used in a species-independent manner by utilizing a monoclonal antibody as a competitor. As recently published (28) these tests have shown a good specificity and higher sensitivity than the HI test. Besides the NP competition ELISA several species-independent cELISAs have been described recently (see introduction). Ideally, the cELISA system is based on an antigen which can be obtained without propagating infectious LPAI. This is generally facilitated by the use of recombinant protein. The use of baculovirus expressed protein for the establishment of diagnostic assays for influenza virus is widely used (3, 10, 27, 29, 45, 47). The generation of a recombinant baculovirus encoding an H6 antigen was used with the goal of eliminating the need for infectious LPAI in the preparation of the antigen. Both components, the antigen and the mAb, can be generated in low biosafety level laboratories in contrast to laboratories which need to propagate LPAI for use in assays. The disadvantage of the described assay is the low sensitivity in comparison to the HI test; however the specificity of the test was 100%. The low sensitivity might be attributed to the region to which mAb H6-2 binds. This region is located in the esterase domain of the HA1 protein, situated between the receptor-binding domain and the fusion domain of the protein subunit (26). Another H6-specific cELISA has been described recently (5) with a very good specificity but used virus preparations as the antigen with which plates were coated. The high specificity of their assay may be caused by the ability of the mAb which was used to inhibit hemagglutination, indicating that the mAb binding region was in the exposed outer portion of the protein. The disadvantage of the assay was that it recognized HA1-directed antibodies, whereas the assay described in this paper showed 100% specificity with respect to non-H6-specific antisera. The observation that the mAb H6-1 was able to neutralize the virus was surprising since the binding region was mapped to an area below the binding pocket for the receptor. Whether or not this is due to steric hindrance needs further investigation. Interestingly, while all four mAbs were found to bind to the same region as indicated by mapping, it would seem that at least mAb H6-1, which displays different properties, binds to a different epitope within this region.

Acknowledgements

This work was funded by the U.S. Department of Agriculture (USDA) Cooperative State Research, Education, and Extension Service (CREES) Avian Influenza Coordinated Agricultural Project (AICAP) program.

References

1. Alexander, D. J. Avian Influenza, p. 2.7.12., Manual of diagnostic tests and vaccines for terrestrial animals. 2004.

2. Alexander, D. J. An overview of the epidemiology of avian influenza. Vaccine. 25:5637-5644. 2007.

3. Avellaneda, G., E. Mundt, C. W. Lee, S. Jadhao, and D. L. Suarez. Differentiation of infected and vaccinated animals (DIVA) using the NS1 protein of avian influenza virus. Avian Diseases. 54(1 Suppl):278-286. 2010.

4. Beare, A. S. and R. G. Webster. Replication of avian influenza viruses in humans. Archives of Virology. 119:37-42. 1991.

5. Chen, Y. T., R. H. Juang, J. L. He, W. Y. Chu, and C. H. Wang. Detection of H6 influenza antibody by blocking enzyme-linked immunosorbent assay. Veterinary Microbiology. 142:205-210. 2010.

6. Chen Y. C., C. H. Chen, and C. H. Wang. H5 antibody detection by blocking enzyme-linked immunosorbent assay using a monoclonal antibody. Avian Diseases. 52:124-129. 2008.

7. Das, A., E. Spackman, D. Senne, J. Pedersen, and D. L. Suarez. 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. 44:3065–3073. 2006.

8. de Boer, G. F., W. Back, and A. D. Osterhaus. An ELISA for detection of antibodies against influenza A nucleoprotein in humans and various animal species. Archives of Virology. 115:47–61. 1990.

9. De Marco, M. A., E. Foni, L. Campitelli, M. Delogu, E. Raffini, C. Chiapponi, G. Barigazzi, P. Cordioli, L. Di Trani, and I. Donatelli. Influenza virus circulation in wild aquatic birds in Italy during H5N2 and H7N1 poultry epidemic periods (1998 to 2000). Avian Pathology. 34:480-485. 2005.

10. Dlugolenski, D., R. Hauck, R. J. Hogan, F. Michel, and E. Mundt. Production of H5 specific monoclonal antibodies and the development of a competitive ELISA for detection of H5 antibodies in multiple species. Avian Diseases. 54: 644–649. 2010.

11. Fouchier, R. A., A. D. Osterhaus, and I. H. Brown. Animal influenza virus surveillance. Vaccine. 21:1754-7. 2003.

12. Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. Journal of Virology. 79:2814-2822. 2005.

13. Fujii, K., C. Kakumoto, M. Kobayashi, S. Saito, T. Kariya, Y. Watanabe, Y. Sakoda, H. Kida, and M. Suzuki. Serological evidence of influenza A virus infection in Kuril Harbor Seals (*Phoca vitulina stejnegeri*) of Hokkaido, Japan. Journal of Veterinary Medical Science. 69:259-263. 2007.

14. Gall, A., B. Hoffmann, T. Harder, C. Grund, R. Ehricht, and M. Beer. Rapid haemagglutinin subtyping and pathotyping of avian influenza viruses by a DNA microarray. Journal of Virological Methods. 160:200-205. 2009.

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

16. Himly, M., D. N. Foster, I. Bottoli, J. S. Iacovoni, and P. K. Vogt. The DF-chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. Virology. 248:295-304. 1998.

17. Hoffmann, E., G. Neuman, Y. Kawaoka, G. Hobom, and R. G. Webster. A DNA transfection system for generation of influenza A virus from eight plasmids. Proceedings of the National Academy of Science. 97:6108-6113. 2000.

18. Hoffmann, E., J. Stech, Y. Guan, R. G. Webster, and D. R. Perez. Universal primer set for the full-length amplification of all influenza A viruses. Archives of Virology. 146:2275-89. 2001.

19. Jahangir, A., Y. Watanabe, O. Chinen, S. Yamazaki, K. Sakai, M. Okamura, M. Nakamura, and K. Takehara. Surveillance of avian influenza viruses in Northern Pintails (Anas acuta) in Tohoku District, Japan. Avian Diseases. 52:49-53. 2008.

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

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

22. Klopfleisch, R., O. Werner, E. Mundt, T. Harder, and J. P. Teifke. Neurotropism of highly pathogenic avian influenza virus A/chicken/Indonesia/2003 (H5N1) in experimentally infected pigeons (Columbia livia f. domestica). Veterinary Pathology. 43:463-470. 2006.

23. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685. 1970.

24. Lee, M. S., P. C. Chang, J. H. Shien, M. C. Cheng, and H. K. Shieh. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. Journal of Virological Methods. 97:13–22. 2001.

25. Lee, M. S., P. C. Chang, J. H. Shien, M. C. Cheng, C. L. Chen, and H. K. Shieh. Genetic and pathogenic characterization of H6N1 avian influenza viruses isolated in Taiwan between 1972 and 2005. Avian Diseases. 50:561-571. 2006.

26. Lin, T., G. Wang, A. Li, Q. Zhang, C. Wu, R. Zhang, Q. Cai, W. Song, and K. Y. Yuen. The hemagglutinin structure of an avian H1N1 influenza A virus. Virology. 392:73-81. 2009.

27. Liu, Y., E. Mundt, A. Mundt, M. Sylte, D. L. Suarez, D. E. Swayne, and M. García. Development and evaluation of an avian influenza, neuraminidase subtype 1, indirect enzyme-linked immunosorbent assay for poultry using the differentiation of infected from vaccinated animals control strategy. Avian Diseases. 54(Suppl. 1):613-621. 2010.

28. Marché, S. and T. van den Berg. Evaluation of different strategies for the use of ELISA tests as first screening tools for serologic surveillance of low pathogenic avian influenza in the Belgian poultry sector. Avian Diseases. 54(Suppl. 1):627–631. 2010.

29. Mueller, M., S. Renzullo, R. Brooks, N. Ruggli, and M. A. Hofmann. Antigenic characterization of recombinant hemagglutinin proteins derived from different avian influenza virus subtypes. PLoS One. 5:e9097. 2010.

30. Mundt, E., L. Gay, L. Jones, G. Saavedra, S. M. Tompkins, and R. A. Tripp. Replication and Pathogenesis Associated with H5N1, H5N2 and H5N3 Low Pathogenic Avian Influenza Virus Infection in Chickens and Ducks. Archives of Virology. 154:1241-1248. 2009.

31. OIE (2009). http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf, adopted version from May 2009
32. Olsen, B., V. J. Munster, A. Wallensten, J. Waldenström, A. D. M. E. Osterhaus, and R. A. M. Fouchier. Global patterns of influenza A virus in wild birds. Science. 312:384-388. 2006.

33. Palese, P., and M. L. Shaw. Orthomyxoviridae: The viruses and their replication pp. 1647-1689. In D. M. Knipe, D. E. Griffin, R. A. Lamb, S. E. Straus, P. M. Howley, M. A. Martin, and B. Roizman (ed.), Fields Virology, 5th ed, vol. 2. Lippincott Williams & Wilkins, Philadelphia, PA. 2007.

34. Pang, Y., H. Wang, T. Girshick, Z. Xie, and M. I. Khan. Development and application of a multiplex polymerase chain reaction for avian respiratory agents. Avian Diseases. 46:691–699. 2002.

35. Phipps, L.P., S. C. Essen, and I. H. Brown IH. Genetic subtyping of influenza A viruses using RT-PCR with a single set of primers based on conserved sequences within the HA2 coding region. Journal of Virological Methods. 122:119–122. 2004.

36. Prabakaran, M., H. T. Ho, N. Prabhu, S. Velumani, M. Szyporta, F. He, K. P. Chan, L. M. Chen, Y. Matsuoka, R. O. Donis, and J. Kwang. Development of epitope-blocking ELISA for universal detection of antibodies to human H5N1 influenza viruses. PLoS One. 4:e4566. 2009.

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

38. Shafer, A. L., J. B. Katz, and K. A. Eernisse. Development and validation of competitive enzyme-linked immunosorbent assay for detection of type A influenza antibodies in avian sera. Avian Diseases. 42:28-34. 1998.

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

40. Sharp, G. B., Y. Kawaoka, D. J. Jones, W. J. Bean, S. P. Pryor, V. Hinshaw, and R. G. Webster. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. Journal of Virology. 71:6128-6135. 1997.

41. Shien, J. H., L. F. Fu, J. R. Wu, M. C. Cheng, H. K. Shieh, and P. C. Chang. Development of blocking ELISA for detection of antibodies against avian influenza virus of the H7 subtype. Journal of Microbiology, Immunology and Infection. 41:369-376. 2008.

42. 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. Development of real-time RT-PCR for the detection of avian influenza virus. Avian Diseases. 47:1079–1082. 2003.

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

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

45. Starick, E., O. Werner, H. Schirrmeier, E. Mundt, B. Köllner, and R. Riebe. Establishment of a competitive ELISA (cELISA) system for the detection of influenza A virus nucleoprotein antibodies and its application to field sera from different species. Journal of Veterinary Medicine Series B. 53:370-375. 2006.

46. Stelzer-Braid, S., B. Wong, P. Robertson, G. W. Lynch, K. Laurie, R. Shaw, I. Barr, P. W. Selleck, C. Baleriola, R. Escott, G. Katsoulotos, and W. D. Rawlinson. A commercial ELISA detects high levels of human H5 antibody but cross-reacts with influenza A antibodies. Journal of Clinical Virology. 43:241-243. 2008.

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

48. Webby, R. J., P. R. Woolcock, S. L. Krauss, D. B. Walker, P. S. Chin, K. F. Shortridge, and R. G. Webster. Multiple genotypes of nonpathogenic H6N2 influenza viruses isolated from chickens in California. Avian Diseases. 47:905-910. 2003.

49. Webster, R., N. Cox, and K. Stoehr. WHO manual on influenza diagnosis and surveillance, 1st ed. World Health Organization, Geneva, Switzerland. 2002.

50. Wu, R., S. Hu, Y. Xiao, Z. Li, D. Shi, and D. Bi. Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus. Veterinary Research Communications. 31:631-41. 2007.

51. Yang, M., A. Clavijo, J. Graham, T. Salo, K. Hole, and Y. Berhane. Production and diagnostic application of monoclonal antibodies against influenza virus H5. Journal of Virological Methods. 162:194-202. 2009.

52. Zhou, E. M., M. Chan, R. A. Heckert, J. Riva, and M. F. Cantin. Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein. Avian Diseases. 42:517-22. 1998.

Table 2.1 Oligonucleotides used for the analysis of HA and NA genes

Name	Sequence[a]	Orientation	Location[b]
H6-pFast-FP	Rsr II GGCGGACCG**ATG***ATTGCA* *ATCATAATACTTGCGATAG*	sense	6-33
H6-pFast-RP	Spe I ggACTAGT**TTA**gtgatggtgatggtgatgagaccc tct*TATACATATCCTGCATTGCATTGAGC*	antisense	1678-1703
H6-1 BsmBI-FP1	BsmBI TATTCGTCTCAGGGGCGGC CGCAGCAAAAGCAGG*GG*	sense	1-2
H6-1 BsmBI-FLAG	BsmBI ATATCGTCTCGTATTTCTAGA**TTA***cttgtcatcgtcg* *tccttgtagtcTCTTGTCTCTGCCTGTGGGACATTTC*	antisense	1012-1037
H6-1 BsmBI-FLAG RP2	BsmBI ATATCGTCTCGTATTTCTAGA**TTA***cttgtcatcgtc* *gtccttgtagtcAGCATCACAATTTTCAATTGGAAGG*	antisense	863-887
H6-1 BsmBI-FLAG RP3	BsmBI ATATCGTCTCGTATTTCTAGA**TTA***cttgtcatcg* *tcgtccttgtagtcATCAATTCGCCCCCTTTGCCC*	antisense	717-737
H6-1 BsmBI-FLAG RP4	BsmBI ATATCGTCTCGTATTTCTAGA**TTA***cttgtcatcg* *tcgtccttgtagtcACCCCAGAAATATAGGATCGG*	antisense	567-587

[a] The used restriction enzyme cleavage sites are named and underlined. Start and stop codons are in bold-face type. Virus-specific sequences are in italics and upper case. The nucleotide sequence encoding the RGS-6x His sequence is shown in lower case whereas the nucleotide sequence coding for the FLAG-epitope is in lower case and italics.

[b] The location of the oligonucleotides are in accordance with the HA6 sequence of A/duck/PA/486/1969 (H6N1), Genbank accession number EU743286

Table 2.2 Reactivity of anti-H6 monoclonal antibodies with several influenza viruses encoding different HA subtypes

Virus strain	Monoclonal antibodies				NP[a]
	6-1	6-2	6-4	6-5	
A/ AGWT/LA/213GW/1987 (H1N1)	-	-	-	-	+
A/AGWT/NC/6423-165/ 2006 (H2N7)	-	-	-	-	+
A/MALL/MN/AI07-4724/ 2007 (H3N8)	-	-	-	-	+
A/MALL/MN/AI07-4714/ 2007 (H4N6)	-	-	-	-	+
A/MUSW/ MI/451072/ 2006 (H5N1)	-	-	-	-	+
A/MALL/MN/AI07-3174/2007 (H6N1)	+	+	+	+	+
A/duck/PA/486/1969 (H6N1)	+	+	+	+	+
A/MALL/MN/AI07-3174/2007 (H6N4)	+	+	+	+	+
A/TY/ORE/1971 (H7N3)	-	-	-	-	+
A/NSHO/MN/A08-24-50/2008 (H8N4)	-	-	-	-	+
A/turkey/Wisconsin/1/1966 (H9N2)	-	-	-	-	+
A/RUTU/NJ/ AI00-1185/2000 (H10N7)	-	-	-	-	+
A/ABDU/NC/674-1066/2006 (H11N9)	-	-	-	-	+
A/RUTU/NJ/ AI07-677/2007 (H12N5)	-	-	-	-	+
A/LAGU/NJ/ AI08-1460/2008 (H13N9)	-	-	-	-	+

[a] Rabbit serum directed against the nucleoprotein of lpAIV (22).

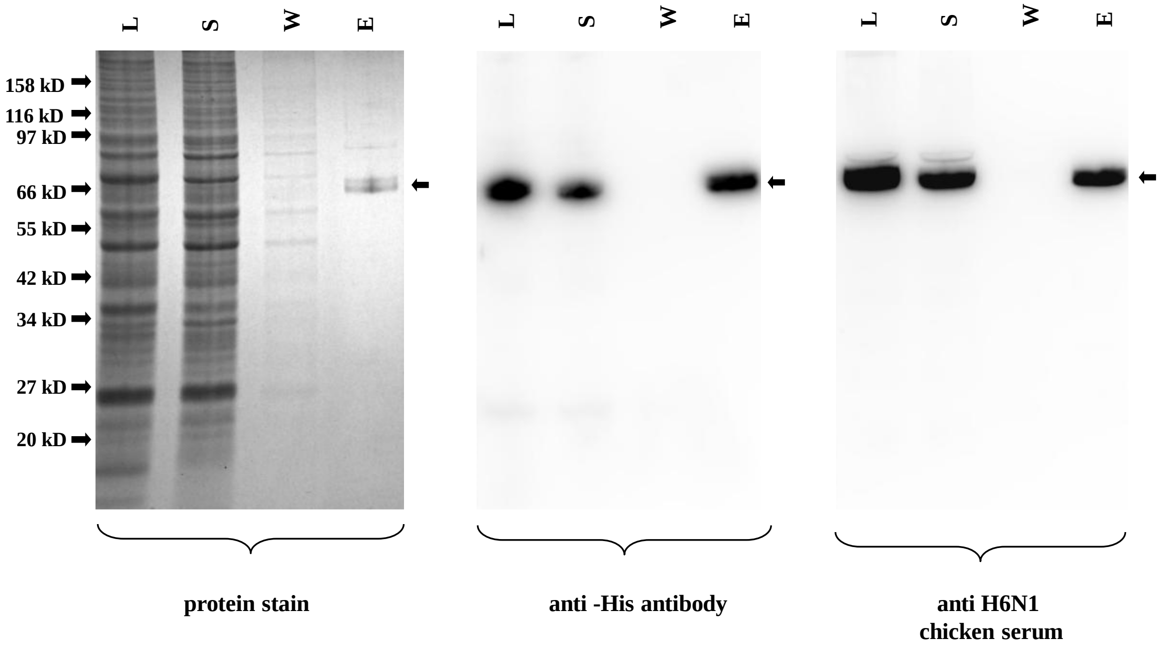


Figure 2.1 Expression and purification of H6 antigen. Samples of lysed cells (L), the supernatant after centrifugation (S), of one of the washing steps (W), and of a protein sample obtained after elution (E) were separated in 12% polyacrylamide gel by SDS-PAGE. The gels were either incubated with Imperial stain to visualize the proteins (protein stain) or processed for Western Blot using anti-His monoclonal antibody (anti-His antibody) or a polyclonal antiserum from a chicken infected with A/duck/PA/486/1969 (H6N1) (anti H6N1 serum). The binding of the mAbs was visualized by chemiluminescence using a peroxidase-labeled anti-species specific goat serum. A molecular mass marker (M) is shown at the left side and the position of the recombinant protein is marked by an arrow at each panel.

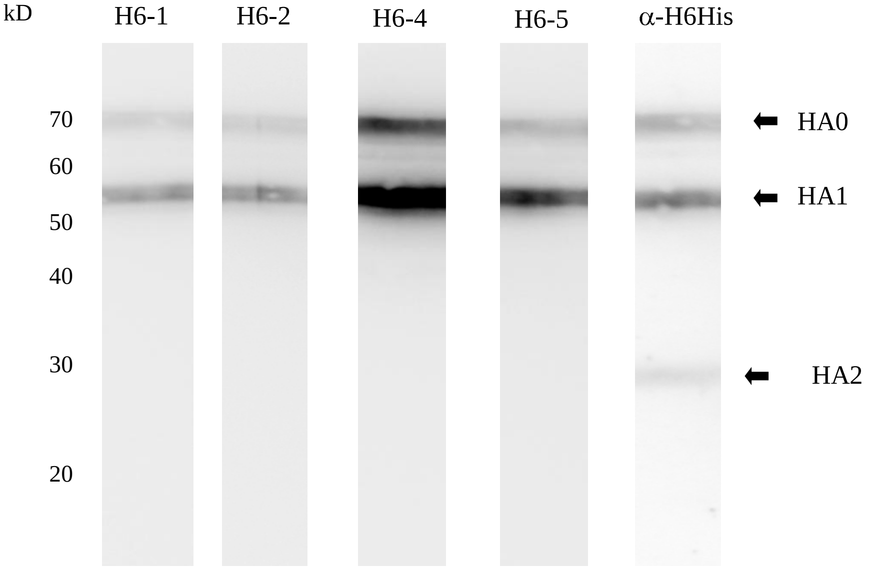


Figure 2.2 Monoclonal antibodies reacting with the HA1 subunit of the protein. Samples of allantoic fluid of A/duck/PA/486/1969 (H6N1) infected SPF eggs were separated in 12% polyacrylamide gel by SDS-PAGE. The gel was processed for Western Blot using the four H6 specific monoclonal antibodies (H6-1, H6-2, H6-4, H6-5) and the antiserum from the mouse which had been immunized with the purified recombinant H6-His antigen. The binding of the mAbs and the polyclonal mouse antibodies was visualized by chemiluminescence using a peroxidase-labeled anti-mouse specific goat serum. A molecular mass marker (M) is shown at the left side and the position of the different HA proteins were marked by an arrow.

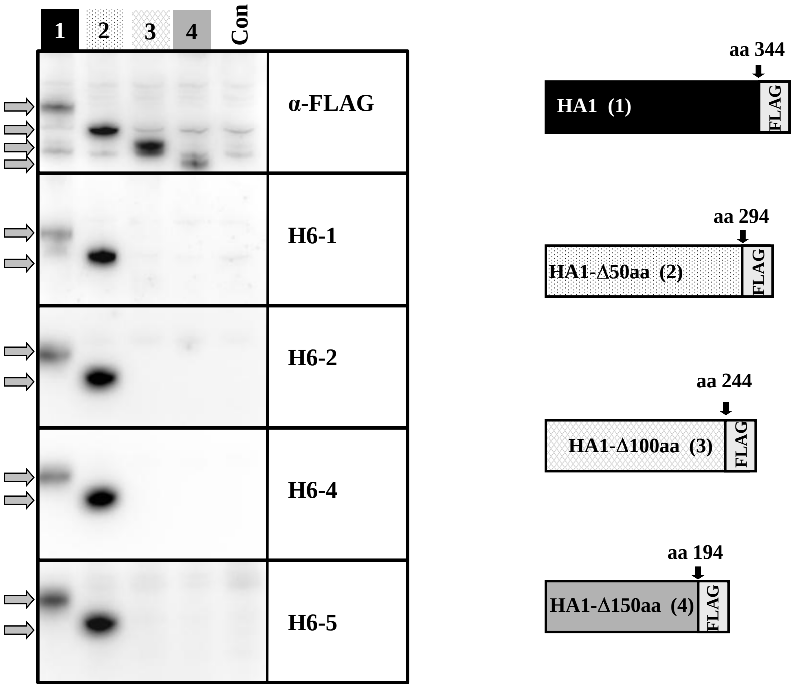


Figure 2.3 The monoclonal antibodies bind to the esterase domain of the HA6 protein. Cellular lysates of transfected DF1 cells were separated in 12% polyacrylamide gel by SDS-PAGE. Cells were transfected with plasmids encoding either the full length HA1 subunit of the H6 protein [HA1 (1)] or HA1 proteins successively truncated at the C-terminus by 50 amino acids [HA1-Δ50 (2)], 100 amino acids [HA1-Δ100 (3)], and 150 amino acids [HA1-Δ150 (4)]. The gels were incubated with the single H6-specific mAbs (H6-1, H6-2, H6-4, H6-5) or a monoclonal antibody specific for the FLAG-tag sequence encoded at the C-terminus of each recombinant protein. The binding of the different mAbs was visualised by chemiluminescence using a peroxidase-labeled anti-mouse specific goat serum. The numbers of the lanes on the gel correspond with the protein as shown in the schematic drawings. Which proteins have been recognized have been highlighted at the left side of the gel by a gray arrow at the appropriate gel.

CHAPTER 5

DISCUSSION

Influenza viruses are undoubtedly a group of significant pathogens for both humans and our domestic animal species. Given the increasingly rapid movement of people and products around the globe, coupled with the expansion of human habitation and animal production facilities into areas occupied by wild reservoir species, the emergence and spread of zoonotic diseases in general is of growing concern. With greater risk comes a greater need for control measures. Surveillance of circulating influenza viruses and institution of effective disinfection programs are two such measures which can curb the spread of avian influenza viruses and also other important pathogens.

We have shown that metam-sodium is able to completely inactivate both AIV, a representative enveloped virus, and IBDV, a representative non-enveloped virus, in contaminated chicken litter within a relatively short period of time. In the case of AIV, the necessary dose for complete inactivation was below that recommended by the manufacturer. These findings are significant because the rapid and safe disposal of poultry litter after a disease outbreak is a persistent problem (2). Although AIV has been categorized as a virus which is readily inactivated (8), it has been observed to persist in poultry litter anywhere from less than an hour (our observation) to several weeks (4, 6). Due to the high variability in AIV survival, it becomes necessary to take certain precautions with contaminated litter in order to ensure that the virus does not persist in the environment after an outbreak. IBDV, in contrast to AIV, is a highly resilient virus which can withstand many harsh chemical and physical treatments, including 0.5% phenol for five hours, incubation at pH 2 for one hour, and incubation at temperatures in excess of 65°C (1, 7). Any substance which can completely inactivate IBDV is therefore an asset.

Due to the highly porous nature of poultry litter, infectious virus particles can become sequestered within its complex matrix, out of reach of conventional disinfectants. Under these circumstances metam-sodium is advantageous because it rapidly forms the gaseous compound MITC upon exposure to moisture (9). MITC is able to thoroughly penetrate the contaminated litter, thus inactivating virus very efficiently in porous materials. Indeed, the value of metam-sodium/MITC has long been recognized by other agricultural sectors. The compound is already approved by the EPA for use as a soil fumigant for the prevention of destructive diseases which afflict major crops such as corn, potatoes, and barley. Metam-sodium could make a valuable addition to the poultry industry's disease-fighting arsenal.

In the area of surveillance, the competitive ELISA (cELISA) has emerged as a useful tool for the rapid and efficient screening of large numbers of serum samples for the presence of antibodies to the nucleoprotein of AIV. Specificities comparable to other antibody detection tests, such as AGPT and HI, have been reported for NP-specific indirect and cELISAs (10, 11). In addition to detecting antibodies raised against type A influenza viruses, it is also possible to tailor cELISAs for the detection of specific HA or NA subtypes (3, 5). We have generated and characterized four monoclonal antibodies raised against the H6 hemagglutinin subtype using a recombinant H6-protein expressed in a baculovirus system. It was determined that all four monoclonal antibodies bind to a region which is located between amino acids 244 and 294 in the HA1 portion of the H6 protein. Furthermore, one of the monoclonal antibodies was evaluated for potential application in an H6-specific cELISA. The cELISA developed with this monoclonal antibody showed a sensitivity of 39.7 % which was is insufficient when compared with the HI test and more work will need to be done in order to improve upon it.

Although a great deal of effort has recently been directed toward measures of avian influenza control, continued worldwide outbreaks indicate that there is yet more work to be done. Expanded AIV surveillance coupled with the development of improved antibody detection tests will allow us to detect outbreaks and potential sources of outbreaks before they have the opportunity to spread. In conjunction with such preventative measures, appropriate decontamination procedures can minimize the detrimental impact of disease.

References

1. Benton, W. J., M. S. Cover, J. K. Rosenberger, and R. S. Lake. Physicochemical properties of the infectious bursal agent (IBA). Avian Diseases. 11:438-445. 1967.

2. De Benedictis, P., M. S. Beato, and I. Capua. Inactivation of avian influenza viruses by chemical agents and physical conditions: a review. Zoonoses and Public Health. 54:51-68. 2007.

3. Dlugolenski, D., R. Hauck, R. J. Hogan, F. Michel, and E. Mundt. Production of H5-specific monoclonal antibodies and the development of a competitive enzyme-linked immunosorbent assay for detection of H5 antibodies in multiple species. Avian Diseases. 53:644-649. 2009.

4. Henzler, D. J., D. C. Kradel, S. Davison, A. F. Ziegler, D. Singletary, P. DeBok, A. E. Castro, H. Lu, R. Eckroade, D. Swayne, W. Lagoda, B. Schmucker, and A. Nesselrodt. Epidemiology, production losses, and control measures associated with an outbreak of avian influenza subtype H7N2 in Pennsylvania (1996-98). Avian Diseases. 47:1022-1036. 2003.

5. Liu, Y., E. Mundt, A. Mundt, M. Sylte, D. L. Suarez, D. E. Swayne, and M. García. Development and evaluation of an avian influenza, neuraminidase subtype 1, indirect enzyme-linked immunosorbent assay for poultry using the differentiation of infected from vaccinated animals control strategy. Avian Diseases. 54(Suppl. 1):613-621. 2010.

6. Lu, H., A. E. Castro, K. Pennick, J. Liu, Q. Yang, P. Dunn, D. Weinstock, and D. Henzler. Survival of avian influenza virus H7N2 in SPF chickens and their environments. Avian Diseases. 47:1015-1021. 2003.

7. Mandeville, W. F., Cook, F. K., and D. J. Jackwood. Heat lability of five strains of infectious bursal disease virus. Poultry Science. 79:838-842. 2000.

8. Prince, H. N. and D. L. Prince. Principals of viral control and transmission. In: Disinfection, sterilization, and preservation, 5th ed. S. S. Block, ed. Williams and Wilkins, Philadelphia, PA. pp. 543-571. 2001.

9. Smelt, J. H. and M. Leistra. Conversion of metham-sodium to methyl isothiocyanate and basic data on the behavior of methyl isothiocyanate in soil. Pesticide Science. 5:401-407. 1974.

10. Wu, R., S. Hu, Y. Xiao, Z. Li, D. Shi, and D. Bi. Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus. Veterinary Research Communications. 31:631-641. 2007.

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