MOLECULAR DETECTION AND IDENTIFICATION OF AVIAN INFLUENZA VIRUSES BY cDNA MICROARRAY

by

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ABSTRACT

Avian influenza (AI) is a respiratory viral pathogen of major concern to poultry producers and public health officials across the world. Rapid detection and subtyping of influenza viruses is necessary in order to control outbreaks and maintain routine surveillance. Microarray technology is a relatively new means of detecting pathogens and characterizing their genomic content. An avian influenza virus-specific cDNA microarray has been created and shown to correctly detect and identify the H5, H7, and H9 hemagglutinin subtypes, the N1, N2, and N3 neuraminidase subtypes, and the matrix gene of AI.

Our prototype AI cDNA microarray contains 16 elements representing the matrix, hemagglutinin, and neuraminidase genes of avian influenza isolates and a negative control from the F gene of Newcastle disease virus. These elements are spotted in duplicate in four subarrays yielding 8 spots per element and 128 spots total. To validate our microarray, an unknown panel comprised of 10 avian influenza isolates was tested. Of the ten isolates, 100% (10/10) were correctly identified as type A influenza viruses and 70% (7/10) were fully subtyped by their hemagglutinin and neuraminidase genes. Further characterization of 100% (4/4) of the H5 isolates was accomplished by quantifying hybridization signal strength between the extensive phylogenetic representation of the H5 hemagglutinin gene sequences on our microarray and the H5 isolates in the unknown panel.

Our results demonstrate the ability of a cDNA microarray to detect, identify, subtype, and phylogenetically/geographically group various avian influenza isolates. Our method as validated here can identify type A influenza via the conserved matrix gene,

differentiate between the H5, H7, and H9 hemagglutinin subtypes, and differentiate between the N1, N2, and N3 neuraminidase subtypes of avian influenza. Furthermore, our AI cDNA microarray demonstrates the ability to determine the phylogenetic/geographic group from which an H5 hemagglutinin subtype originates based on hybridization signal strength.

This method can be applied to clinical situations pending further validation experiments to determine the sensitivity of the array and increasing the number of representative HA and NA subtypes on the array (i.e. H1-4, H6, H8, and H10-16 and N4-9). Also, other viral and/or bacterial pathogens could be added to the array to increase its diagnostic power and aid the medical community in differential diagnoses.

Chapter 1

INTRODUCTION

Influenza viruses are named by their antigenic type (A, B, or C), host of origin (except human), geographic location, strain number, and year of isolation, followed by the HA and NA subtype designation. For example, an influenza type A virus isolated from a chicken in New York in 1994 that was subtyped as an H7N2 is officially an "A/chicken/New York/13142-5/94 (H7N2)".

Avian influenza is a type A influenza. There are 16 hemagglutinin subtypes and nine neuraminidase subtypes, all of which have been isolated from birds (H2, H3, H4, H5, H6, H7, and H9 hemagglutinin subtypes and N1, N2, N3, N4, N6, N7, and N9 neuraminidase subtypes have been isolated from chickens (Sharp *et al.*, 1997)). The HA and NA subtypes are not equally distributed: the H3, H4, H6 and the N2, N6, and N8 subtypes are the most commonly isolated subtypes from birds (Sharp *et al.*, 1997).

Avian influenza is highly contagious and affects many species of birds throughout the world. It was first recognized in the 1870s as "fowl plague" and the agent was found to be a virus in 1901 (Alexander, 2000). At the First International Symposium on Avian Influenza in 1981, the disease was officially termed "highly pathogenic avian influenza" (HPAI).

In the field, influenza A viruses infecting poultry flocks are divided into two groups based on their apparent pathogenicity: HPAI and low pathogenicity avian influenza (LPAI). HPAI is the group of AIV formerly known as "fowl plague" and can result in flock mortality as high as 100%. HPAI viruses are composed of the H5 and H7 hemagglutinin subtypes although not all viruses of these subtypes cause HPAI. LPAI viruses can belong to any hemagglutinin subtype and usually present as a much milder respiratory disease, causing depression and a decrease in egg production, but in conjunction with secondary viral and/or bacterial infections or poor environmental conditions, severe disease with high mortality may be seen.

The most important biological distinction between HPAI and LPAI virus is that HPAI replicates systemically and LPAI replicates mucosally. This phenomenon occurs because the HA protein of HPAI can be cleaved by ubiquitous endogenous proteases, found throughout the host system causing a systemic infection, whereas the HA protein of LPAI can only be cleaved by trypsin-like proteases, which confines replication to the respiratory and enteric tracts (Suarez and Schultz-Cherry, 2000). The HA protein differs between HPAI and LPAI by the addition of multiple basic amino acid residues at the HA cleavage site (Steinhauer, 1999).

Highly pathogenic avian influenza (HPAI) is defined by the Office International des Epizooties (O.I.E.) the World Organization for Animal Health based on an isolates' intravenous pathogenicity index (IVPI) and the amino acid sequence at the HA cleavage site (Tollis *et al.* 2002). The O.I.E defines AIV as HPAI if it meets one of three criterion: (1) it kills at least six of eight (75%) experimentally infected susceptible four to six week old chickens; (2) any H5 or H7 subtype that kills less than six of eight chickens, but has a

multiple basic amino acid sequence at the HA cleavage site indicative of HPAI; (3) any other HA subtype (not H5 or H7) which kills one to five, out of eight, chickens and grows in cell culture absent of trypsin (Tollis *et al.* 2002).

The European Union (EU) defines HPAI as "an infection of poultry caused by any influenza A virus which has an IVPI in six-week-old chickens greater than 1.2 or any infection with influenza A viruses of H5 or H7 subtype for which nucleotide sequencing detects the presence of multiple basic amino acids at the HA cleavage site (Council Directive, 1992).

Ultimately, the majority of H5 and H7 viral isolates are confirmed by nucleotide sequence analysis of the HA cleavage site. The time line for positively diagnosing AI is that once a sample has had a positive AI result by a molecular diagnostic, the results are then confirmed by isolating the virus is embryonating chicken eggs or cell culture. The virus is then subtyped and if it is H5 or H7, the HA cleavage site is generally sequenced to determine the pathogenicity of a strain (HP or LP), and selected viruses are testing by animal inoculation. In depth analysis of all eight gene segments can be used to look at other virulence factors, the presence of reassortants, and the progression of genetic drift due to mutations in the viral genome (Ellis and Zambon, 2001).

1.1 Clinical Signs and Pathology

In general, LPAI in poultry is characterized by a mild respiratory disease (coughing, sneezing, rales), decreased egg production in layers and breeders, and signs such as depression, decreased physical activity, decreased feed conversion rates, ruffled feathers, huddling, and diarrhea (Swayne and Halvorson, 1997). Gross lesions can also

be extremely variable. Lesions frequently occur in the respiratory tract, characterized by various forms of inflammation. The tracheal mucosa is often edematous with congestion and hemorrhages, and tracheal exudates ranging from serous to caseous, occasionally causing asphyxiation. Air sacculitis is also common and may be accompanied by fibrinopurulent inflammation, indicative of a secondary bacterial infection. In addition, there may be mucus or mucopurulent nasal discharge present in inflamed infraorbital sinuses. The peritoneal cavity may have catarrhal to fibrinous inflammation and catarrhal to fibrinous enteritis may also be noted in the ceca and intestine. Inflammatory exudates are often observed in the oviducts of layers. The eggs from infected hens are often misshapen and fragile due to the lower levels of calcium present in their shells (Swayne and Halvorson, 1997). The lesions from field infected birds are complicated by infections of other pathogens or poor environmental conditions. Infections of specific pathogen free birds typically shows only mild to no clinical lesions.

HPAI, in poultry, can cause peracute mortality. But the classical signs includes nervous signs such as tremors of the head and neck, torticollis, and opisthotonus. Respiratory distress (coughing, sneezing, rales) may also be observed. Depression, decreased physical activity, and decreases in food and water intake are also common.

In wild birds, AIV is usually subclinical because of the virus' inability to replicate efficiently. One documented exception to this general rule involved common terns in South Africa during an HPAI (H5N3) outbreak where sudden death occurred without any other clinical signs of disease (Becker, 1966). Gross lesions are often not observed when death is peracute. For less acute infections, a wide variety of lesions are observed. HPAI causes swelling of the head, face (especially the periorbital area), neck, and feet due to

subcutaneous edema. Petechial and ecchymotic hemorrhages may also accompany the swelling. Wattles and combs may also appear discolored due to necrotic foci, hemorrhage, and cyanotic tissue in those areas.

1.2 AI History and Outbreaks in the United States

The history of AI is divided into three chronological periods: 1) early reports of "Fowl Plague" (i.e. HPAI), 2) identification of LPAI, and 3) isolation of AIV from asymptomatic wild bird populations. In 1878, Perroncito first reported HPAI in Italy as "fowl plague" (Stubbs, 1948). In 1901, Centanni and Savonuzzi identified the cause of HPAI as being a filterable agent (i.e. virus), but it was not until 1955 that the agent, or virus, was identified as influenza (Stubbs, 1948). The first reported outbreak of HPAI in the United States occurred in 1924 in the live bird market (LBM) of New York and rapidly spread to New Jersey and Philadelphia, Pennsylvania (Stubbs, 1948). By 1925, the virus had spread to Connecticut, West Virginia, Indiana, Illinois, Michigan, and Missouri (Stubbs, 1948). Eventually, HPAI was eradicated from the United States by strategic quarantine, depopulation, cleaning, and disinfection (Stubbs, 1948). The strains involved in these initial US outbreaks were identified as belonging to the H7N1 and H7N7 subtypes (Easterday *et al.*, 1997).

Subsequent outbreaks of HPAI in 1959 and 1961 were caused by the H5N9 and H5N3 subtypes, which led investigators to conclude that all H5 and H7 AIV subtypes were highly pathogenic. This conclusion was later found to be false when in 1966 an LPAI of the H5 subtype was found to be responsible for causing a mild respiratory disease of chickens in Canada and the United States (Wisconsin) (Smithies *et al.*, 1969).

Further evidence for the existence of less pathogenic H5 or H7 subtypes was found in 1971 when an H7N3 causing mild respiratory disease and diarrhea (LPAI) was isolated from a flock of turkeys in Oregon (Beard and McCauley, 1972).

Since the 1970s, studies looking for Newcastle disease virus have isolated LPAI from asymptomatic free-flying wild bird populations (Slemons *et al.*, 1974). These healthy wild birds are now known to be the natural reservoir for AIV.

HPAI was once considered to be a rare occurrence in domestic poultry, with only 17 outbreaks reported between 1959 and 1998 (Alexander, 2001). Between 1998 and 2004, however, there have been eight episodes of HPAI involving 12 countries. In the past seven years, there has also been an increase in LPAI infections caused by the H5 and H7 subtypes (Capua and Alexander, 2004)

1.2.1 Pennsylvania 1996-1998

This outbreak involved an LPAI virus of the H7N2 subtype. From December 1996 to April 1998, over 2.5 million layer chickens on 24 farms and 47 flocks became infected and were consequently depopulated (Capua and Alexander, 2004). Only 25% of the infected birds showed signs of an LPAI infection, mainly upper respiratory signs and a temporary decrease in egg production (Davison *et al.*, 2003). Sequence analysis of the HA cleavage site determined the amino acid sequence at the HA cleavage site to be PENPKTR*GLF. This outbreak probably resulted from contact with the live bird markets (LBM) in New York City (Kleven, 1998).

1.2.2 Virginia 2002

An LPAI virus of the H7N2 subtype caused an outbreak in Virginia and parts of North Carolina and West Virginia in 2002. The outbreaks affected primarily turkey farms and some chicken farms. On a molecular basis, this strain was almost identical to the H7N2 AIV strain that had been circulating in the LBMs of the northeastern United States for the previous 8 years (Akey, 2002). The outbreak cost $149 million dollars and resulted in the depopulation of almost 5 million birds. Investigators found additional basic amino acid residues at the HA cleavage site, but the virus never mutated into HPAI (Spackman and Suarez, 2003).

1.2.3 Connecticut 2003

An LPAI virus of the H7N2 subtype was confirmed in New London County, Connecticut in March of 2003. Two separate outbreaks, involved 2.9 million table-egg layers from two commercial operations run by the same company. The control strategy implemented involved vaccination of hens that were initially infected but recovered, and the vaccination of replacement pullets (Capua and Alexander, 2004). The source of the outbreaks was thought to be from the LBMs based on sequence analysis. This case was unusual in that a vaccination strategy to control the outbreak was used instead of customary depopulation. Due to the enormity of the costs associated with potential depopulation, loss of income, indemnities, trade embargoes, and increased table egg prices totaling an estimated $194.2 million, the Connecticut Department of Economic and Community Development decided to, instead, employ a $16 million vaccination program (Regan and Prisloe, 2003). An inactivated oil-emulsion H7N2 vaccine was injected into

replacement layers (naïve pullets). The vaccination protocol required two injections, one month apart and the second vaccination was given two weeks before the replacement layers were placed in the layer house. Meanwhile, the vaccination of exposed layer hens was carried out by a single injection with the same vaccine. At the time, this process involved over 3.5 million birds in 38 layer houses in various stages of production. In addition to vaccination, 100 sentinel layer hens were placed in each house to serve as biological indicators of AI. Each week, 20 sentinels from each house were blood tested for the presence of AIV and overall flock morbidity/mortality was noted. Infected spent layers were replaced with vaccinated pullets after the layer house was thoroughly cleaned, disinfected, and tested for the presence of AIV. The vaccination program saved approximately 4 million layer hens and was therefore considered a clinical and economic success (Connecticut Annual Report of Accomplishments FY 2004). The 2003 Connecticut AI outbreak was unique in several ways. It involved a large number of layers, which is more costly than an outbreak in broilers because the production cycle of chick to pullet to layer takes 15 months. Most importantly, however, it was the first time vaccination was used to control an LPAI outbreak in the Northeastern USA.

1.2.4 Delaware, New Jersey, and Maryland 2004

An LPAI virus of the H7N2 subtype was confirmed by quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) on two farms in Delaware in February 2004. One farm was a non-commercial farm with approximately 11,000 chickens, which supplied the LBMs in northern New Jersey. It was in this region of northern New Jersey where the virus was eventually found on four farms (Capua and

Alexander, 2004). The other farm was a commercial operation with 85,800 broilers. AI was suspected because the birds were showing signs of respiratory illness (coughing, rales, sneezing). The Maryland outbreak occurred in March 2004 and was detected due to increased surveillance following the Delaware outbreak. The Maryland H7N2 virus was a considered to be a separate introduction because sequencing of the HA and NA genes showed that the two strains, Delaware and Maryland, were significantly different at the nucleotide level. In total, 424,800 birds were depopulated and the outbreak did not spread past the three areas (Capua and Alexander, 2004). The Delaware/Maryland AI outbreaks represent a classical case of LPAI and the strategies implemented to control it. The exceptional feature of this outbreak was that it was contained so rapidly and efficiently. Within two months University diagnostic laboratories, had tested 4,247 poultry houses representing 1,739 farms. The immediate quarantine, increased biosecurity and surveillance, and rapid depopulation were credited for the control of this outbreak.

1.2.5 HPAI

An HPAI virus of the H5N2 subtype was confirmed in a broiler flock in Gonzales, Texas in February, 2004. Clinically, this strain of AIV initially appeared to be of low pathogenicity due to its low mortality rate, but upon sequence analysis, the cleavage site in the HA protein was shown to be indicative of HPAI. The farm was depopulated shortly after HPAI confirmation. The source of this infection and its potential relatedness to H5N2 HPAI virus found in nearby Mexico is unknown. The Texas 2004 outbreak was unusual because it was the first time the USA had experienced

HPAI since 1984 and mostly because the virus was atypical in its presentation. The clinical signs of HPAI were not noted and the virus had a clinical presentation indicative of LPAI, causing low mortality and mild to severe respiratory disease. It was only upon nucleotide sequence analysis of the cleavage site that the virus seemed characteristic of an HPAI. This finding highlights the need for reliable and rapid clinical diagnostics in order to confirm or refute hypotheses based on clinical symptoms.

The H5N2 Mexico LPAI problem started in 1993 and continues to plague parts of Mexico. In 1993, an LPAI H5N2 virus was isolated from Puebla and Jalisco, Mexico. The LPAI virus eventually mutated into an HPAI virus with hemagglutinin cleavage site sequence of RKRKTR*GLF (Capua and Alexander, 2004). Mexico responded slowly and in 1997 employed a vaccination strategy to try to control the outbreaks. Over 2 billion doses of an inactivated H5N2 vaccine combined with a recombinant fowl Pox-H5 vaccine were used. Although the H5N2 HPAI virus has been eradicated in Mexico, the vaccination strategy did not work completely and LPAI H5N2 continues to circulate and infect poultry flocks in Mexico.

The last time HPAI was found in the United States was in Pennsylvania and Virginia in 1983. In April 1983, an LPAI H5N2 virus was isolated from commercial poultry operations in Pennsylvania. The virus spread to flocks throughout Pennsylvania causing low mortality (0-15%), decreased egg production and mild to severe respiratory illness. However, in October 1983 clinical signs indicative of HPAI (high mortality) were being noted and laboratory tests confirmed that the virus was now indeed an HPAI virus with an hemagglutinin cleavage site sequence of PQKKKR*GLF (Alexander, 2000). Depopulation was used as a control strategy, but the virus was persistent and

spread to Virginia, with a case being confirmed in July of 1984. In total, 17 million birds were slaughtered with direct costs totaling over $63 million and indirect costs estimated at over $250 million (Alexander, 2000) (WHO Update 31, 2004).

The reason for the rise in the number of AI outbreaks in the past seven years is not clear. The apparent increase in the occurrence of LPAI could be simply due to improved diagnostic tools and surveillance programs. Alternatively, these outbreaks could be due to climate changes the migration patterns of affecting free-flying wild bird populations, which could introduce new strains of the virus into susceptible populations. Another theory notes the growth in the number of commercial poultry operations that raise their chickens on open range, leaving them susceptible to interactions with wild birds (Capua and Alexander, 2004). The most likely explanation for a rise in the number of LPAI outbreaks is several factors having to do with antigenic shift and drift of the virus, changing environmental conditions, and an increase in poultry density.

1.2.6 Economic Significance

The economic significance of AI cannot be understated. In general, economic losses associated with AI correlate to the pathogenicity of the virus, the species of bird infected, the number of farms involved, and the speed with which control measures are implemented. The greatest losses occur when an HPAI outbreak occurs in a densely populated farming area. Significant costs are associated with the cleaning and disinfection of poultry houses, vehicles, processing plants, caging, and farm equipment. Direct costs are associated with depopulation, quarantine, indemnities, mortality, surveillance, and the loss of foreign export markets.

As examples, the 1924-5 HPAI outbreak in the United States cost over $1 million dollars in direct losses (Stubbs, 1948). Direct eradication costs from the aforementioned Pennsylvania 1983-1984 HPAI (H5N2) outbreak were over $63 million dollars, involving more than 17 million poultry and 449 commercial farms (Fichtner, 1987). The consumers were not spared from the costs and experienced a 30% increase in egg prices totaling $349 million (Swayne and Halvorson, 1997). The economic significance of LPAI outbreaks is slightly less due to lower mortality rates, alternative control measures (vaccination instead of depopulation), and the lack of trade embargoes/restrictions due to the non-reportable (internationally) nature of the disease. An example, the 1978 LPAI H6N1 outbreak in turkeys in Minnesota involved 141 flocks and cost producers $5 million while the 1995 LPAI H7N3 outbreak in turkeys in Utah involved 220 flocks and cost $2 million. The cost differential in the two aforementioned examples is that the turkeys in the Minnesota outbreak were depopulated using a controlled marketing program whereas the turkeys in the Utah outbreak were controlled similarly, but a vaccination control strategy was also employed (Halvorson *et al.*, 2003). A controlled marketing program is simply a plan to take flocks to market (slaughter) regardless of age in an attempt to accomplish depopulation without a complete loss of income.

To prevent outbreaks of AI, strict biosafety measures must be in place. Surveillance is probably the most important biosafety tool when it comes to disease prevention. Ideal surveillance requires routine sampling of live bird markets (LBMs), poultry operations, and wild bird populations. In reality, current surveillance methods are not routine and are usually in response to outbreaks in the area. Once samples are collected, scientists can characterize influenza strains and subtypes and begin to track

virus evolution due to antigenic shift/drift. Phylogenetic analysis based on the nucleic acid sequences of AIV has been useful in determining the rate of mutation, the geographic region from which a virus has originated, and the relatedness of human and avian influenza strains (Liu *et al.*, 2003). Phylogenetic sequence analysis was useful in characterizing the various sublineages of AIV present during the 1996-2001 H9N2 AI outbreak in China (Liu *et al.*, 2003).

In poultry management, prevention and control are the two most critical biosafety measures in combination with surveillance. The reservoir for AIV is wild birds, usually migratory waterfowl, and contact with free-flying birds or their feces represents a significant source of transmission. Although, ideally such contact should be nonexistent, or at least rare and limited, this is difficult to accomplish when commercial birds are raised on open range or when complexes are located on major migratory routes. Control can be accomplished by strict biosecurity measures, depopulation of infected flocks, in-house composting of carcasses, and cleaning and disinfection of farm equipment. Vaccination is considered a control measure in some cases of LPAI and will be discussed in detail later.

LBMs are a major source of AIV infection to the integrated poultry system. Transmission occurs mainly through the mechanical movement of fomites on equipment, clothing, or the shoes of people with direct contact between the LBM and commercial poultry operation. Evidence that AIV transmission from LBMs to commercial farms is mechanical and not airborne, airborne dissemination of AIV was evaluated in a large-scale air sampling study during the 1983-1984 HPAI (H5N2) outbreak in the United States. The results found that no influenza virus was in air samples collected 45 meters

downwind of AIV infected farms (Brugh and Johnson, 1987). This study illustrates the importance of controlling AIV infection via strict biosecurity measures in order to prevent introduction of the virus onto commercial farms.

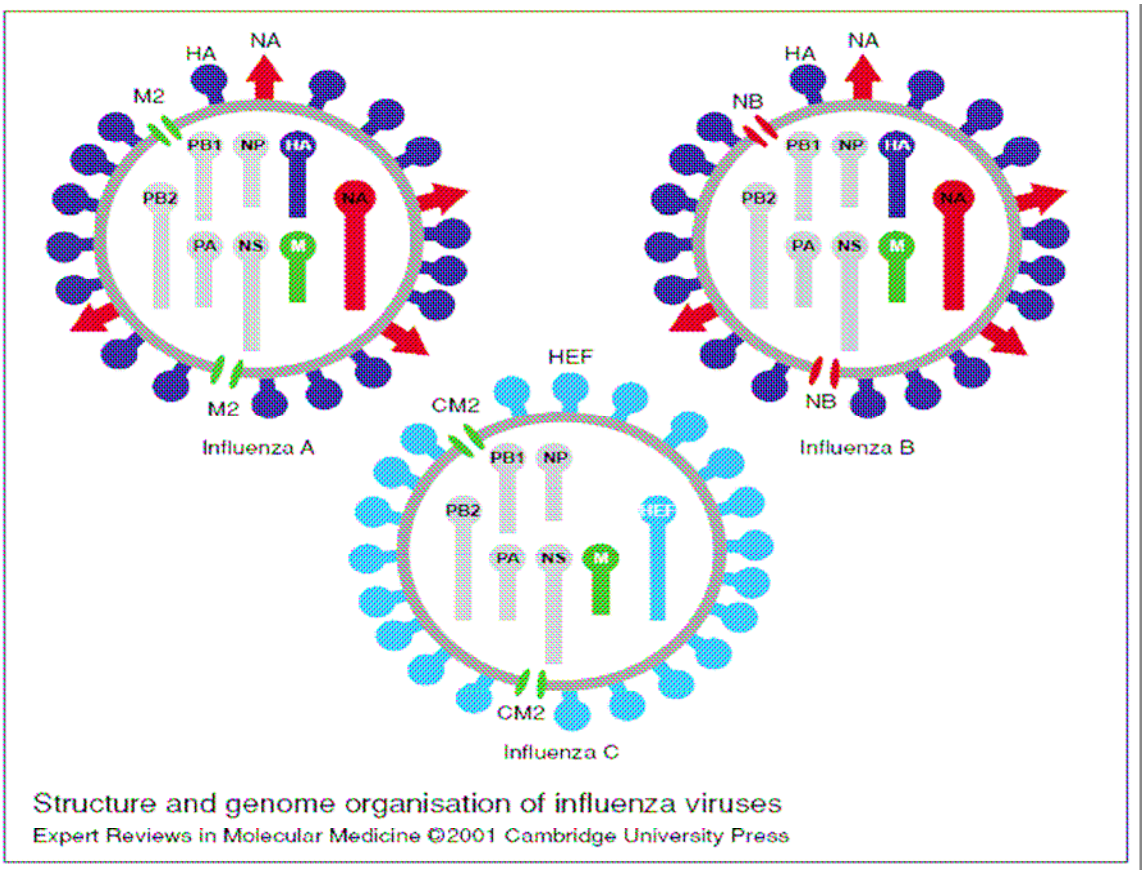
In summary, the economic significance of AI can be devastating to both the producer and the consumer. Examples of the recent outbreaks in the United States highlight this fact and although surveillance is expensive, it is a critical component of disease prevention and is necessary in order to properly manage this pathogen.

1.3 Biology of AI

1.3.1 Virus Characteristics

Influenza viruses are enveloped, single-stranded RNA viruses belonging to the *Orthomyxoviridae* family (Lamb and Krug, 2001). The influenza genome is comprised of eight negative-sense RNA segments. There are three types of influenza viruses: A, B, and C that are differentiated antigenically in their nucleoprotein and matrix proteins. Influenza A viruses are the most common and widespread, infecting many animal species, including humans. Types B and C mainly cause disease in humans, but recently, type B influenza has been isolated from seals (Osterhaus *et al.*, 2000). Type C influenza differs from the other two types of influenza in that its genome consists of seven segments due to the fact that it encodes HEF protein (HEF), which performs the functions of both the hemagglutinin and neuraminidase proteins (Figure 1.1).

Figure 1.1 Types A, B, and C Influenza: Structure and Genome Organization. This figure illustrates the different types, A, B, and C of influenza virus both structurally and in terms of genome organization.



Structure and genome organisation of influenza viruses

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Avian influenza is a type A influenza virus. Influenza A viruses are comprised of eight gene segments of negative sense single stranded RNA. The avian influenza viral genome is approximately 13,588 nucleotides in length and the complete influenza gene and protein function is summarized in Table 1.1. Each genome segment encodes one protein except segments 7 and 8, which encode two proteins via an alternative splicing event. The ten viral proteins can be divided into three categories based on their location within the virus. The surface proteins include the hemagglutinin (HA), the neuraminidase (NA), and matrix 2 (M2) proteins. The internal proteins include the four proteins that comprise the RNA-dependent RNA transcriptase complex: PA, PB1, and PB2, the nucleoprotein (NP), matrix protein 1 (M1), and nonstructural protein 2 (NS2). Finally, NS1 is a nonstructural protein that is the only protein not packaged into the virion but it is produced in large quantities in infected host cells. NS1 is an RNA binding protein responsible for inhibiting the processing of host mRNA. It also is responsible for regulating viral pre-mRNA splicing, translation, and polymerase activity, and inhibiting host anti-viral response via interferon pathways (Swayne and Halvorson, 1997).

The virion, 50-120 nm in diameter, is enveloped and pleomorphic. The envelope has surface projections embedded within its membrane, comprised of the antigenic determinants, HA and NA. The HA antigen is a homotrimeric protein that is proteolytically cleaved into the HA1 and HA2 subunits. It is the receptor that binds the virus to sialic acid residues on the surface of host cells thus allowing attachment of the virus to the cell. The NA antigen is a tetrameric protein, which serves as a receptor destroying enzyme (Brown, 2000). These receptor-destroying enzymes cleave a terminal

sialic acid residue from host cells and virion glycoproteins to prevent clumping of released virions due to HA binding. This activity has been suggested to be involved in initiating infection either by promoting fusion activity (Huang *et al*., 1980, 1985) or by releasing sialic acids near the receptor-binding site that could interfere with HA binding to cellular receptors (Ohuchi *et al*., 1995). NA also digests mucin, which enables the virus to reach target epithelium. Mucins are heavily glycosylated proteins secreted on mucosal surfaces that are saturated with oligosaccharides, thereby making them resistant to proteolysis and consequently providing a protective barrier at the mucosal surface. The full activity of the HA and NA proteins is discussed in greater detail later.

The matrix 2 (M2) integral membrane protein also exists as a tetramer and functions as a cation channel to acidify the endosome and dissociate M1 from the viral ribonucleocapsid (vRNP) (Brown, 2000). Each virion RNA (vRNA) segment is associated with nucleocapsid protein (NP) and with RNA polymerase complexes to form vRNP. At the interface between the cytoplasmic tails of the HA, NA, and M2 membrane proteins and the envelope interior, matrix 1 (M1) and non-structural protein 2 (NS2) are complexed with the vRNP (Brown, 2000).

The vRNA segments are transcribed into messenger RNA (mRNA) by a virally encoded transcriptase (PB1), which is then translated into the ten viral proteins. vRNA replication is conducted through an intermediate, complementary RNA (cRNA), which differs from the mRNA by lacking the 5' cap and 3' poly A tail. Influenza is a relatively unstable virus in the environment. The virus can be inactivated by heat, extreme changes in pH, and desiccation. Virus inactivation is vital to the control of AIV. Heating buildings to 37°C for seven days, complete removal of litter, proper litter disposal

Table 1.1. Influenza virus gene and protein information*.

Genome		Protein				
Segment	**Length**	**Name**	**Size**	**Localization**	**Type**	**Function**
	(nt)		(aa)			
1	2341	PB1	759	Virion interior, infected cell nuclei	Polymerase complex	Transcriptase
2	2341	PB2	757	Virion interior, infected cell nuclei	Polymerase complex	Endonuclease
3	2233	PA	716	Virion interior, infected cell nuclei	Polymerase complex	Replicase, proteolytic activity
4	1778	Hemagglutinin (HA)	566	Virion envelope, infected cell surface	Integrated type I membrane glycoprotein	Virus attachment to sialic acid containing receptors on host cells, penetration of virus genome into host cell cytoplasm, envelope fusion, antibody-mediated viral neutralization,hemagglutinating activity, major antigenic determinant
5	1565	Nucleoprotein (NP)	498	Virion interior, associated with the polymerase complex and vRNA	Major structural protein	Cytoplasmic to nuclear transport of vRNP, role in vRNA replication, role in virus maturation/packaging, antigen target for cytotoxic T lymphocytes
6	1413	Neuraminidase (NA)	454	Virion envelope, infected cell surface	Integrated type II membrane glycoprotein	Sialidase receptor-destroying enzyme, digests mucin enabling virus to reach target epithelium, facilitates release of infectious progeny, antibody-mediated virus neutralization restricting virus spread
7	1027	Matrix 1 (M1)	252	Beneath virion envelope, associated with the vRNPs in mature virion to form nucleocapsid	Non-glycosylated structural protein	Central role in replication, virus assembly/formation/budding, nuclear transport of vRNP, most abundant protein
		Matrix 2 (M2)	97	Virion envelope, infected cell surface	Integrated type III membrane glycoprotein	Membrane cation channel activity, raises pH of golgi to protect pH-sensitive HA, may permit acidification of virus interior during passage through endosomal pathway to dissociate vRNP from M1
8	890	Non-structural 1 (NS1)	230	Infected cell nuclei	RNA binding protein	Binds RNA, inhibits porcessing of host mRNA, regulates viral pre-mRNA splicing, translation, and polymerase activity, inhibits host anti-viral response via interferon pathways
		Non-structural 2 (NS2)	121	Associated with core components of virion, cytoplasm of infected cells	Nuclear export protein	Mediates nuclear export of vRNP

*Adapted from Baigent and McCauley, 2003 and Swayne and Halvorson, 1997.

(composting, incineration, or burial), cleaning and disinfecting buildings/equipment, and two to three week delays before restocking aids in the elimination of AIV (Halvorson, 1987). The virus is sensitive to sodium desoxycholate and sodium dodecylsulfate, organic solvents and detergents (Swayne and Halvorson, 1997). AIV is also susceptible to chemical inactivants such as formaldehyde, beta-propiolactone, and binary ethylenimine (Swayne and Halvorson, 1997). Chemical disinfectants such as phenolics, quaternary ammonium, sodium hypochlorite, and hydroxylamine can also inactivate AIV (Swayne and Halvorson, 1997).

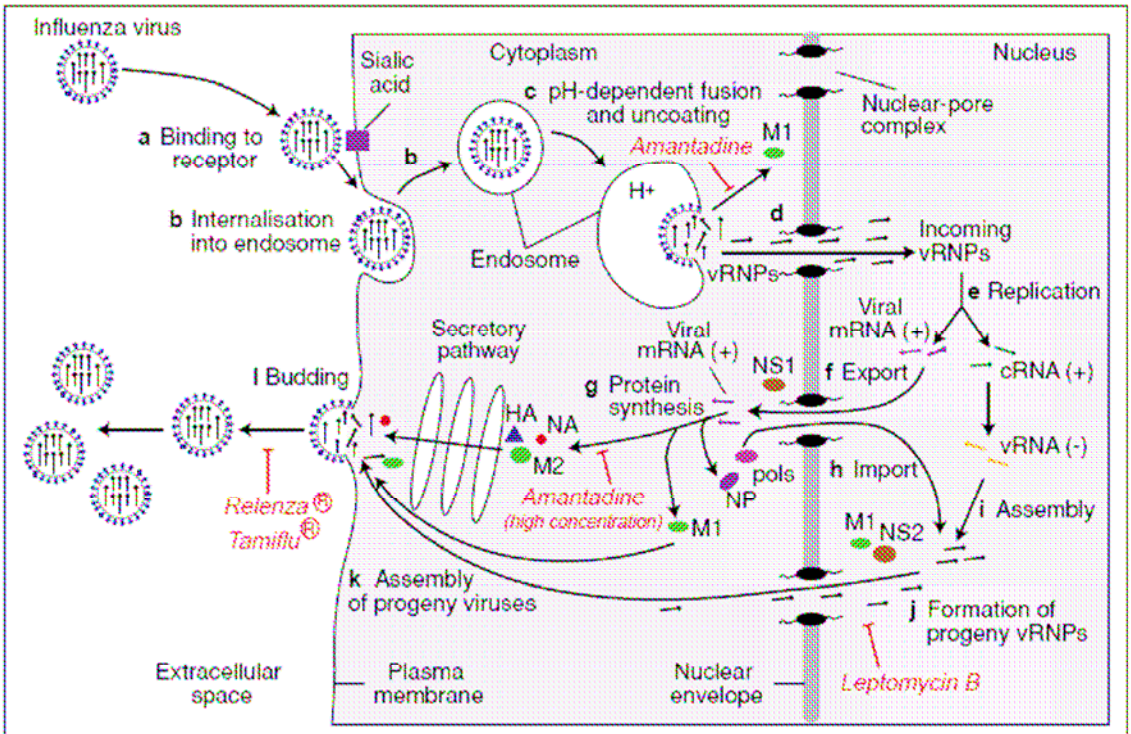
1.3.2 Infection, Replication, and Export

Influenza virus attaches to host cell glycoprotein receptors containing 5-*N*-acetyl neuraminic acid (sialic acid) via its HA spike. Some influenza viruses bind preferentially to terminal sialic acids containing α-(2,6) linkages and others prefer α-(2,3) linkages (Steinhauer and Wharton, 1998). Receptor binding specificity is correlated to amino acid position 226 of the HA protein. HAs containing a leucine at position 226 specifically bind α-(2,6) linkages, preferentially binding to human host cell receptors. HAs containing a glutamine at that position, specifically bind α-(2,3) linkages, preferentially binding avian and equine host cell receptors. Swine are considered the best reservoirs for the transmission of AIV between avian and human hosts because their tracheal epithelial cells contain both α-(2,3) and α-(2,6) sialic acid linkages. This makes swine susceptible to both human and avian/equine strains of influenza, resulting in potential coinfection and the possibility of recombination between the two viruses.

The infection, replication, and export of AIV particles is illustrated in Figure 1.2. Once the viral HA protein fuses with the host cell sialic acid receptor (Fig. 1.2a), the virus enters the cell via receptor-mediated endocytosis and is internalized into an endosome (Fig. 1.2b). There, the HA undergoes a conformational change triggered by the lowering of the pH that is characterized by the formation of a coiled coil comprised of alpha helices. This change exposes the previously buried hydrophobic fusion peptide located on HA2, which then inserts into the endosomal membrane (Fig. 1.2c) (Hernandez *et al.*, 1996). The low pH of the endosome acidifies the interior of the virion via the M2 membrane protein that channels protons into the virion thereby dissociating M1 from the vRNP. The vRNP is then released into the cytoplasm and transported to the nucleus (Fig. 1.2d) (Brown, 2000).

The vRNP, in the nucleus, is then replicated (Fig. 1.2e). There are two positive sense RNA transcripts synthesized in the nucleus, a messenger RNA (mRNA) for translation and a complementary RNA (cRNA) for replication. During transcription, mRNA is synthesized by an RNA-dependent RNA polymerase (consisting of PB1, PB2, and PA). Prior to transcription, the endonuclease activity of PB2 causes the excision of host mRNA 5' terminal nucleotides. This "cap-stealing" results in the removal of 10-13 nucleotides of the hosts' 5' cap, consisting of a methylated guanosine residue, which is then used to prime vRNA synthesis (Ortega *et al.*, 2000). The resulting viral mRNA is polyadenylated due to the presence of a poly-A signal in the 5' portion of the vRNA. During replication, RNA-dependent RNA polymerase is used to create full-length, positive-sense cRNA. The transition from transcription to replication is signaled by

Figure 1.2 Influenza virus replication. This figure illustrates the cycle of influenza virus replication (a-l) (Whittaker, 2001).



Replication cycle of an influenza virus

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increased levels of NP protein in the nucleus. cRNA never becomes capped or polyadenylated, it simply remains in the nucleus to serve as the template for further vRNA replication. Export of the eight viral mRNA segments from the nucleus is selectively mediated by NS1 (Chen and Krug, 2000). Upon exit from the nucleus (Fig. 1.2f), the mRNA fragments travel to the cytoplasmic ribosomes for translation (Fig. 1.2g). The mRNA of the M and NS genes are spliced into M1, M2, and NS1 and NS2 mRNAs and then translated to obtain a total of ten viral proteins. Six of the proteins (PB1, PB2, PA, M1, NS2, and NP) are imported into the nucleus to assist in vRNA replication (Fig. 1.2h) and the assembly of the vRNP (Fig. 1.2i). The HA and NA proteins are glycosylated in the rough endoplasmic reticulum and transported to the plasma membrane via the Golgi apparatus. Once in the Golgi apparatus, the HA glycoprotein is proteolytically cleaved from an uncleaved precursor (HA0) into HA1 and HA2 subunits, a step crucial to establishing fusion and infectivity (Swayne and Halvorson, 1997). Mature vRNPs form and leave the nucleus (Fig.1.2j). HA1, HA2, NA, and M2 are now embedded in the plasma membrane, and are joined by the eight viral gene segments and the internal viral proteins: PB1, PB2, PA, NP, and M1, which completes the assembly of progeny virions (Fig. 1.2k).

The M1 protein is responsible for budding of the progeny virions from the plasma membrane (Fig. 1.2l). M1, via electrostatic interactions, binds to the vRNP, the plasma membrane, and other M1 proteins in order to form a layer beneath the plasma membrane, which eventually forms the virus envelope (Ruigrok *et al.*, 2000; Whittaker, 2001). The location along the plasma membrane where budding occurs is referred to as a detergent-insoluble glycolipid-enriched domain (DIG) (Whittaker, 2001). Virus formation at these

sites is dependent on the cytoplasmic tails of the viral glycoproteins HA and NA, along with M1, M2, and host cell factors (actin cytoskeleton). These factors influence virus morphology: spherical or filamentous (Roberts and Compans, 1998) (Jin *et al.*, 1997) (Roberts *et al.*, 1998).

Release of the virus is dependent on the NA protein, which removes sialic acid viral receptors from the surface of host cells (Colman, 1989). If this step did not occur, the progeny virions would immediately clump to receptors and would not be released (Whittaker, 2001).

HA glycoprotein processing is a very important step in the infectious cycle. HA is synthesized in the rough endoplasmic reticulum as a precursor molecule (HA0). It is then proteolytically cleaved into two active subunits, HA1 and HA2, which are held together by disulfide bonds (Whittaker, 2001). Without this cleavage, acidification of the endosome would have no effect on the HA molecule, i.e. would not cause a conformational change, and would not expose HA fusion peptide necessary for insertion into the endosomal membrane and subsequent infection. The virion would be non-infectious. The proteolysis is dependent upon a certain amino acid sequence at the HA0 cleavage site. Most influenza strains have a single basic amino acid, arginine (R), at the cleavage site, e.g. HA1-PQVR*GLF-HA2. The enzyme that cleaves at that site is a tryptase Clara, which is a protease present in the epithelial cells lining the respiratory tract (Whittaker, 2001). Because the cleavage site is specific and the necessary tryptase is only present in the respiratory tract, most influenza viruses are limited in their infectivity to the upper respiratory tract. In avian influenza the situation is a bit different.

HPAI viruses have a multiple basic amino acid sequence at the HA0 cleavage site (RXR/KR*GLF motif) consisting of arginine (R) and lysine (K) amino acid residues (the star represents the cleavage site between HA1 and HA2). Conversely, LPAI virus strains have only two basic amino acids at the HA0 cleavage site at positions -1 and -4 for the H5 subtype and -1 and -3 for the H7 subtype (Wood *et al.*, 1993). The multiple basic amino acid sequence present at the HA cleavage site of HPAI viruses allows HA0 to be cleaved by ubiquitous proteases. Furin is an example of an enzyme that is present in the Golgi apparatus of all cell types and can cleave at the HPAI virus HA0 cleavage site (Whitakker, 2001). This feature allows HPAI to replicate systemically while LPAI replication is limited and localized because it's HA0 protein can only be cleaved by trypsin-like proteases present in the respiratory and enteric tracts.

1.3.3 Epidemiology

There are three major factors that determine an animal's relative susceptibility to AI: 1) likelihood of exposure to the virus, 2) virus virulence, and 3) the ability of the host to counteract pathogenic mechanisms of the virus. Other considerations include the host species, its age, sex, and environmental factors. Commercial poultry operations are often densely populated which makes the likelihood of exposure high for each individual bird if one bird in the house becomes infected or even if a nearby farm becomes infected. Exposure is also increased by the presence of free-flying wild birds, AIVs natural reservoir, in and around the farm. Virulence is always a concern due to the devastating effects of an HPAI outbreak and its ability to cause up to 100% mortality by natural infection and because depopulation is the primary control measure.

The incubation period varies by infective dose, age/species of bird, route of infection, and environmental conditions. AIV has an incubation period as short as three hours in intravenously (IV) inoculated birds to 14 days in a flock naturally infected with LPAI (Easterday *et al.*, 1997). Typically, in a naturally infected bird, AIV has an incubation period of approximately three days.

AIV is spread from the infected birds' nares, mouth, conjunctiva, and cloaca. Virus can be transmitted through aerosolized virus or virus-contaminated fomites (Easterday *et al.*, 1997). An initial outbreak is often caused by fomites that are contaminated with the feces of infected birds and transmitted by humans between farms, live-bird markets (LBMs) (Easterday *et al.*, 1997). Once one bird is infected, the most significant mode of transmission is via aerosolized virus from the respiratory tract of infected chickens because of the high viral concentrations present in the respiratory tract.

Intraspecies transmission of AIV occurs most readily, but interspecies transmission is also possible, occurring most often among closely related species within the same taxonomic order (chickens, turkeys, quail, guinea fowl). Interspecies transmission has also been shown to occur among different orders within the same class, e.g. free-flying ducks (*Anseriformes*) to chickens (*Galliformes*) (Swayne, 2000). Finally, interspecies transmission from chickens to humans has been documented (Swayne, 2000). Influenza readily adapts to a wide range of hosts, but is still limited by geographic host distribution, age and density of birds, various environmental conditions, and the cohabitation of different species.

1.3.4 Host Immune Response to AI

The host response to AIV varies greatly by species. Some strains of AIV that are highly pathogenic for chickens create no signs of disease in ducks (Alexander *et al.*, 1978). The difference in clinical signs is most probably related to the pathogenesis of AIV in different species, i.e. tissue tropism, and the different immune responses unique to each species. For example, replication of AIV in ducks is primarily enteric, while in chickens it is mainly respiratory (Webster *et al.*, 1978).

Immunity to AIV is controlled by both cell-mediated and humoral antibody responses. Natural infection with AIV elicits a humoral immune response in chickens at both the systemic and mucosal levels. The systemic antibody response is measured by IgM production five days post-infection (PI) and IgG and IgY production shortly thereafter. Antibody levels measured in response to AIV infection indicate that antibody titers are greatest for chickens and then pheasants, turkeys, quail, ducks in decreasing order (Suarez and Schultz-Cherry, 2000). The mucosal immune response has yet to be fully characterized and the protective effect of maternal antibodies against HA and NA has not been studied (Suarez and Schultz-Cherry, 2000).

Antibodies are produced against the AIV surface proteins HA and NA. These antibodies are capable of neutralizing the virus either by inhibiting its binding to the host cell by blocking its subsequent entry into cells. Antibody to the M2 protein has been shown to provide incomplete protection, but it does reduce the amount of viral shedding and produces some protection from disease (Suarez and Schultz-Cherry, 2000). Antibodies are also made to the nucleoprotein (NP) and matrix 1 (M1) proteins and are

used in two important diagnostic tests, the agar gel immunodiffusion test (AGID) and the enzyme-linked immunosorbent assay (ELISA).

The host immune response to AIV is complicated by the fact that influenza viruses have a high frequency of antigenic variation in their glycoproteins (HA and NA) due to antigenic shift and drift. In LBMs and wild bird populations, many different strains of AIV circulate and coinfection is suspected to be a common occurrence (Swayne and Halvorson, 1997). The immunological consequences to the host of a mutating virus are that the host is always susceptible to infection from a virus that has changed its antigenicity.

An important cell in the avian immune response is the macrophage. An avian macrophage cell line, HD11, supports the replication of thirteen of the fifteen HA subtypes, leading ultimately to macrophage cell death (Lyon and Hinshaw, 1991). AIV can also be isolated from splenic macrophages of infected birds suggesting that these cells became infected in vivo (Campen *et al.*, 1989). Influenza infected avian macrophages produce less nitric oxide (NO) than control cells and they fail to produce NO in response to lipopolysaccharide (LPS) stimulation, a known inducer of NO in avian macrophages (Lyon and Hinshaw, 1993). These are important findings, because NO is responsible for microbiocidal activity and for triggering the inflammatory response. LPAI infection was also shown to suppress the ability of pulmonary macrophages to phagocytize and to induce microbiocidal activity (Kodihalli *et al.*, 1994). In vivo and in vitro studies showing decreased macrophage function in AIV infections may also help explain why AIV infected birds are more susceptible to secondary bacterial infections.

1.3.5 Natural, Experimental, and Laboratory Hosts

AIV has been isolated from more than 90 species of birds across 17 orders (Table 1.2). Experimental studies have shown that AIV has the ability to infect pigs, mice, rats, rabbits, ferrets, guinea pigs, cats, mink, primates, and humans (Easterday *et al.*, 1997). Laboratory host systems are limited for propagating AIV. Chicken embryo fibroblast (CEF) and Madin-Darby canine kidney cell cultures can be used for plaque assays or virus neutralization tests. In CEF cultures incubated with LPAI, trypsin must be added to the agar in order for the cells to cleave the hemagglutinin precursor protein (HA0) (Easterday *et al.*, 1997). HPAI in cell culture does not require the addition of trypsin for HA0 cleavage and consequent infectivity.

1.4 Treatment and Vaccination

There are limited treatment options for avian influenza. Supportive care and the use of antibiotics to prevent secondary bacterial infection are sometimes employed. Two classes of antiviral drugs have been created in order to manage influenza infection. Amantadine and rimantadine are M2 protein inhibitors and oseltamivir and zanamivir are neuraminidase inhibitors (Nicholson *et al.*, 2003). Amantadine has been shown to reduce mortality, but this drug is not approved for use in food animals and amantadine-resistant viruses are rapidly produced in response to its application (Beard *et al.*, 1987). For the most part, infected flocks are depopulated and cleaning and disinfection is done to prevent further spread of the disease.

The use of vaccines to control AIV outbreaks is the cause of much debate. The general consensus is that vaccination should be avoided. The major drawbacks

Table 1.2 Natural, Experimental, and Laboratory Hosts for Avian Influenza. Seventeen taxonomic orders of birds are capable of becoming infected with avian influenza virus.

1) Anseriformes (ducks, geese, swans)
2) Casuariiformes (emu)
3) Charadriiformes (turnstones, sandpipers, gulls, terns, puffins, guillemots)
4) Ciconiiformes (herons, ibis)
5) Columbiformes (doves)
6) Falconiformes (raptors)
7) Galliformes (chickens, turkeys, quail, pheasant, guineafowl, partridges)
8) Gaviiformes (loons)
9) Gruiformes (coots, moorhen)
10) Passeriformes (mynahs, finches, weaverbirds)
11) Pelecaniformes (cormorant)
12) Piciformes (woodpecker)
13) Podicipediformes (grebe)
14) Procellariiformes (shearwater)
15) Psittaciformes (parrots, cockatoos, parakeets)
16) Rheiformes (rhea)
17) Struthioniformes (ostrich)

(Stallknecht and Shane, 1988) (Easterday *et al.*, 1997)

associated with vaccination are the inability to distinguish infected from vaccinated birds by serological methods, the observation that virus can persist in the absence of clinical signs, the variable protective quality of vaccines, and the belief that vaccination puts selective pressure on the virus to mutate (as has been shown for human influenza virus) (Tollis and Trani, 2002). The positive aspects to vaccination are associated with the decreased cost of the outbreak because eradication is not necessary and birds can still be sold. Vaccination is routinely used in turkey production in the Midwestern United States and was successfully used to control an outbreak of LPAI in Connecticut in 2003. Conversely, a vaccination program utilized in Mexico in 1995, was unsuccessful at eradicating LPAI. In this case, vaccinations were administered in an attempt to control an LPAI outbreak that had become an HPAI outbreak. The program was unsuccessful because the LPAI virus continues to circulate and mutate in Mexico to this day.

1.4.1 Whole Virus Vaccines

Vaccination of chickens with inactivated influenza vaccines has been shown to be an effective tool in the control of AI, although this leads to seropositive birds, which hinders surveillance and epidemiological studies. To counter this, the Differentiation of Infected from Vaccinated Animals (DIVA) system has been developed (Lee *et al.*, 2004). This method of vaccination uses a live reassorted influenza virus that contains a neuraminidase (NA) gene that does not normally exist in poultry in a certain country or region (e.g. N1 and N8 in the United States) while maintaining the hemagglutinin (HA) gene of the challenge virus. Lee *et al.* (2004) reported that reassorted DIVA influenza vaccines (rH5N1 and rH7N8) produce similar antibody profiles and protection rates to

vaccines made from parent H5N2 and H7N2 viruses. More importantly, sera from infected and vaccinated birds can be differentiated by neuraminidase inhibition tests and indirect immunofluorescent antibody assays (Lee *et al.*, 2004).

The DIVA strategy was successfully employed in Italy during a 2000-2002 AI outbreak. An H7N3 (A/CK/Pakistan/95) vaccine was used in combination with biosecurity control measures in the eradication of an H7N1 LPAI virus. These results are promising in that there now exists a way to vaccinate birds in order to treat/control an AI outbreak without compromising surveillance. The drawbacks to this method are that the HA type must closely match the outbreak HA type, and the NA subtypes of vaccine and challenge viruses must differ (Lee *et al.*, 2004). A vaccine bank has been proposed in order to provide different HA and NA types for vaccine creation.

Vaccination was also effective in interrupting virus transmission in chickens during a HPAI H5N1 outbreak in Hong Kong in 2002 (Ellis *et al.*, 2004). During the outbreak, infected flocks were depopulated and biosecurity was increased, while three surrounding unaffected farms were vaccinated with a killed H5N2 vaccine. Infection spread to two of the vaccinated farms but low mortality rates were observed 9-18 days post-vaccination (Ellis *et al.*, 2004). Asymptomatic shedding of the H5N1 virus was not detected via viral culture or real-time reverse-transcription polymerase chain reaction (qPCR). This study illustrates that a killed H5 vaccine can stop virus transmission and shedding, both major goals of vaccination strategies.

An inactivated avian influenza H7N2 virus vaccine was also evaluated using a Virginia LPAI isolate from 2002. The inactivated vaccine was prepared from a A/Chicken/Pennsylvania/21342/97 (H7N2) virus, a strain very similar to the field virus

(A/Turkey/Virginia/158512/02 (H7N2)). The vaccine significantly reduced viral shedding but did not prevent infection (Tumpey *et al.*, 2004). The benefit of using this vaccine was that by reducing viral shedding, bird-to-bird transmission also decreased. The drawback of this method of vaccination was the creation of seropositive birds that hindered surveillance and epidemiological studies.

1.4.2 Subunit Vaccines and Short Interfering RNAs

Most subunit vaccines target the HA gene. This includes various DNA vaccines, plant based vaccines, and recombinant or vectored vaccines (Suarez and Schultz-Cherry, 2000). The advantage of HA subunit vaccination is that serological surveillance is not disturbed because serum from HA subunit vaccinated birds does not react to the AGID test which detects antibodies to the matrix or NP, and can therefore be distinguished from naturally infected birds (Swayne *et al.*, 2000).

The use of short interfering RNAs (siRNAs) to prevent and treat influenza virus infection has been studied in humans. siRNAs are short (20-25) nucleotide double-stranded RNA molecules that induce a phenomenon known as RNA interference (RNAi). RNAi involves the sequence-specific degradation of messenger RNA (mRNA). siRNAs recognize and cleave target viral mRNAs, therefore interfering with viral gene expression in a sequence-specific manner (Elbashir *et al.*, 2001). siRNAs targeting toward regions of the influenza viral genome have proven to be powerful inhibitors of viral replication in cell lines and embryonating chicken eggs (Ge *et al.*, 2004). Influenza virus is a good candidate for siRNA technology because it is an RNA virus with multiple targets and

siRNAs can be administered via inhalation, targeting the site of entry for influenza virus (Ge *et al.*, 2004). However, this approach is unlikely to ever be cost effective in poultry.

1.5 Avian Influenza Diagnostics

Methods for detecting and diagnosing influenza rely on direct and indirect detection. Indirect methods detect an antibody to different influenza proteins, which may lack specificity, while direct methods depend on detecting the actual virus particle or a portion of the viral genome. Diagnostic methods for influenza differ in their cost, complexity, specificity, and sensitivity.

1.5.1 Indirect Methods of Detection

1.5.1.1 Serology. The HA protein can be detected by an indirect antibody test, the hemagglutination inhibition assay (HI). The specific attachment of HA antibody, present in the sera of infected birds, to the HA antigen molecule interferes with the binding between the HA protein and receptors on erythrocytes. This test is performed by co-incubating the allantoic fluid from AIV infected embryonated chicken eggs with individual standard reference sera (containing HA antibodies) for each of the HA subtypes and chicken erythrocytes. A positive result is indicated by the inhibition of hemagglutination of the erythrocytes because the reference sera antibodies were able to neutralize the HA antigen in the allantoic fluid and therefore prevent hemagglutination. This test is used to subtype the 16 different HA types by measuring the ability of reference serum to block the hemagglutination of a constant amount of virus (Suarez and Schultz-Cherry, 2000). A similar serological method is employed to identify the nine

different neuraminidase subtypes. The advantage to this method of subtyping is the fact that results are almost instantaneous; a disadvantage is the potential cross reactivity of one subtype to another based on nonspecific antigen-antibody interactions.

1.5.1.2 Agar Gel Immunodiffusion. The basic science behind the Agar Gel Immunodiffusion (AGID) test is the concurrent migration of antigen (nucleoprotein (NP) or matrix 1 (M1) protein) and antibodies toward each other through an agar gel matrix. When the antigen and specific antibodies come in contact, they combine to form a precipitate that produces a visible line in the agar. Differences in the relative concentration of the antigen or antibodies will shift the location of the line towards the well with the lowest concentration or result in the absence of a precipitin line (Beard, 1970). AGID is performed using serum from an infected bird or with allantoic fluid from infected embryonating eggs. The main advantage inherent in this method is its simplicity. The disadvantage is that it is not as sensitive as other commercially available methods like the ELISA and HI tests and when used for subtyping with reference sera, cross-reactivity is often observed (Meulemans *et al.* 1987).

1.5.1.3 Immunofluorescence. Immunofluorescence (IF) detects viral antigens (matrix, nucleoprotein, hemagglutinin) or virus-specific (IgG, IgA, or IgM) antibodies in clinical specimens. IF can be conducted using nasopharyngeal aspirates, nose and throat swabs, or bronchoalveolar lavage. Monoclonal antibodies to HA, MA, or NP are conjugated with a fluorochrome, such as fluorescein, and applied to a clinical specimen on a slide (Petric *et al.*, 2006). Examination with a fluorescence microscope reveals the

labeled antibody bound to infected cells expressing antigen or antibody. Most commercially available tests contain antibodies that are directed towards the matrix protein. The advantage of IF is its ability to be performed directly on clinical material, therefore bypassing the need for eggs or cell culture. It is also a rapid assay, taking 2 hours to complete. The drawback to this method is that it is low throughput and low sensitivity.

1.5.1.4 Enzyme Linked Immunosorbent Assay. The Enzyme Linked Immunosorbent Assay (ELISA) is an immunological technique that is used to detect the presence of antibody or the presence of antigen in a sample. ELISA uses two antibodies to detect antigen, one antibody is specific for the antigen and the other reacts to antigen-antibody complexes and is linked to an enzyme (Goldsby *et al.*, 2003). In other words, ELISA uses enzymes instead of fluorochromes to label antibodies directed against the influenza nucleoprotein (NP) or matrix protein (M1). Typical enzyme labels are alkaline phosphatase, horseradish peroxidase, and β-galactosidase. ELISAs usually use a change in color to signify a positive result, which requires specialized equipment to quantitate the amount of antigen. ELISAs can either be used to detect antibody, which is called the indirect ELISA, or to detect antigen, which is called the direct ELISA. ELISA can be performed on serum, nasopharyngeal aspirates, nose and throat swabs, and bronchoalveolar lavage to detect influenza. There are numerous advantages to this diagnostic method including its sensitivity, rapidity (15 min to 24 hr), high throughput, and low skill requirement. The disadvantage is that no virus is recoverable and it can be costly.

1.5.2 Direct Methods of Detection

1.5.2.1 Virus Isolation. The gold standard for detecting AIV is virus isolation via viral culture in embryonating chicken eggs followed by subtyping using the hemagglutination inhibition (HI) assay and neuraminidase serology. Virus isolation can be conducted from nasopharyngeal aspirates, nose and throat swabs, bronchoalveolar lavage, or environmental samples (Ellis and Zambon, 2002). The sample is inoculated into the amniotic cavity of 10-12 day old embryonating chicken eggs and incubated at 37°C. AIV replicates in the amniotic membrane and is released into the allantoic-amniotic fluid. Allantoic fluid is harvested 48-72 hours post-inoculation. The presence of virus particles in the allantoic fluid is confirmed by the hemagglutination test in which the allantoic fluid, containing virus, is coincubated with chicken erythrocytes (red blood cells). A positive result is indicated by the hemagglutination of the erythrocytes.

AIV will also replicate in chick embryo fibroblasts (CEFs), Madin-Darby canine kidney cells (MDCKs), or primary rhesus monkey kidney cells (PMKs). AIV produces a limited cytopathic effect (CPE) in these cell culture systems and is therefore hard to characterize. As previously mentioned, to detect LPAI in CEF cell culture, trypsin must be added, whereas with HPAI, no trypsin is needed.

Virus isolation is a sensitive and reliable method of AIV identification. The advantages are that the virus is recovered. The main drawback to virus isolation is the fact that it can only detect live virus and requires higher levels of biosecurity. Some of the other disadvantages of this method are that it is time intensive and costly.

1.5.2.2 Reverse Transcription Polymerase Chain Reaction. Reverse transcription polymerase chain reaction (RT-PCR) amplifies a specific region of AIV RNA using synthetic oligonucleotides (primers) in vitro. Genome specific primers designed for the gene of interest (M, HA, NA) are combined with reverse transcriptase, dNTPs, buffer, and template (vRNA) (Offringa *et al.*, 2000). The first step in the reaction is a reverse transcription reaction followed by a polymerase chain reaction step. Then a three-step cycling process occurs. The steps in the cycling process denature the template cDNA, anneal the primers to the template and extend the polymerase transcription on the template. The final product is a double stranded cDNA derived from the RNA.

RT-PCR can amplify the nucleic acids of the influenza genome from nasopharyngeal aspirates, nose and throat swabs, bronchoalveolar lavage, and post-mortem tissue (Ellis and Zambon, 2002). RT-PCR has been used to detect influenza A viruses from many different species by amplifying the highly conserved matrix gene (Fouchier *et al.*, 2000). This method is advantageous because it can detect all influenza A viruses and is high throughput. RT-PCR is extremely sensitive and specific, and results can be obtained within 8 hours (Cattoli *et al.*, 2004). The disadvantage to this method is that it requires specialized equipment. Due to the sensitive nature of RT-PCR, great care must be taken to avoid contaminating DNA or RNA. Another disadvantage is that RT-PCR requires multiple reactions with different primer sets in order to detect and amplify the HA gene due to the highly variable nucleotide composition of HA influenza genes.

1.5.2.3 Real-Time Reverse Transcription Polymerase Chain Reaction. Real time reverse transcription polymerase chain reaction (RRT-PCR), also called quantitative PCR (qPCR), is technically similar to RT-PCR but is a quantitative technique. It is a method that detects the presence and quantifies the amount of specific nucleic acid sequences in real-time. Gene specific primers are still used, just as in RT-PCR, but in qPCR an additional primer is labeled with a fluorophore at the 5' end, and a quencher dye is located at the 3' end of the probe. This method is based on the detection and quantitation of the fluorescent reporter. During the extension phase of PCR, the probe is cleaved by the 5'-3' exonuclease activity of *Taq* polymerase and the fluorophore and the quencher dye separate. This results in emitted fluorescence that is proportional to the amount of accumulated PCR product. The qPCR technology detects fluorescence in real time. qPCR is unique because the higher the starting copy number of the nucleic acid target, the sooner a detectable increase in fluorescence is observed, allowing for rapid results. The advantage of qPCR is its speed, limited sample handling, sensitivity, specificity, and high throughput ability (Spackman *et al.*, 2002). The disadvantage to this method is that it requires specialized equipment, and cannot account for sequence variation in gene segments because of the specificity of the genome specific primers.

A qPCR assay based on the AIV matrix gene has been developed and is capable of detecting all type A influenza viruses. qPCR primers have also been developed to detect North American avian H5 and H7 subtypes. qPCR was compared to VI and HI using 1,550 tracheal and cloacal swabs from various species of bird and environmental swabs taken from the LBMs of New York and New Jersey. The qPCR results correlated with VI in 89% of the samples and the rest of the samples were positive with either VI or

HI (Spackman *et al.*, 2002). Unfortunately, in 11% (165/1550) of the samples, the qPCR and VI results differed. In these 165 samples, 101 tested positive by VI and negative by qPCR while the remaining 64 samples tested positive by qPCR and negative by VI. These results illustrate the necessity of using multiple testing methods in order to corrobrate any diagnostic test. The power of this method of AIV detection lies in its rapidity (<24 hours) and it is amenable to high throughput techniques and is cheaper on a cost-per-sample basis than VI (Spackman *et al.*, 2002).

1.5.2.4 Nucleic Acid Sequence-Based Amplification. Nucleic acid sequence-based amplification (NASBA) is an alternative and robust amplification technique. NASBA technology utilizes reverse transcriptase, T7 RNA polymerase, and RNase H to yield a single-stranded RNA product. A target-specific capture probe in real time detects this RNA product. This probe has a 5' fluorescent dye and a 3' quencher molecular and works similarly to qPCR in that, in the presence of a complementary target sequence, the probe hybridizes to the target separating the reporter dye from the quencher molecular resulting in a measurable increase in fluorescence. NASBA has been successfully used in detecting HIV-1, HCV, rhinoviruses, and enteroviruses (Ellis and Zambon, 2002). Recently, this technology has been applied to AIV, detecting H5 subtypes isolated from a Eurasian lineage (Collins *et al.*, 2002). Generic and virus-specific primers were used to amplify LP and HP H5 HA sequences. The advantage of this method is that it is rapid (<4 hr), specific, and has the potential to detect multiple strains within a subtype. Also, the NASBA-based pathogenic H5 test has one primer located at the cleavage site of the HA0 gene, which can give a rapid confirmation that a newly isolated H5 influenza virus

is likely to be a HPAIV (Collins *et al.*, 2002). The disadvantage to this method is that it is highly sequence specific, which limits its ability to detect a virus that is constantly mutating at the nucleotide level. In dealing with type A influenza, qPCR has its limitations. Due to the high sequence homology within subtypes, strain differentiation is not possible. Also, due to the changing nucleotide composition of AI, qPCR primers must be constantly reevaluated. qPCR can be multiplexed, but has yet to be able to detect more than two genes per assay and is therefore limited for fully subtyping AI. Usually, qPCR must be combined with sequencing in order to confirm results or discriminate between subtypes. This is labor-intensive, time-consuming, and adds to the overall costs of diagnosis.

1.5.2.5 Microarrays. A microarray is an ordered array of nucleic acids (complementary DNA) fixed to a glass slide in known positions. cDNA on a slide (probe) and fluorescently labeled DNA or RNA (target) hybridize based on their complementary nucleotide sequences and this fluorescence is then detected and measured by a laser or white-light scanner. Microarrays are usually used to quantify gene expression levels on a whole genome level, but when used as a detection method, microarrays identify the presence of viral genes within a sample. Specifically, avian influenza gene probes representing the matrix, hemagglutinin, and neuraminidase genes are spotted onto a glass slide. The DNA probes are then hybridized to a clinical sample (blood, allantoic fluid, tracheal swab) from an infected bird. This clinical sample is usually RNA isolated from an infectious sample that is reverse transcribed into cDNA, which is then fluorescently labeled. The difference between microarray technology and

many other diagnostics is that microarrays have the ability to detect hundreds of nucleic acid sequences simultaneously, whereas most systems can only detect one virus or just one viral gene at a time.

Previous studies using microarrays as a method to detect and subtype influenza virus isolates have been successful and are gaining popularity as another assay to confirm established laboratory tests. Microarray detection of viral pathogens using long oligonucleotides (70-mer) has been used to detect a wide variety of human respiratory pathogens (Wang *et al.*, 2002). Researchers at the Howard Hughes Medical Institute were able to detect respiratory syncytial virus (RSV), parainfluenza3, adenovirus 12, and human RVs 1b, 2, 14, 16, 21, 62, 65, and 72, and poliovirus1 (Wang *et al.*, 2002).

There is also a commercially available DNA Flow-Thru Chip (synonymous to a DNA microarray) that has been used to type and subtype human influenza viruses (Kessler *et al.* 2004). The DNA Flow-Thru chip is a three-dimensional bio-chip that contains immobilized oligonucleotide probes for the influenza A and B genes in microchannels of silicon wafers. The chip is able to detect the influenza A virus matrix protein gene; the influenza B virus NS gene; the H1, H3, and H5 hemagglutinin genes; and the N1 and N2 neuraminidase genes from human influenza isolates (Kessler *et al.*, 2004).

In a study conducted by Li *et al.* (2001), human influenza viruses were subtyped using a DNA microarray. This study used primers capable of amplifying 26 different portions of the influenza A and B virus H1, H2, H3, N1, N2, N3, and M genes. The amplified portions of influenza were approximately 500 base pairs in length and were spotted onto a glass slide. The clinical samples used to hybridize to the array were the

same human influenza virus isolates that were used for probe design. These isolates were inoculated into 10-day-old embryonating chicken eggs and vRNA was extracted via a Trizol® extraction. An RT-PCR reaction incorporating fluorescently labeled nucleotides (Cy3- and/or Cy5-dCTP) was used to create labeled cDNA. Hybridizations were conducted at 58-62°C overnight. The results showed the ability of a DNA microarray to differentiate between type A and B influenza and to differentiate H1, H2 or H3 hemagglutinin subtypes and N1 or N2 neuraminidase subtypes.

Microarray technology has also recently been applied to the detection of equine influenza A viruses (Sengupta *et al.*, 2003). In this study, 476 influenza virus-specific oligonucleotides, 21 base pairs in length, were spotted onto a slide. The oligonucleotides were chosen from VirOligo, an internet-based database of published virus-specific oligonucleotides (Onodera and Melcher, 2002). Target cDNAs, consisting of three equine, two human, and one lab strain of influenza, were labeled and hybridized to the oligonucleotide microarray. Preparation of the sample for final hybridization to the array involved harvesting allantoic fluid from influenza-infected embryonating chicken eggs 3 days post-inoculation, isolating RNA via Trizol® extraction, and amplifying all segments of the influenza genome via an RT-PCR reaction that incorporated amino allyl modified deoxynucleoside triphosphates. The resulting cDNA was labeled with Cy3 or Cy5 cyanine dyes and then hybridized to the array for 1 hour at 22°C. The results were highly reproducible and demonstrated the ability to subtype and differentiate H1 from H3 and N1 from N2 subtypes using target strains homologous to the probe.

1.6 Rationale and Objectives

Diagnosis of AI can be difficult when clinical signs are generalized, such as respiratory distress. By detecting viral nucleic acid, the qPCR assay has revolutionized AI diagnosis by increasing the sensitivity, specificity, and speed of diagnosis compared to viral isolation or viral antigen-based assays. qPCR technology is constantly being improved in order to detect more than one virus in a single reaction. Still, the utility of qPCR is limited because only a small number of virus subtypes can be detected (Elnifro *et al.*, 2000).

The poultry industry is in need of a reliable, specific, sensitive, and rapid method of AIV detection in order to provide adequate surveillance methods to prevent the spread of disease and obtain important epidemiological information about AI outbreaks. Currently, qPCR is serving as the industries best diagnostic, but it is has inherent limitations. DNA microarray technology offers a promising new means of AIV surveillance, detection, and diagnosis. The objective of this project was to develop an avian influenza microarray for diagnostic and surveillance purposes. Our objectives were twofold:

1) Develop a cDNA-based Avian Influenza microarray that can
    a. Identify type A influenza via the conserved matrix gene
    b. Differentiate H5, H7, and H9 subtypes
    c. Differentiate N1, N2, and N3 subtypes

2) Differentiate phylogenetic clades of the H5 hemagglutinin subtype.

Chapter 2

MATERIALS AND METHODS

2.1 Amplification of AIV M, H, and N Genes

Avian influenza gene sequences from the matrix (M), hemagglutinin (H), and neuraminidase (N) genes were amplified by a PCR reaction in order to provide enough material for spotting onto microarray slides. AIV sequence information for thousands of isolates of all three genes were generously provided by Dr. David L. Suarez from USDA ARS SEPRL, Athens, GA (24 matrix gene sequences, 114 H5, 157 H7, 81 H9, 137 N1, 418 N2, and 156 N3). These sequences were entered into DNASTAR MegAlign (DNASTAR; Madison, WI). Regions of high homology (>95% nucleotide identity) among isolates within the same M, H, and N type were used to create 12-23 nt PCR primers to amplify full-length genes. PCR primers were synthesized by Sigma Genosys (The Woodlands, TX) and resuspended in DEPC water at a 100 μM concentration and stored at -20°C.

AIV gene segments from various subtypes and strains of AI were amplified in a One-Step RT-PCR reaction (Qiagen; Valencia, CA). The primers used are represented in Table 2.1. The 3' and 5' designation are based on the 3' and 5' ends of the cDNA sequences of the AIV isolates. AIM3' and AIM5' were used to amplify the matrix gene,

Table 2.1 PCR Primers Used to Amplify the Matrix (M), Hemagglutinin (HA), and Neuraminidase (NA) Genes of AIV and the Fusion (F) Gene of NDV. Primers used to amplify the matrix, hemagglutinin, and neuraminidase genes of AIV. Tm, melting temperature of primer in °C.

Primer	Gene	Tm	Sequence 5'-3'
AIM5'	M	43.2	AGCAAAAGCAGG
AIM3'	M	61.1	GACGATCAAGAATCCACAATA
USA AIH7-5'	HA	62	AGCCAAAAGCAGGGGA
AIH-3'	HA	46.6	AGTAGAAACAAGGGTG
AIH5-5'	HA	60.8	AGCCAAAAGCAGGGGT
USA1 AIH5-5'	HA	59.6	TCTGCATTGGTTATCATGC
USA1 AIH5-3'	HA	60.6	TATTGCTCCAAATAGGCCTC
USA3 AIH5-5'	HA	56.9	GGTTATCATGCAAACAATTC
USA3 AIH5-3'	HA	59.3	TATTGCTCCAAACAGACCTC
Eurasia AIH5-5'	HA	67	CAGATTTGCATTGGTTACCATGC
Eurasia AIH5-3'	HA	64.1	GATTTCACRTATTTGGGGCATTC
N1-3'	NA	61.9	GAATGGCAACTCAGCACC
N2-5'	NA	59.2	ATGAATCCAAATCAGAAGATAATAAC
N2-3'	NA	69.2	CCATCAGGCCATGAGCCTG
N3-5'	NA	64.3	AGCAAAAGCAGGTGCGAG
N3-3'	NA	58.9	CGATCCAGGTTCATTGTC
NDV3-5'	F		CCTTGGTGAITCTATCCGIA
NDV4-3'	F		CTGCCACTGCTAGTTGIGATAATCC

the sequences of which were kindly provided by Dr. Suarez. AIH3' is a primer common to the 3' end of all HA subtypes and was used with specific 5' primers for the H5 and H7 HA subtypes. The H9 HA subtype was amplified using a mixture of AIH3', AIH5-5' and AIH7-5'. Finally, the neuraminidase subtypes were amplified using NA subtype specific 5' and 3' primers (N1 primer set used an N1-3' and the N2-5'). Newcastle Disease virus (NDV) RNA was generously provided by Dr. Jack Gelb's laboratory, as were the primers.

The reverse transcription reaction was carried out at 50°C for 30 min followed by activation of the DNA polymerase at 95°C for 15 min. Then, a 40 cycle, three step PCR cycle was performed at 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min, followed by a final 72°C for 10 min extension. PCR products were purified using a Qiagen QIAQuick PCR Purification Kit per the manufacturer's instructions (Qiagen; Valencia, CA). To confirm correct amplification of desired gene segments, agarose gel electrophoresis was employed. The concentration of purified RT-PCR products was determined by UV-Vis Spectrophotometeric analysis (NanoDrop Technologies; Wilmington, DE) and 1 μg of cDNA was removed, evaporated to dryness, and resuspended in 10 μL spotting solution (0.01% SDS, 3X SSC), placed in a Genetix 384-well, V-bottom plate (Genetix; Boston, MA) and stored at -20°C until use.

2.2 Microarray Printing, Processing and Quality Control

Silanated amine glass microarray slides (VAE-25C) (CEL Associates; Pearland, TX) were spotted using an OmniGrid Accent robotic spotter (Gene Machine; San Carlos, CA) and one Telechem Stealth microarray printing pin (Cat.No. SMP3) (Telechem;

Sunnyvale, CA). The Omnigrid spotter contains a robotic printhead and the pin is sonicated for 30 sec, washed in Millipore water for 30 sec, and then vacuum dried for 30 sec between each dipping cycle into the 384-well plate. Spotting was conducted at room temperature and 65% humidity. After spotting, the slides were left to dry at room temperature and humidity for 30 min and then UV cross linked in a Stratagene Stratalinker 2400 (Stratagene; La Jolla, CA) at 400 mJ. The UV cross-linking covalently bonds the spotted cDNA to the amine-reactive glass microarray slide so that the probe will not wash off in subsequent steps. Slides were blocked at 55°C in a solution of 1% bovine serum albumin (BSA), 3.5X SSC, and 10% SDS for 20 min followed by three washes in ddH_2O and centrifugation at 1000 x g to dry the slide. The blocking step inactivates free amine groups on the slide and therefore eliminates nonspecific binding of the labeled target to the slide. A brief dip in boiling ddH_2O followed by snap cooling in 100% ice-cold ethanol for 5 sec denatured the double stranded cDNA and created single stranded cDNA, available for hybridization.

Slide quality, the presence of DNA and spot morphology, was confirmed using SYBR Green II dye (Invitrogen; Carlsbad, CA). A Nunc mSeries LifterSlip (Nunc Brand; Rochester, NY) was placed on top of a blocked slide. SYBR Green II stain was diluted 10,000-fold in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0), pipetted under the lifterslip, and incubated at room temperature for 2–3 min. The slide was washed 3 times with TBE buffer and centrifuged to dry. The slide was then scanned at 495nm. Scanning of the slide was conducted in an arrayWoRx^e Biochip Reader (Applied Precision; Issaquah, WA). This machine emits light from a white light source and passes it through an excitation filter. The fluorescence is dispensed through 19 fiber optic

strands and is uniformly distributed onto the slide. A scientific grade charge-coupled device (CCD) camera captures and relays the image to a computer connected to the scanner.

2.3 Preparation and Hybridization of Fluorescently-labeled aRNA

AIV RNA was isolated via Trizol® extraction at the USDA, Southeast Poultry Research Lab (SEPRL) in Athens, Georgia. Viral RNA was stored in 50 DEPC-treated water at -80°C. The Ambion Amino Allyl Message Amp II aRNA Amplification Kit (Ambion; Austin, TX) was modified and used to create indirectly labeled aRNA. Normally, the first step in the aRNA process is a reverse transcription reaction using an oligo(dT) primer to create cDNA from mRNA. Instead of using this primer, we used a primer (5'-AAACGACGGCCAGTGAATTGTA ATACGACTCACTATAGGCGCAGC AAAAGCAGG-3') containing a T7 promoter sequence and Uni3, a primer previously developed to amplify all influenza gene segments due to their conserved 5' end (Offringa *et al.*, 2000). The creation of sufficient quantities of aRNA requires two rounds of amplification, the first round yields aRNA and the second round yields amino-allyl dUTP aRNA. Eleven μL of vRNA (of any concentration of vRNA) in DEPC water was mixed with 1 μL of the modified T7 Oligo(dT) primer. The solution was incubated at 70°C for 10 min.

A reverse transcription reaction to synthesize first strand cDNA was accomplished by the addition of 8 μL of the reverse transcription master mix (10X first strand buffer, dNTP mix, RNase inhibitor, and an RT enzyme, ArrayScript). Once the

master mix was added to the sample of vRNA and the modified T7 oligo(dT) primer, the solution was incubated at 42°C for 2 h.

Second strand cDNA synthesis was conducted to convert the single-stranded cDNA into a double-stranded cDNA template for transcription. This reaction used 80 µL of a second strand master mix (nuclease-free water, 10X second strand buffer, dNTP mix, DNA polymerase, and RNaseH to degrade the RNA intermediate and synthesize the second strand cDNA in a 16°C incubation for 2 h. This reaction was followed by cDNA purification on a column membrane to remove the RNA, primers, enzymes, and salts that could inhibit the subsequent in vitro transcription (IVT) reaction.

IVT reaction creates aRNA from the double-stranded cDNA templates. Double stranded cDNA was incubated for 14 h at 37°C with ATP, CTP, GTP, UTP, T7 10X reaction buffer, and a T7 enzyme mix. The following day, aRNA was purified in order to remove the unincorporated NTPs, enzymes, salts, and inorganic phosphates. At this step, spectrophotometric analysis of aRNA quantity was performed using a UV-Vis Spectrophotometer (NanoDrop Technologies; Wilmington, DE). aRNA concentrations after the first round of amplification varied from 17 ng/µL to 53 ng/µL in 100 µL total volume.

A second round of aRNA amplification was performed in order to maximize aRNA yield. This involved mixing the purified aRNA from the first IVT reaction (concentrated to a 10 µL volume) with 2 µL of Ambion's proprietary second round primers and then incubating the mixture for 10 min at 70°C. The addition of 8 µL of the reverse transcription master mix (containing the same reagents used in the first round of amplification) and incubation at 42°C for 2 h completed the reverse transcription reaction

and first strand cDNA synthesis. Reverse transcription reaction was followed by the addition of 1 µL of RNaseH for 30 min at 37°C. The next step was the addition of 5 µL of the modified T7 Oligo(dT) primer in a 10 min incubation at 70°C. The second strand reaction was conducted at 16°C for 2 h in the presence of water, 10X second strand buffer, dNTP mix, and DNA polymerase. The double stranded cDNA was column purified and then placed in a second IVT reaction, except this second round included the addition of aaUTP in the master mix to provide binding sites for the amine reactive fluorescent dye. This mixture was incubated at 37°C for 14 h to create amino-allyl aRNA and was then purified on a column in preparation for dye coupling. aRNA synthesis was assessed using a UV-Vis Spectrophotometer. aRNA concentrations after performing the second amplification varied from 1462 ng/µL to 2990 ng/µL in 100 µL total volume.

Ten µg of aRNA was concentrated to 5 µL and mixed with 3 µL of labeling buffer (25mg/mL sodium bicarbonate). The fluorescent dye, AlexaFluor 555 (Molecular Probes; Eugene, OR) resuspended in 2 µL of DMSO, was mixed with the aRNA in labeling buffer (25mg/mL sodium bicarbonate) for 2 h at room temperature and kept in the dark. This protocol was adapted from the Molecular Probes protocol instead of the Ambion protocol, which is optimized for cyanine dyes, and not NHS ester dyes like the AlexaFluor series of fluorescent dyes. Purification of labeled aRNA was performed according to Ambion protocol and involved a column purification step using aRNA binding buffer, 100% ethanol, a wash step, and an elution with 50°C nuclease-free water. Dye incorporation efficiency was validated using a UV-Vis Spectrophotometer. The Nanodrop, on the microarray setting, measures the absorbancy at 260nm and 555nm

wavelength. The absorbencies at 260 and 555nm are entered into an online calculator (http://probes.invitrogen.com/resources/calc/basedyeratio.html), which calculates the number of dye molecules incorporated per base of aRNA. The calculator determines the absorbance of the labeled base of aRNA by utilizing the formula: $A_{base} = A_{260} - (A_{dye} * CF_{260})$, where A_{260} is the absorbance at 260nm, A_{dye} is the absorbance at 555nm, and CF is the correction factor for the dye (0.04). To determine the dye:base ratio, the calculator utilizes the formula: dye:base = $100/(A_{base} * \varepsilon_{dye})/(A_{dye} * \varepsilon_{base})$, where ε_{dye} is the extinction coefficient of the dye (150000 cm^{-1} M^{-1}), and ε_{base} is the average extinction coefficient of the base (8250 cm^{-1} M^{-1}). Labeling efficiency, the amount of dye molecules incorporated into the bases during transcription, or base:dye ratios varied from 89.7 to 156.3 bases/dye molecule.

Purified, dye-labeled aRNA was placed in a Thermo Savant DNA 110 SpeedVac™ Dessicator (Thermo Savant; Farmingdale, NY) and concentrated to 2 µL. The solution was then incubated at 95°C for 1 min and resuspended in 18 µL of Telechem UniHyb Hybridization buffer (Telechem; Sunnyvale, CA) prewarmed to 65°C. Hybridizations were performed in the Advalytix ArrayBooster™ (Advalytix; Brunnthal, Germany) containing 65°C Tris-EDTA (TE) buffer in the metal wells. Purified, dye-labeled, concentrated aRNA was pipetted under a Nunc mSeries Lifter Slip onto the slide (Nunc Brand; Rochester, NY). The ArrayBooster™ was then set to the desired hybridization temperature (50°C) and hybridization proceeded overnight. Post-hybridization washes were performed to remove excess solution and prepare the slide for scanning. The slide was removed from the ArrayBooster™ and placed in wash solution one (0.5X SSC, .01% SDS) for 5 sec. The slide was then transferred to wash two (0.2X

SSC, 0.2% SDS) for 15 min with moderately vigorous up and down agitation. The slide was then transferred to wash three (0.2X SSC) and rinsed twice in that solution. This was followed by three washes with ddH_2O and an immediate centrifugation to dry the slide. Before scanning, the slide was dried with pressurized nitrogen (N_2) gas to remove dust particles. Scanning of the slide was conducted in an arrayWoRx^e Biochip Reader (Applied Precision; Issaquah, WA) as previously described.

2.4 Data Analysis

A tag image file format (TIFF) computer file of scanned fluorescent spot intensity values was transferred from the arrayWoRx^e Biochip Reader to SoftWoRx Tracker (Applied Precision; Issaquah, WA) for data analysis. (Silicon Genetics, Redwood City, CA). Abnormal spots (dust, bubbles) were manually eliminated from analysis. Background intensity was determined using the SoftWoRx Tracker cell method. This method draws a two pixel (1 pixel = 10 micron) margin around each 200-micron spot and then calculates everything else in between spots to be background. On each slide, spot intensities were normalized to that slide's mean background subtracted spot intensity. The raw values for spot mean intensity, background mean intensity, and background standard deviation were extracted from SoftWoRx Tracker and exported into Microsoft Excel 2000 version 9.0 (Microsoft; Seattle, WA). Spots with mean intensity values less than 2.5X mean background intensity values were eliminated from analysis as negatives. Elements on the array were considered positive if $\geq$ 75% (6/8) of the spots on the array passed the aforementioned analysis. Hybridizations that resulted in more than one

element on the array being rendered positive were further analyzed by performing one-way ANOVA analysis ($p=0.01$) of average spot normalized intensity values.

Chapter 3

RESULTS

3.1 Microarray Design and Construction

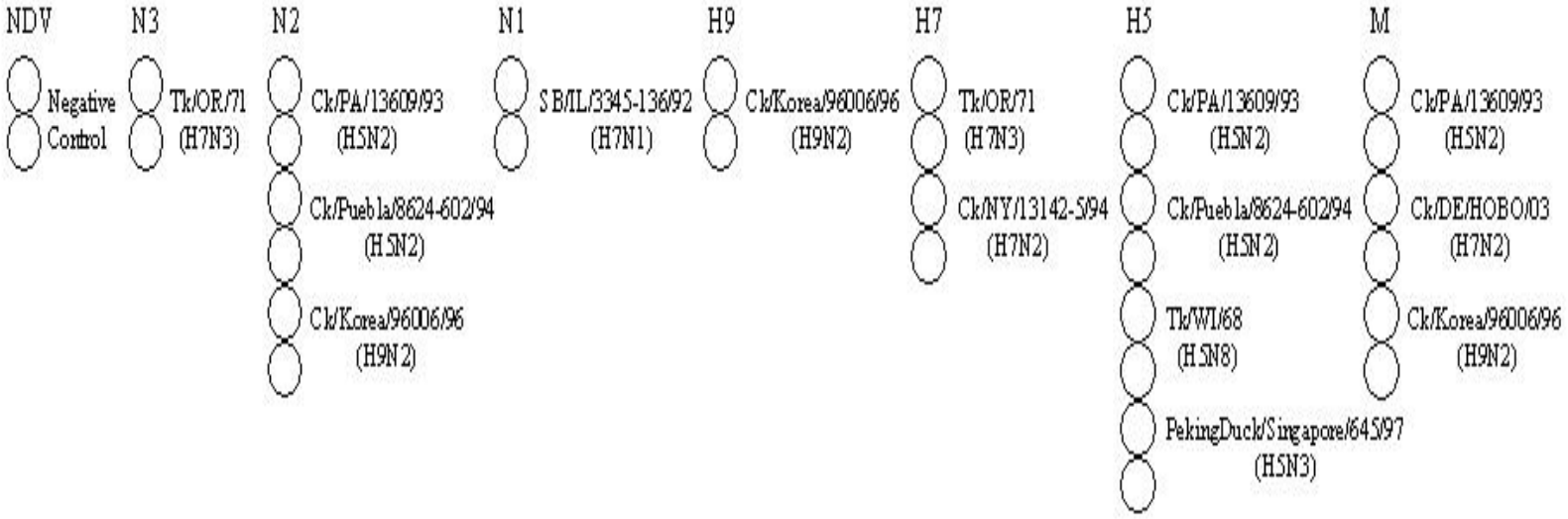
The matrix, hemagglutinin, and neuraminidase genes from various strains of AI were successfully amplified as revealed by agarose gel electrophoresis (data not shown). The strains and subtypes of AI used to amplify the M, HA, and NA genes are listed in Table 3.1 along with their predicted cDNA product lengths. The primer sequences are listed in Table 2.1 in the Materials and Methods.

The prototype AI microarray is illustrated in Figure 3.1. There are 32 spots per subarray and 4 subarrays on each slide for a total of 128 spots representing 16 elements. Three matrix genes from three different HA subtypes of AIV were spotted onto the microarray. The matrix genes, RT-PCR amplified, from Ck/PA/13609/93 (H5N2), Ck/DE/HOBO/03 (H7N2), and Ck/Korea/96006/96 (H9N2) are placed on the array to identify Type A influenza. The primer pair used to RT-PCR amplify the matrix gene is AIM-5' and AIM-3' which yields an 849 base pair (bp) amplicon. Due to the conserved nature of the matrix gene in type A influenza viruses, PCR product from the M gene of any subtype will react with all other AI subtypes (Lamb and Choppin, 1983). A negative control element was placed on the array containing cDNA from the Newcastle Disease

Table 3.1 Avian Influenza Strains and Genes on the Array. The AI strains and genes present on the microarray. The cDNA product lengths of each gene are listed in their respective order. The primers used to amplify each gene are listed in Table 2.1 in the Materials and Methods.

Gene	Virus Isolate	Subtype	Primers	PCR product (bp)
M	Ck/PA/13609/93	H5N2	AIM5' / AIM3'	849
M	Ck/DE/HOBO/03	H7N2	AIM5' / AIM3'	849
M	Ck/Korea/96006/96	H9N2	AIM5' / AIM3'	849
H5	Ck/PA/13609/93	H5N2	USA1 AIH5-5' / USA1 AIH5-3'	990
H5	Ck/Puebla/8624-602/94	H5N2	AIH5-5' / AIH-3'	995
H5	Tk/WI/68	H5N8	USA3 AIH5-5' / USA3 AIH5-3'	982
H5	PekingDuck/Singapore/645/97	H5N3	Eurasia AIH5-5' / Eurasia AIH5-3'	920
H7	Tk/OR/71	H7N3	USA AIH7-5' / AIH-3'	1726
H7	Ck/NY/13142-5/94	H7N2	USA AIH7-5' / AIH-3'	1726
H9	Ck/Korea/96006/96	H9N2	AIH5-5' / USA AIH7-5' / AIH-3'	1726
N1	ShoreBird/IL/3345-136/92	H7N1	N2-5' / N1-3'	1385
N2	Ck/PA/13609/93	H5N2	N2-5' / N2-3'	1382
N2	Ck/Puebla/8624-602/94	H5N2	N2-5' / N2-3'	1382
N2	Ck/Korea/96006/96	H9N2	N2-5' / N2-3'	1382
N3	Tk/OR/71	H7N3	N3-5' / N3-3'	1383
F	NewCastle Disease Virus	n/a	NDV3-5' / NDV4-3'	600

Figure 3.1 Array Design with AI Strain Designation and Subtype. Each subarray contained 16 elements spotted in duplicate, yielding 32 spots. The subarrays were spotted in quadruplicate, 2 horizontal and 2 vertical, for a total of 128 spots on the array. The isolate name and subtype information is located to the right of the two spots representing that element. The gene abbreviation is located above each row of spots.


NDV
Negative Control
N3
Tk/OR/71 (H7N3)
N2
Ck/PA/13609/93 (H5N2)
Ck/Puebla/8624-602/94 (H5N2)
Ck/Korea/96006/96 (H9N2)
N1
SB/IL/3345-136/92 (H7N1)
H9
Ck/Korea/96006/96 (H9N2)
H7
Tk/OR/71 (H7N3)
Ck/NY/13142-5/94 (H7N2)
H5
Ck/PA/13609/93 (H5N2)
Ck/Puebla/8624-602/94 (H5N2)
Tk/WI/68 (H5N8)
PekingDuck/Singapore/645/97 (H5N3)
M
Ck/PA/13609/93 (H5N2)
Ck/DE/HOBO/03 (H7N2)
Ck/Korea/96006/96 (H9N2)

Virus (NDV) F gene. The primer pair used to RT-PCR amplify the NDV F gene and the NDV RNA was kindly provided by Dr. Gelb's laboratory at the University of Delaware.

Thanks to Dr. David Suarez (USDA, SEPRL, Athens, GA), 114 H5 hemagglutinin gene sequences were obtained and evaluated using DNASTAR MegAlign software (DNASTAR; Maidson, WI) multiple sequence alignments. Those sequences were used to create the phylogenetic tree represented in Figure 3.2. Clades were arbitrarily assigned and named according to the geographical region associated with the clade.

The four H5 hemagglutinin elements on the microarray represent four of the five designated clades. The Ck/PA/13609/93 (H5N2) is contained within the USA1 clade, Ck/Puebla8624-602/94 (H5N2) belongs to the Mexico and Central America clade, Tk/WI/68 (H5N8) is contained within the USA3 clade and finally, PekingDuck/Singapore/645/97 (H5N3) belongs to the Eurasia clade. The USA2 (wildfowl) clade does not have a corresponding element on the array and is therefore not represented.

Dr. David Suarez also provided 157 H7 hemagglutinin gene sequences which were evaluated using DNASTAR MegAlign software (DNASTAR; Maidson, WI) multiple sequence alignments. Those sequences were used to create the phylogenetic tree represented in Figure 3.3. Clades were arbitrarily assigned and named according to the geographical region associated with the clade.

The H7 HA elements are RT-PCR amplified from Ck/NY/13142-5/94 (H7N2) and Tk/OR/71 (H7N3) and are both contained within the USA clade. This illustrates the

Figure 3.2 Phylogenetic Analysis of H5 Hemagglutinin Gene Sequences. The phylogenetic tree was created using DNASTAR MegAlign multiple sequence alignment program. The sequences and alignments were generously provided by Dr. David Suarez (USDA, SEPRL, Athens, GA). The clades are arbitrarily assigned to 5 clades and named to aid identification.

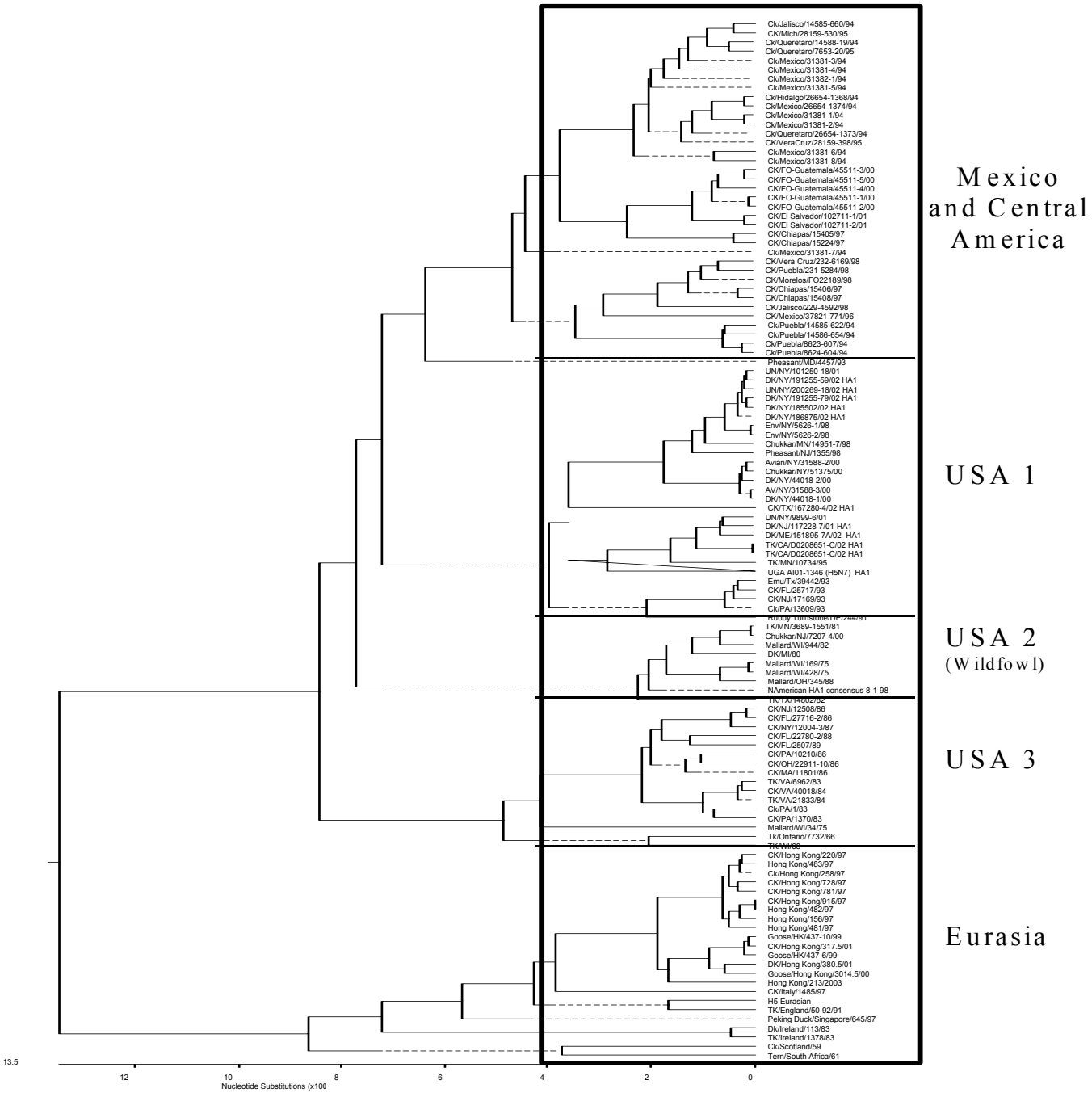
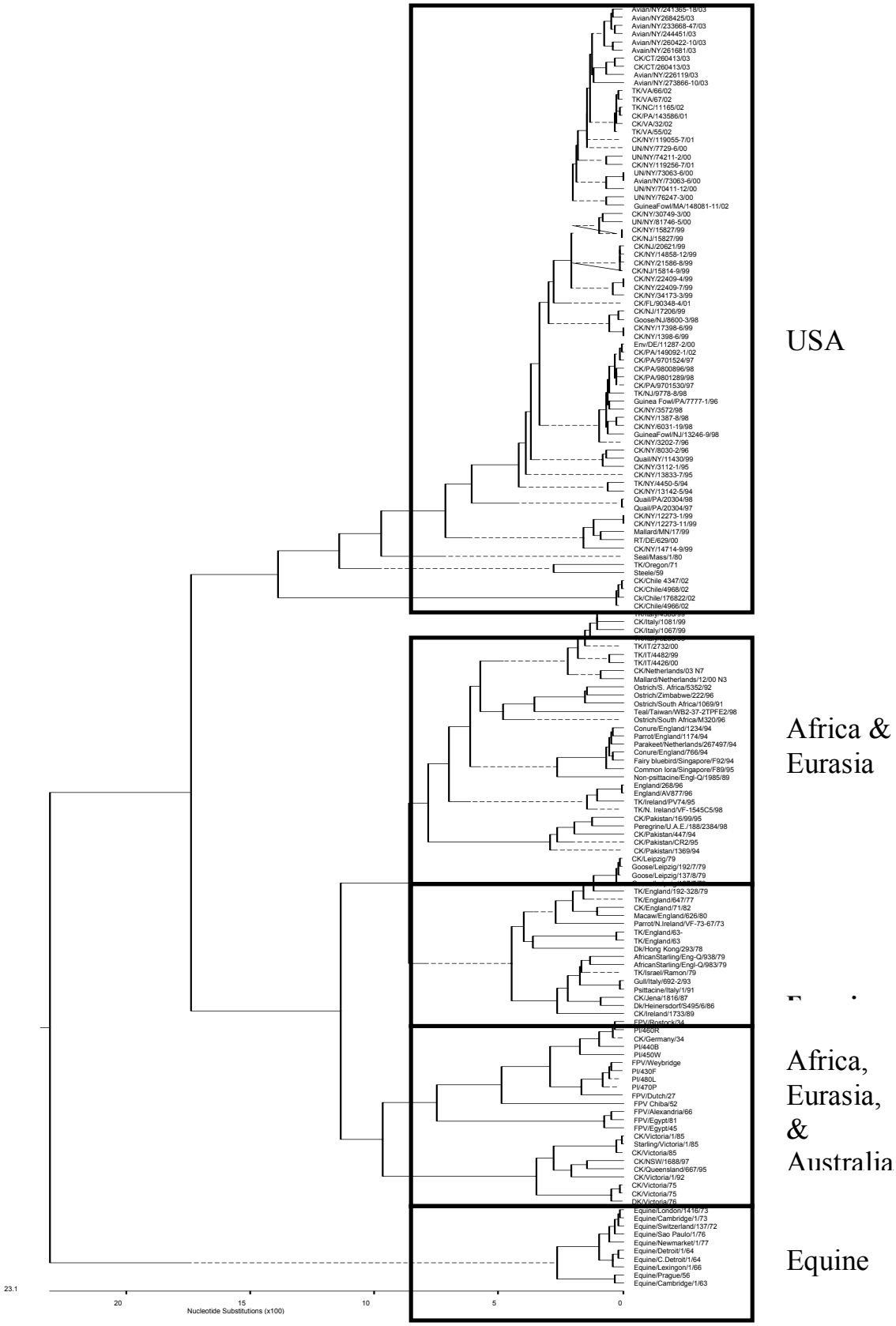

Ck/Jalisco/14585-660/94
CK/Mich/28159-530/95
Ck/Queretaro/14588-19/94
Ck/Queretaro/7653-20/95
Ck/Mexico/31381-3/94
Ck/Mexico/31381-4/94
Ck/Mexico/31382-1/94
Ck/Mexico/31381-5/94
Ck/Hidalgo/26654-1368/94
Ck/Mexico/26654-1374/94
Ck/Mexico/31381-1/94
Ck/Mexico/31381-2/94
Ck/Queretaro/26654-1373/94
CK/VeraCruz/28159-398/95
Ck/Mexico/31381-6/94
Ck/Mexico/31381-8/94
CK/FO-Guatemala/45511-3/00
CK/FO-Guatemala/45511-5/00
CK/FO-Guatemala/45511-4/00
CK/FO-Guatemala/45511-1/00
CK/FO-Guatemala/45511-2/00
CK/El Salvador/102711-1/01
CK/El Salvador/102711-2/01
CK/Chiapas/15405/97
CK/Chiapas/15224/97
Ck/Mexico/31381-7/94
CK/Vera Cruz/232-6169/98
CK/Puebla/231-5284/98
CK/Morelos/FO22189/98
CK/Chiapas/15406/97
CK/Chiapas/15408/97
CK/Jalisco/229-4592/98
CK/Mexico/37821-771/96
Ck/Puebla/14585-622/94
Ck/Puebla/14586-654/94
Ck/Puebla/8623-607/94
Ck/Puebla/8624-604/94
Pheasant/MD/7457/93
UN/NY/101250-18/01
DK/NY/191255-59/02 HA1
UN/NY/200269-18/02 HA1
DK/NY/191255-79/02 HA1
DK/NY/185502/02 HA1
DK/NY/186875/02 HA1
Env/NY/5626-1/98
Env/NY/5626-2/98
Chukkar/MN/14951-7/98
Pheasant/NJ/1355/98
Avian/NY/31588-2/00
Chukkar/NY/51375/00
DK/NY/44018-2/00
AV/NY/31588-3/00
DK/NY/44018-1/00
CK/TX/167280-4/02 HA1
UN/NY/9899-6/01
DK/NJ/117228-7/01-HA1
DK/ME/151895-7A/02 HA1
TK/CA/D0208651-C/02 HA1
TK/CA/D0208651-C/02 HA1
TK/MN/10734/95
UGA AI01-1346 (H5N7) HA1
Emu/Tx/39442/93
CK/FL/25717/93
CK/NJ/17169/93
Ck/PA/13609/93
Ruddy Turnstone/DE/244/91
TK/MN/3689-1551/81
Chukkar/NJ/7207-4/00
Mallard/WI/944/82
DK/MI/80
Mallard/WI/169/75
Mallard/WI/428/75
Mallard/OH/345/88
NAmerican HA1 consensus 8-1-98
TK/TX/14802/82
CK/NJ/12508/86
CK/FL/27716-2/86
CK/NY/12004-3/87
CK/FL/22780-2/88
CK/FL/2507/89
CK/PA/10210/86
CK/OH/22911-10/86
CK/MA/11801/86
TK/VA/6962/83
CK/VA/40018/84
TK/VA/21833/84
Ck/PA/1/83
CK/PA/1370/83
Mallard/WI/34/75
Tk/Ontario/7732/66
TK/MN/68
CK/Hong Kong/220/97
Hong Kong/483/97
Ck/Hong Kong/258/97
CK/Hong Kong/728/97
CK/Hong Kong/781/97
CK/Hong Kong/915/97
Hong Kong/482/97
Hong Kong/156/97
Hong Kong/481/97
Goose/HK/437-10/99
CK/Hong Kong/317.5/01
Goose/HK/437-6/99
DK/Hong Kong/380.5/01
Goose/Hong Kong/3014.5/00
Hong Kong/213/2003
CK/Italy/1485/97
H5 Eurasian
TK/England/50-92/91
Peking Duck/Singapore/645/97
Dk/Ireland/113/83
TK/Ireland/1378/83
Ck/Scotland/59
Tern/South Africa/61
13.5
12
10
8
6
4
2
0
Nucleotide Substitutions (x100
Mexico and Central America
USA 1
USA 2
(Wildfowl)
USA 3
Eurasia

Figure 3.3 Phylogenetic Analysis of H7 Hemagglutinin Gene Sequences. The phylogenetic tree was created using DNASTAR MegAlign multiple sequence alignment program. The sequences and alignments were generously provided by Dr. David Suarez (USDA, SEPRL, Athens, GA). The clades are arbitrarily assigned to 5 clades and named to aid identification. The last clade contains H7 HA gene sequences from equine isolates.


Avian/NY/241365-18/03
Avian/NY268425/03
Avian/NY/233668-47/03
Avian/NY/244451/03
Avian/NY260422-10/03
Avain/NY/261681/03
CK/CT/260413/03
CK/CT/260413/03
Avian/NY/225119/03
Avian/NY/273866-10/03
TK/VA/66/02
TK/VA/67/02
TK/NC/11165/02
CK/PA/143586/01
CK/VA/32/02
TK/VA/55/02
CK/NY/119055-7/01
UN/NY/7729-6/00
UN/NY/74211-2/00
CK/NY/119256-7/01
UN/NY/73063-6/00
Avian/NY/73063-6/00
UN/NY/70411-12/00
UN/NY/78247-3/00
GuineaFowl/MA/148081-11/02
CK/NY/30749-3/00
UN/NY/81746-5/00
CK/NY/15827/99
CK/NJ/15827/99
CK/NJ/20621/99
CK/NY/14858-12/99
CK/NY/21586-8/99
CK/NJ/15814-9/99
CK/NY/22409-4/99
CK/NY/22409-7/99
CK/NY/34173-3/99
CK/FL/90348-4/01
CK/NJ/17206/99
Goose/NJ/8600-3/98
CK/NY/17398-6/99
CK/NY/1398-6/99
Env/DE/11287-2/00
CK/PA/149092-1/02
CK/PA/9701524/97
CK/PA/9800896/98
CK/PA/9801289/98
CK/PA/9701530/97
TK/NJ/9778-8/98
Guinea Fowl/PA/7777-1/96
CK/NY/3572/98
CK/NY/1387-8/98
CK/NY/6031-19/98
GuineaFowl/NJ/13246-9/98
CK/NY/3202-7/96
CK/NY/8030-2/96
Quail/NY/11430/99
CK/NY/3112-1/95
CK/NY/13833-7/95
TK/NY/4450-5/94
CK/NY/13142-5/94
Quail/PA/20304/98
Quail/PA/20304/97
CK/NY/12273-1/99
CK/NY/12273-11/99
Mallard/MN/17/99
RT/DE/629/00
CK/NY/14714-9/99
Seal/Mass/1/80
TK/Oregon/71
Steele/59
CK/Chile 4347/02
CK/Chile/4968/02
Ck/Chile/176822/02
CK/Chile/4966/02
CK/Italy/1081/99
CK/Italy/1067/99
TK/IT/2732/00
TK/IT/4482/99
TK/IT/4426/00
CK/Netherlands/03 N7
Mallard/Netherlands/12/00 N3
Ostrich/S. Africa/5352/92
Ostrich/Zimbabwe/222/96
Ostrich/South Africa/1069/91
Teal/Taiwan/WB2-37-2TPFE2/98
Ostrich/South Africa/M320/96
Conure/England/1234/94
Parrot/England/1174/94
Parakeet/Netherlands/267497/94
Conure/England/766/94
Fairy bluebird/Singapore/F92/94
Common Iora/Singapore/F89/95
Non-psittacine/Engl-Q/1985/89
England/268/96
England/AV877/96
TK/Ireland/PV74/95
TK/N. Ireland/VF-1545C5/98
CK/Pakistan/16/99/95
Peregrine/U.A.E./188/2384/98
CK/Pakistan/447/94
CK/Pakistan/CR2/95
CK/Pakistan/1369/94
CK/Leipzig/79
Goose/Leipzig/192/7/79
Goose/Leipzig/137/8/79
TK/England/192-328/79
TK/England/647/77
CK/England/71/82
Macaw/England/626/80
Parrot/N.Ireland/VF-73-67/73
TK/England/63-
TK/England/63
Dk/Hong Kong/293/78
AfricanStarling/Eng-Q/938/79
AfricanStarling/Engl-Q/983/79
TK/Israel/Ramon/79
Gull/Italy/692-2/93
Psittacine/Italy/1/91
CK/Jena/1816/87
Dk/Heinersdorf/5495/6/86
CK/Ireland/1733/89
FPV/Rostock/34
PI/460R
CK/Germany/34
PI/440B
PI/450W
FPV/Weybridge
PI/430F
PI/480L
PI/470P
FPV/Dutch/27
FPV Chiba/52
FPV/Alexandria/66
FPV/Egypt/81
FPV/Egypt/45
CK/Victoria/1/85
Starling/Victoria/1/85
CK/Victoria/85
CK/NSW/1688/97
CK/Queensland/667/95
CK/Victoria/1/92
CK/Victoria/75
CK/Victoria/75
DK/Victoria/76
Equine/London/1416/73
Equine/Cambridge/1/73
Equine/Switzerland/137/72
Equine/Sao Paulo/1/76
Equine/Newmarket/1/77
Equine/Detroit/1/64
Equine/C.Detroit/1/64
Equine/Lexingon/1/66
Equine/Prague/56
Equine/Cambridge/1/63
23.1
20
15
10
5
0
Nucleotide Substitutions (x100)
USA
Africa &
Eurasia
Africa,
Eurasia,
&
Australia
Equine

lack of representative coverage of the H7 hemagglutinin gene phylogeny because only one of the four (avian) clades is represented on the microarray.

There is one H9 HA element present on the microarray and it is RT-PCR amplified from Ck/Korea/96006/96 (H9N2). NA subtypes on the array are from N2, and N3. Namely, the N2 NA elements are RT-PCR amplified from Ck/PA/13609/93 (H5N2), Ck/Puebla/8624-602/94 (H5N2), and Ck/Korea/96006/96 (H9N2). The N3 NA element is RT-PCR amplified from Tk/OR/71 (H7N3). During the process of obtaining new AIV isolates, an N1 neuraminidase gene element was added to the microarray from Softbill/IL/33445-136/92 (H7N1).

Slide quality and spot morphology were evaluated using a SYBR stain. The spot morphology was consistent and the array showed relatively uniform fluorescence as illustrated in Figure 3.4.

Each element on the array was tested for its ability to hybridize. Isolates present on the microarray were also used in an aRNA amplification, labeling, and hybridization to evaluate the level of fluorescent signal using a sample with 100% homology to the isolate on the microarray. An example of a homologous hybridization is illustrated in Figure 3.5, in which fluorescently-labeled Ck/PA/13609/93 (H5N2) aRNA was hybridized to the array. The matrix, hemagglutinin, and neuraminidase genes from Ck/PA/13609/93 (H5N2) (noted in the white boxes) are present on the microarray and illustrate the fluorescent signal intensities associated with homologous hybridizations.

Figure 3.4 Evaluation of Spotting Efficiency using SYBR Green II Stain. SYBR Green II stain of one subarray on the prototype AI cDNA microarray. Each element is spotted in duplicate. Spotted RT-PCR products follow the pattern displayed in Figure 3.1.

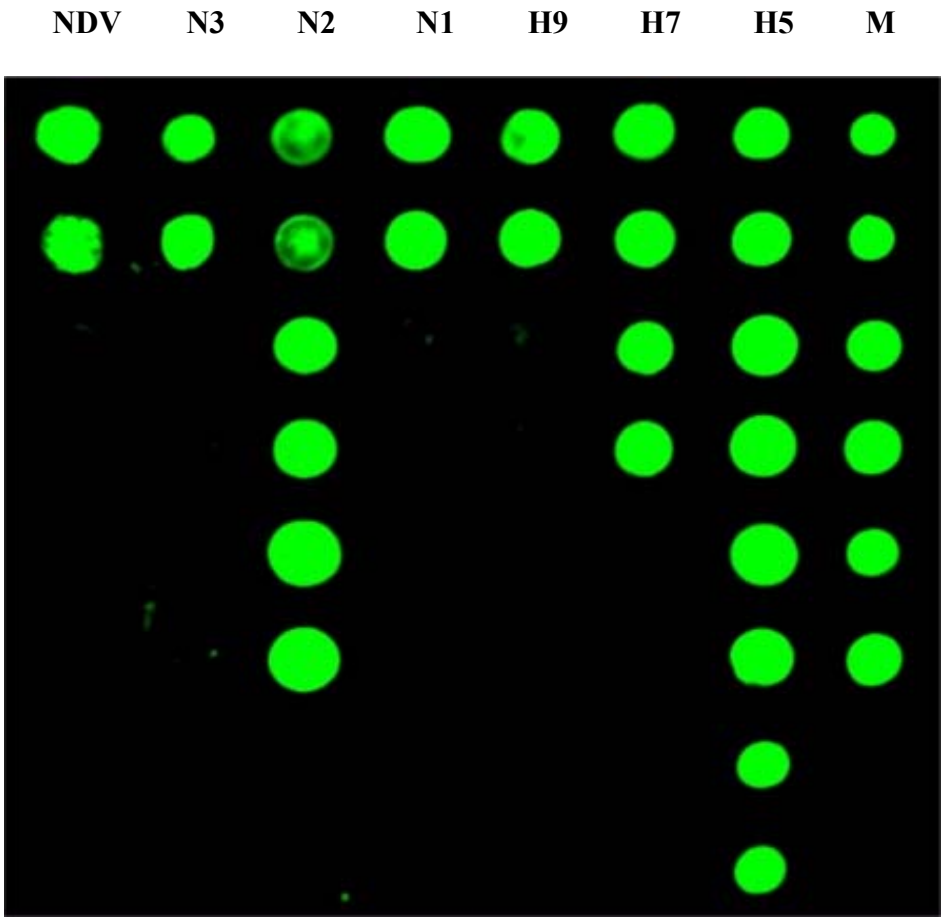
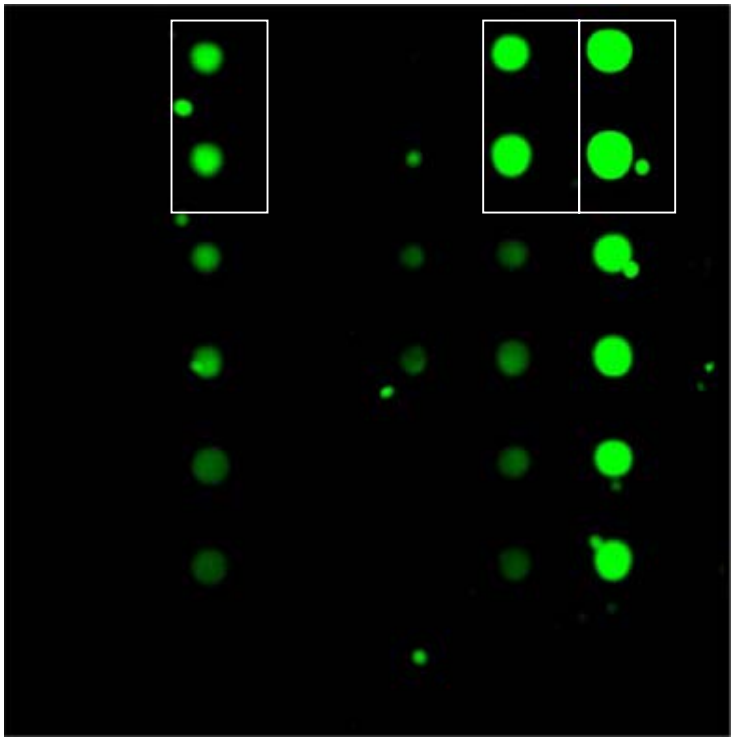

NDV
N3
N2
N1
H9
H7
H5
M

Figure 3.5 Hybridization of Ck/PA/13609/93 (H5N2) to its Corresponding Matrix, Hemagglutinin, and Neuraminidase Genes on the Microarray. One of the four subarrays is illustrated with the homologous matrix, hemagglutinin, and neuraminidase gene cDNAs in the white boxes.

NDV N3 N2 H9 H7 H5 M



3.2 Evaluation of an Unknown AIV Panel

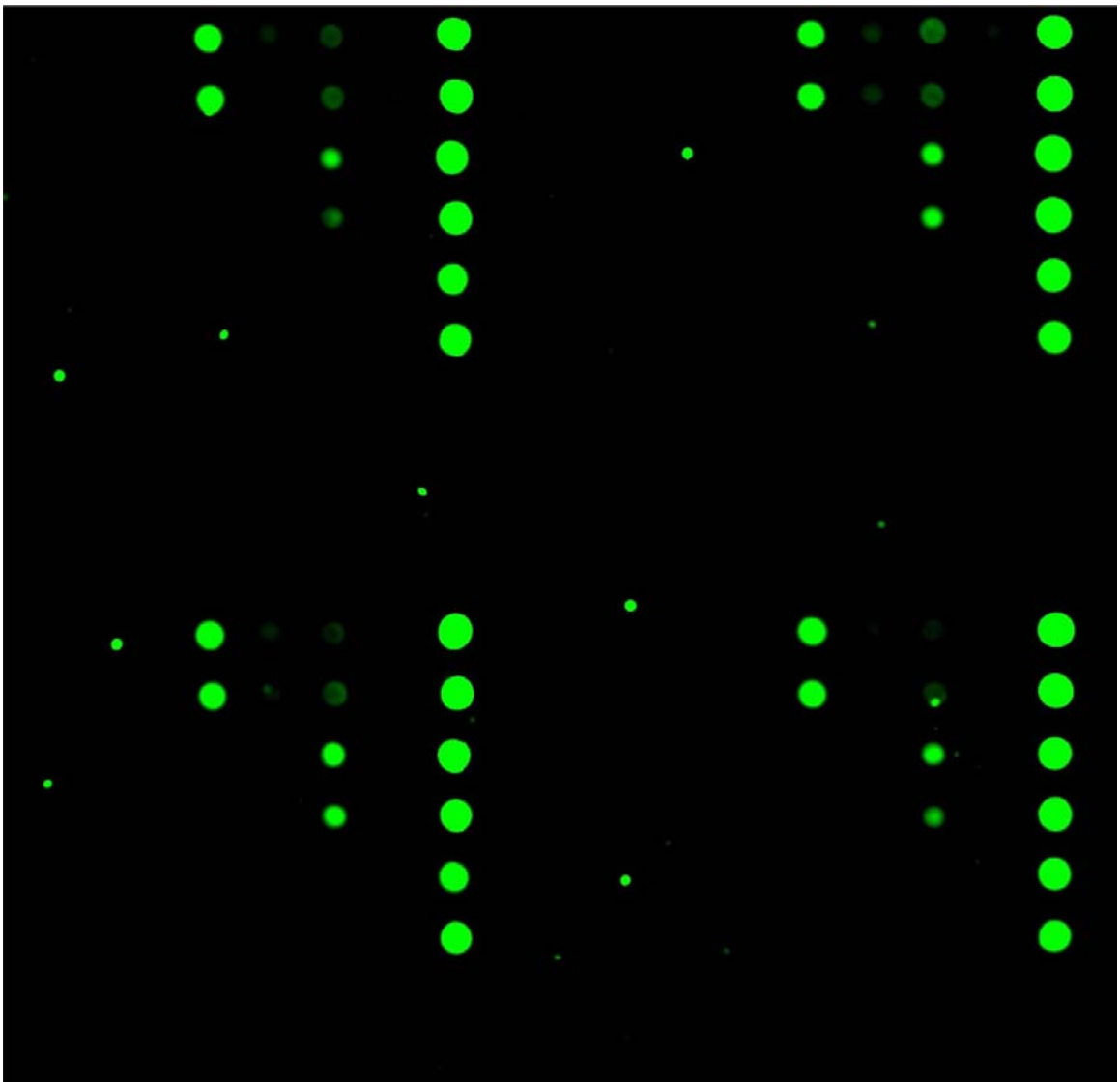
An unknown panel of AIV isolates was kindly provided by Dr. David Suarez (USDA, SEPRL, Athens, GA) along with a master key placed in a sealed envelope. Briefly, each sample of AIV RNA was used in an aRNA amplification reaction, indirectly labeled with fluorescent dye and used in a hybridization to the AI microarray. The hybridizations were conducted as previously described in Materials and Methods employing a 3 hr 50°C hybridization using 10μg of labeled aRNA. The images produced by the arrayWoRx Biochip reader, or scanner, were entered into the data acquisition software, SoftWoRx Tracker. Abnormal spots on the array, caused by dust or bubbles, were manually flagged as abnormal and eliminated from further analysis. The mean fluorescent intensity values of each normal spot and its mean background intensity value was determined by the software and entered into an Excel spreadsheet. Spots with mean intensity values less than 2.5 times the mean background intensity values were eliminated from analysis and considered negatives (the element did not hybridize to that sample). Elements on the array were considered positive if ≥ 75% of the eight spots on the array passed the aforementioned analysis. Hybridizations that resulted in more than one element of a different HA subtype being rendered positive were further analyzed by performing a one-way ANOVA analysis on the average spot normalized intensity values.

3.2.1 Unknown A

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample A is show in Figure 3.6. All 24 matrix gene

Figure 3.6 Scanned Image of Unknown A Hybridized to the AI Microarray. In a hybridization experiment with unknown A, 10 μg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix, HA7 and NA1 elements.

NDV N3 N2 N1 H9 H7 H5 M



elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. Similarly, 100% of the N1 gene elements had positive signals (>2.5X mean background intensity) allowing it to be neuraminidase subtyped as N1. Data for the HA elements is represented in Table 3.2. The only elements to pass the criteria for hybridization were those elements representing the H7 subtype. Consequently, microarray analysis indicated that the sample was an H7N1 Type A influenza.

This analysis was found to be partially correct as unknown sample A was found to be an H1N1 Type A influenza (Dk/NJ/7717-70/95). Although correctly identified as a type A influenza virus and subtyped as N1, the hemagglutinin subtype was found to be incorrect. Figure 3.7 compares the average spot normalized intensity values for the H7 elements to the matrix and N1 elements. The H7 spot intensity values can be seen to be significantly lower than the matrix and N1 values. Our hypothesis is that hybridization values associated with an H1 element (none are present on the array) would have more closely approximated the N1 and matrix values and led to the proper HA subtype classification of unknown A.

3.2.2 Unknown B

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample B is shown in Figure 3.8. All 24 matrix gene elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. Similarly 100% of the N1 gene elements had

Table 3.2 Data Analysis of Unknown A. This table illustrates the percentage of spots present on the AI microarray that exhibit positive signal (>2.5X mean background intensity).

Microarray Element	% Spots Pass 2.5X Mean Background	PASS/Fail 2.5X Mean Background?
H5 Ck/PA/13609/93 (H5N2)	0.0%	Fail
H5 Ck/Puebla/8624-602/94(H5N2)	0.0%	Fail
H5 PekingDuck/Singapore/645/97 (H5N3)	0.0%	Fail
H5 TK/WI/68 (H5N8)	0.0%	Fail
H7 Ck/NY/13142-5/94(H7N2)	100.0%	PASS
H7 TK/OR/71(H7N3)	87.5%	PASS
H9 Ck/Korea/96006/96(H9N2)	50.0%	Fail

Figure 3.7 Unknown A Average Spot Normalized Intensity Values for the Elements on the Microarray Exhibiting Positive Signal. The matrix, NA1 and HA7 elements on the AI microarray exhibited positive signal.

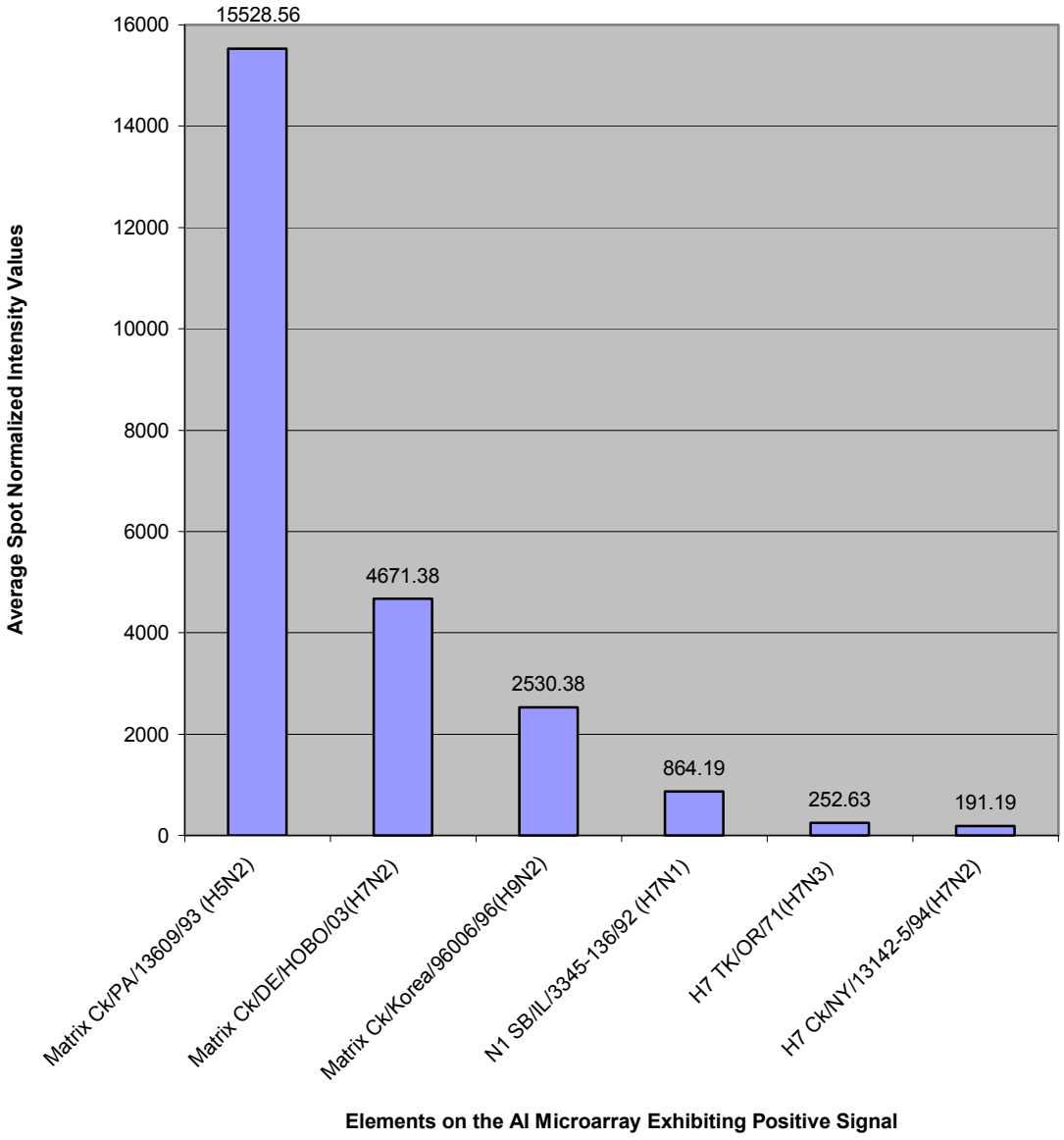
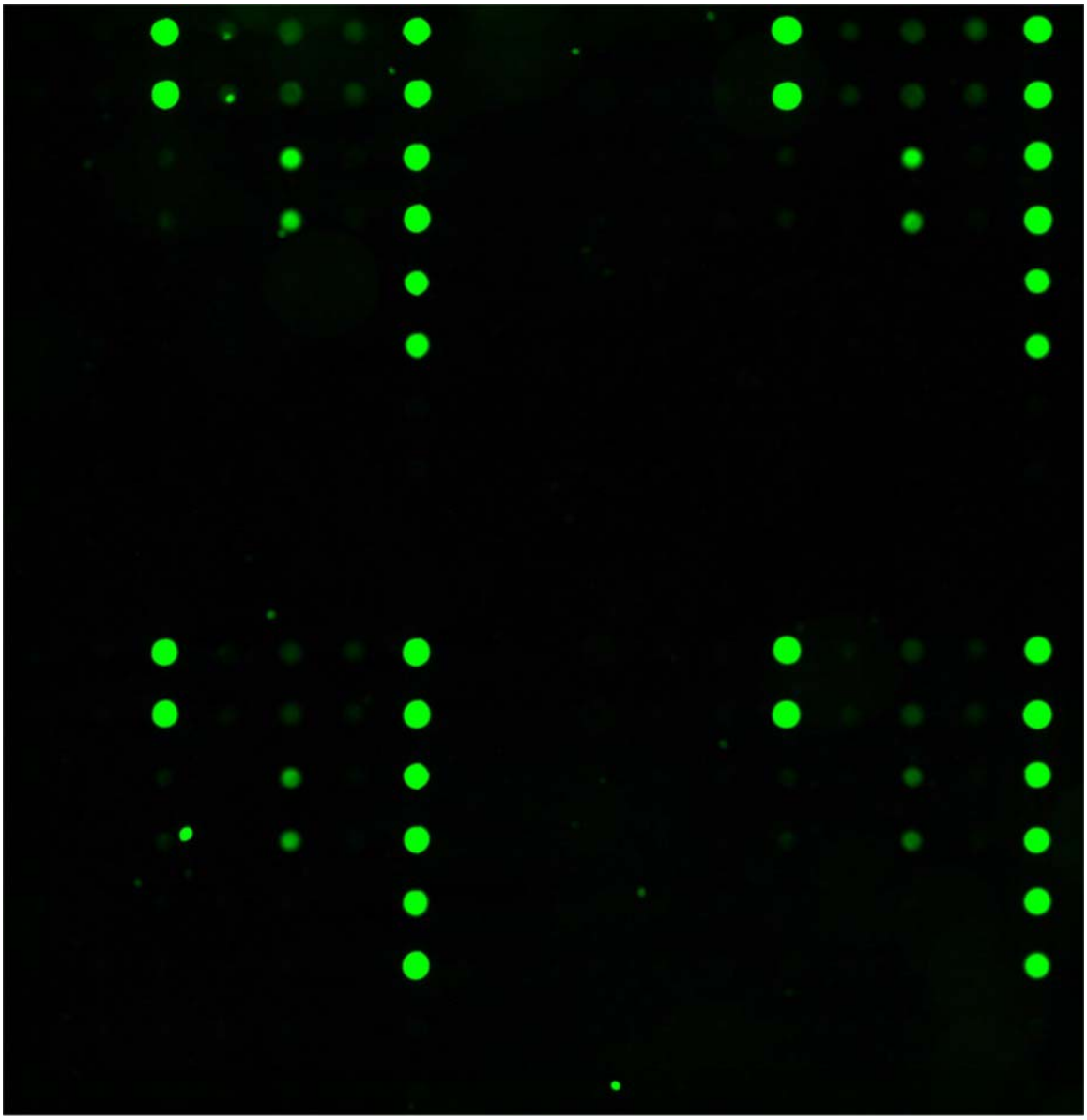

15528.56
4671.38
2530.38
864.19
252.63
191.19
16000
14000
12000
10000
8000
6000
4000
2000
0
Average Spot Normalized Intensity Values
Matrix Ck/PA/13609/93 (H5N2)
Matrix Ck/DE/HOBO/03(H7N2)
Matrix Ck/Korea/96006/96(H9N2)
N1 SB/IL/3345-136/92 (H7N1)
H7 TK/OR/71(H7N3)
H7 Ck/NY/13142-5/94(H7N2)
Elements on the AI Microarray Exhibiting Positive Signal

Figure 3.8 Scanned Image of Unknown B Hybridized to the AI Microarray. In a hybridization experiment with unknown B, 10 µg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix, HA7 and NA1 elements.

NDV N3 N2 N1 H9 H7 H5 M



positive signals (>2.5X mean background intensity) allowing it to be neuraminidase subtyped as N1. The hemagglutinin gene spots from the H7 Tk/OR/71 (H7N3) element on the array had 75% of the spots exhibiting positive signal (>2.5X mean background intensity) allowing it to be hemagglutinin subtyped as H7. This analysis was found to be correct as unknown sample B was found to be an H7N1 Type A influenza (Softbill/IL/33445-136/92).

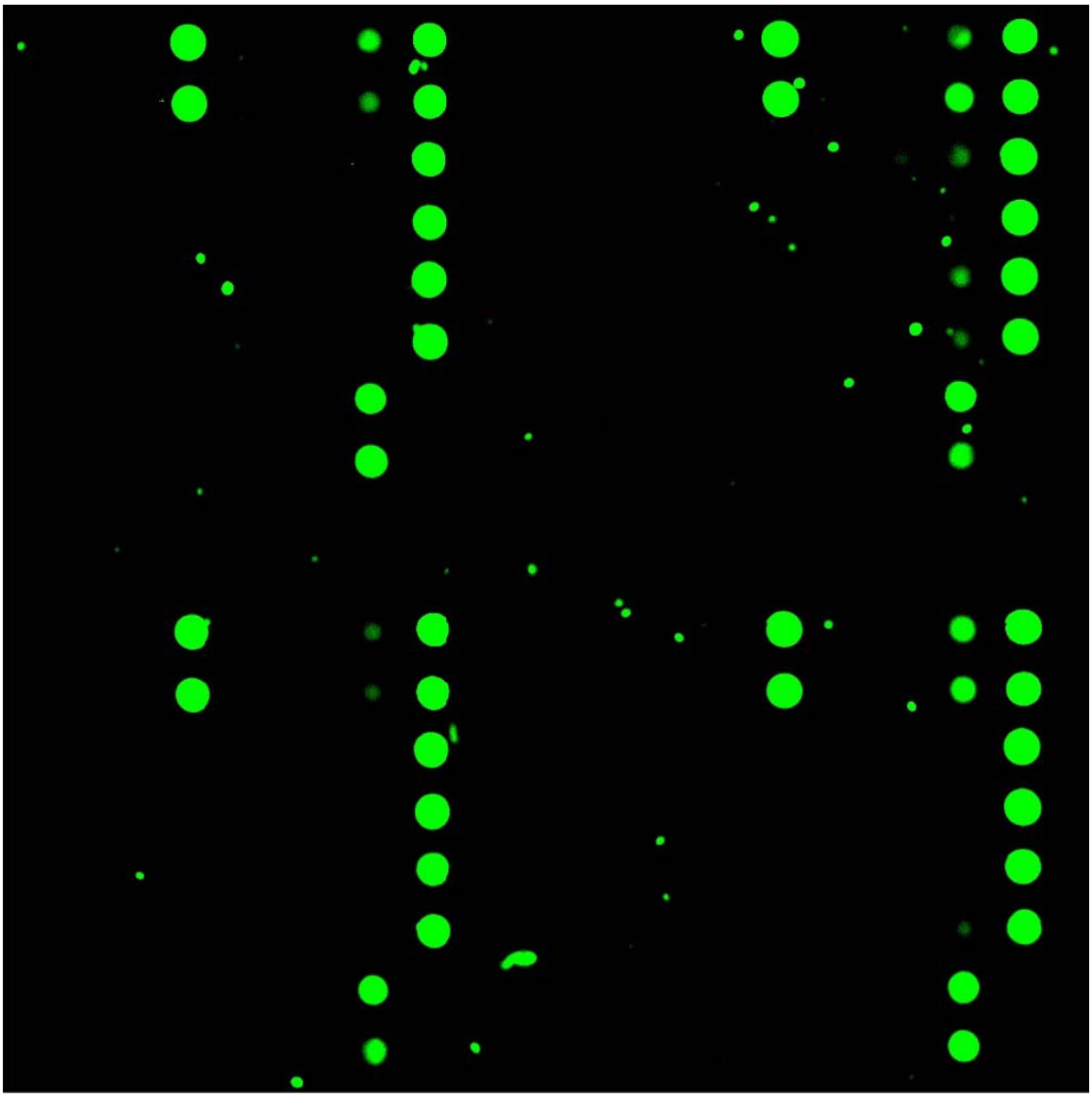
3.2.3 Unknown C

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample C is show in Figure 3.9. All 24 matrix gene elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. Similarly, 100% of the N1 gene elements had positive signals (>2.5X mean background intensity) allowing it to be neuraminidase subtyped as N1. The only HA elements to pass the criteria for hybridization were those elements representing the H5 subtype. Consequently, microarray analysis indicated that the sample was an H5N1 Type A influenza. This analysis was found to be correct as unknown sample C was found to be an H5N1 Type A influenza (Ck/HongKong/220/97).

Furthermore, the phylogenetic clade from which the isolate belonged was determined to be the H5 HA Eurasian clade. Due to the fluorescent signal intensity of the H5 HA element on the microarray from PekingDuck/Singapore/645/97 (H5N3) to unknown sample C, it was determined that the hemagglutinin gene from unknown C was not only subtyped as H5, but was also most homologous to AIV isolates belonging to the Eurasian H5 HA phylogenetic clade.

Figure 3.9 Scanned Image of Unknown C Hybridized to the AI Microarray. In a hybridization experiment with unknown C, 10 µg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix, HA5 and NA1 elements. The hybridization pattern is consistent with a type A influenza belonging to the H5N1 subtype and the Eurasian clade.

NDV N3 N2 N1 H9 H7 H5 M



3.2.4 Unknown D

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample D is show in Figure 3.10. All 24 matrix gene elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. Similarly, 100% of the N2 gene elements had positive signals (>2.5X mean background intensity) allowing it to be neuraminidase subtyped as N2. The HA elements to pass the criteria for hybridization were elements representing the H5 subtype and the H7 subtype. Due to the significant cross-hybridization, one-way ANOVA analysis ($p<0.01$) was performed on the average spot normalized intensity values from each of the HA elements on the microarray to the element on the array exhibiting the most intense fluorescent signal intensity (Ck/Puebla/8624-602/94 (H5N2)). The average spot normalized intensity values of the HA elements exhibiting positive signal is shown in Figure 3.11. Consequently, microarray analysis indicated that the sample was an H5N2 Type A influenza. This analysis was found to be correct as unknown sample D was found to be an H5N2 Type A influenza (Ck/Puebla/8624-602/94).

Furthermore, the phylogenetic clade from which the isolate belonged was determined to be the H5 HA Mexico and Central America clade. Due to the fluorescent signal intensity of the H5 HA element on the microarray from Ck/Puebla/8624-602/94 (H5N2) to unknown sample D, it was determined that the hemagglutinin gene from unknown D was not only subtyped as H5, but was also most homologous to AIV isolates belonging to the Mexico and Central America phylogenetic clade.

Figure 3.10 Scanned Image of Unknown D Hybridized to the AI Microarray. In a hybridization experiment with unknown D, 10 µg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix, HA5 and NA2 elements. The hybridization pattern is consistent with a type A influenza belonging to the H5N2 subtype and the Mexico and Central America clade.

NDV N3 N2 N1 H9 H7 H5 M

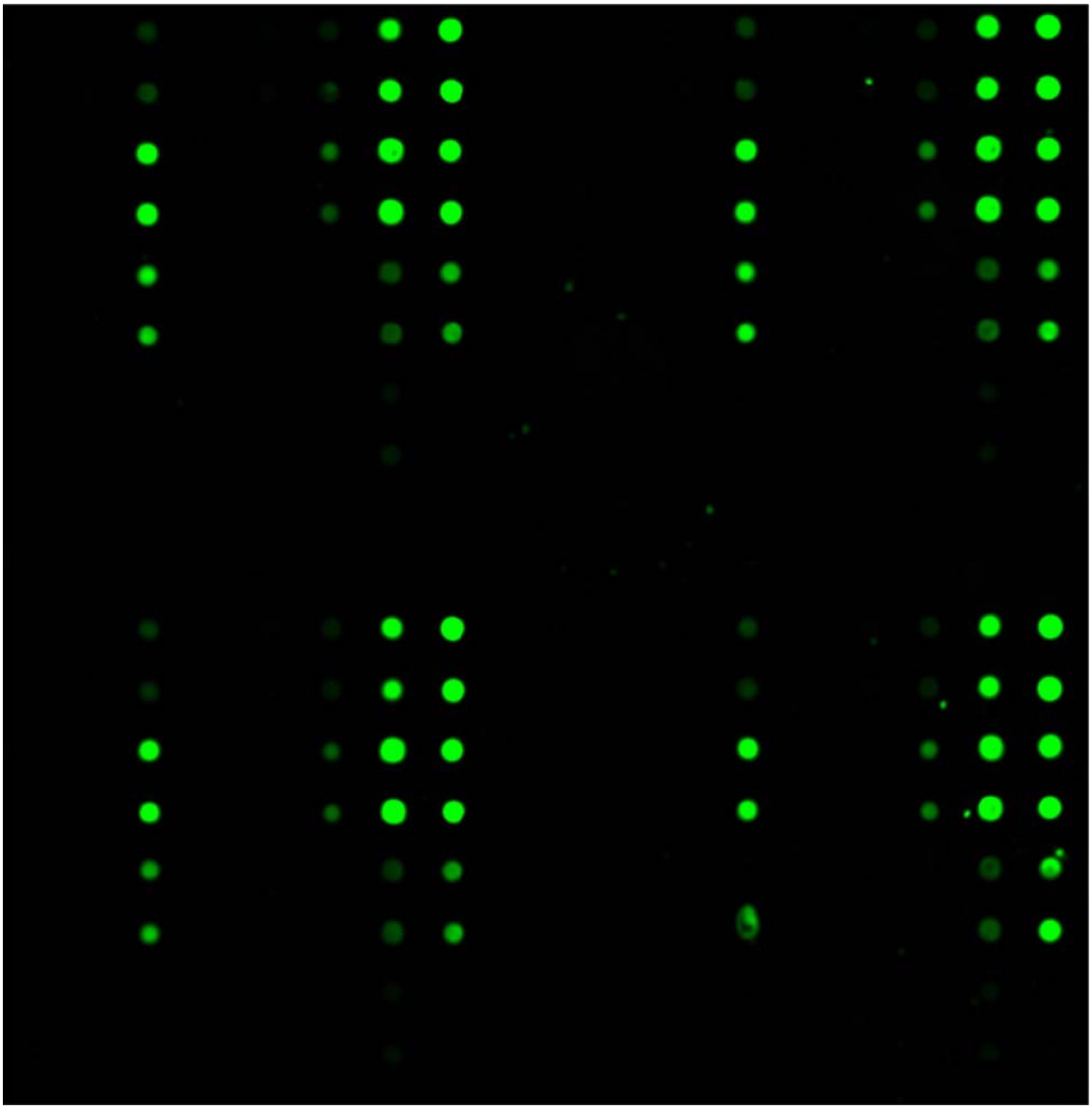
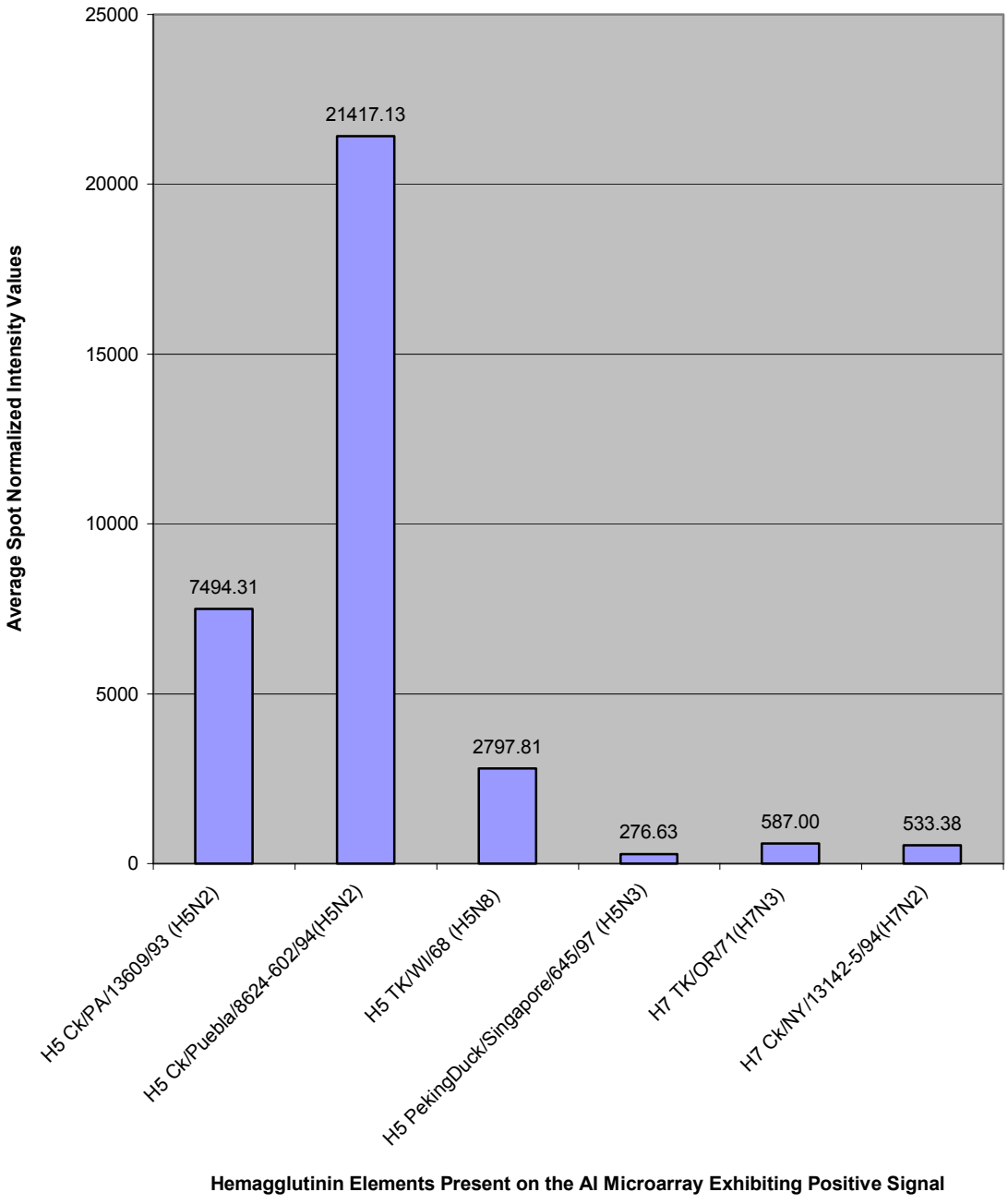


Figure 3.11 Unknown D Average Spot Normalized Intensity Values for the HA Elements on the Microarray Exhibiting Positive Signal. The four HA5 and two HA7 elements on the AI microarray exhibited positive signal. The signal intensity exhibited by the hybridization of unknown sample D to the HA5 element on the microarray, Ck/Puebla/8624-602/94 (H5N2), is clearly illustrated.


25000
20000
15000
10000
5000
0
Average Spot Normalized Intensity Values
21417.13
7494.31
2797.81
276.63
587.00
533.38
H5 Ck/PA/13609/93 (H5N2)
H5 Ck/Puebla/8624-602/94(H5N2)
H5 TK/WI/68 (H5N8)
H5 PekingDuck/Singapore/645/97 (H5N3)
H7 TK/OR/71(H7N3)
H7 Ck/NY/13142-5/94(H7N2)
Hemagglutinin Elements Present on the AI Microarray Exhibiting Positive Signal

3.2.5 Unknown E

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample E is shown in Figure 3.12. All 24 matrix gene elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. Similarly 100% of the N2 gene elements had positive signals (>2.5X mean background intensity) allowing it to be neuraminidase subtyped as N2. The only element to pass the criteria for hybridization was the element representing the H9 subtype. Consequently, microarray analysis indicated that the sample was an H9N2 Type A influenza. This analysis was found to be correct as unknown sample E was found to be an H9N2 Type A influenza (Ck/NJ/12220/97).

3.2.6 Unknown F

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample F is shown in Figure 3.13. All 24 matrix gene elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. However, none of the neuraminidase gene elements had positive signals (>2.5X mean background intensity) and none of the hemagglutinin gene elements had positive signals (>2.5X mean background intensity). Consequently, microarray analysis indicated that the sample was a Type A influenza of undetermined subtype

This analysis was found to be partially correct as unknown sample A was found to be an H7N7 Type A influenza (Ck/VIC/85). Although correctly identified as a type A influenza virus, the hemagglutinin and neuraminidase subtype was undetermined. Our

Figure 3.12 Scanned Image of Unknown E Hybridized to the AI Microarray. In a hybridization experiment with unknown E, 10 μg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix, NA2 and HA9 elements. The hybridization pattern is consistent with a type A influenza belonging to the H9N2 subtype.

NDV N3 N2 N1 H9 H7 H5 M

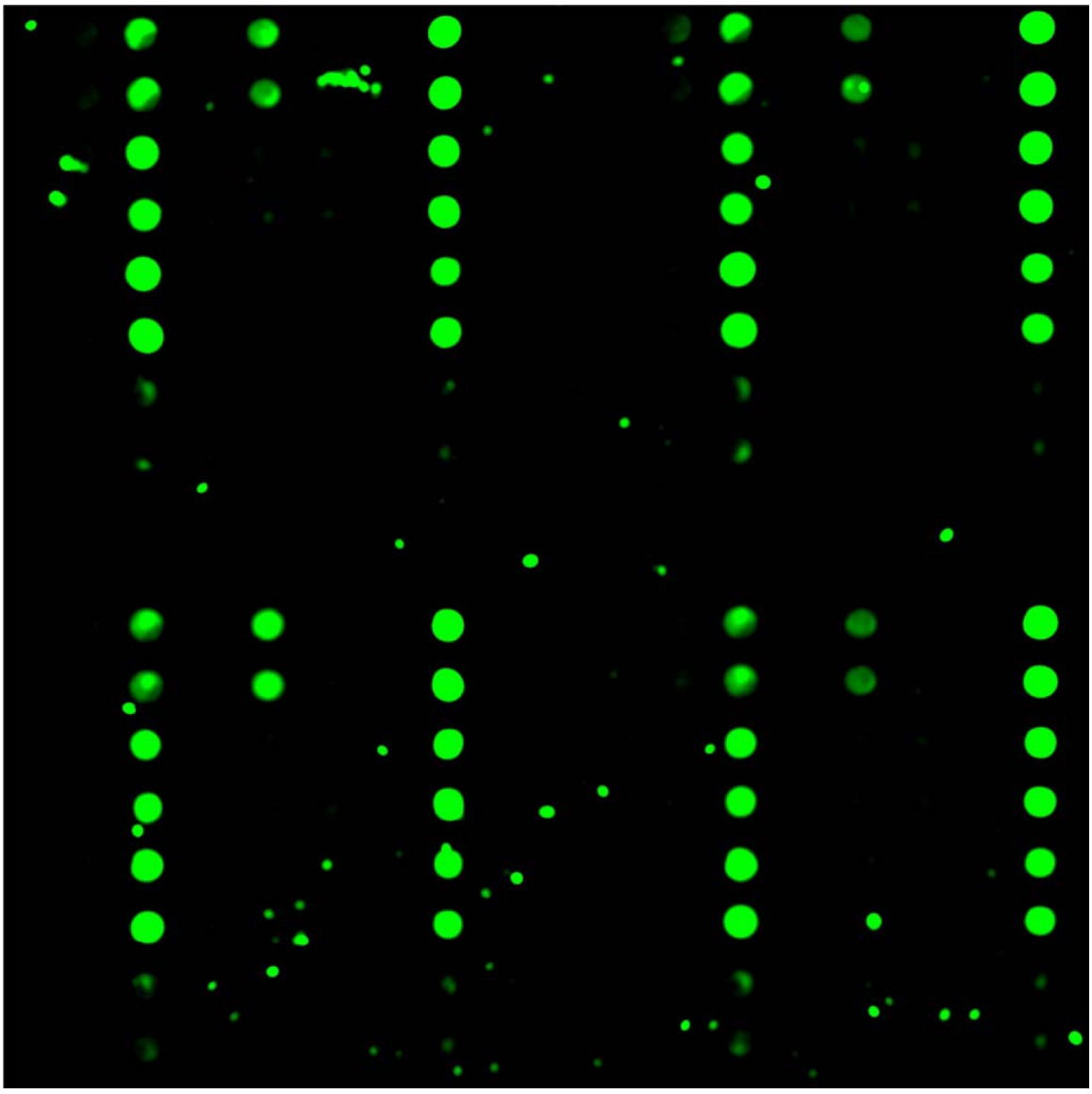
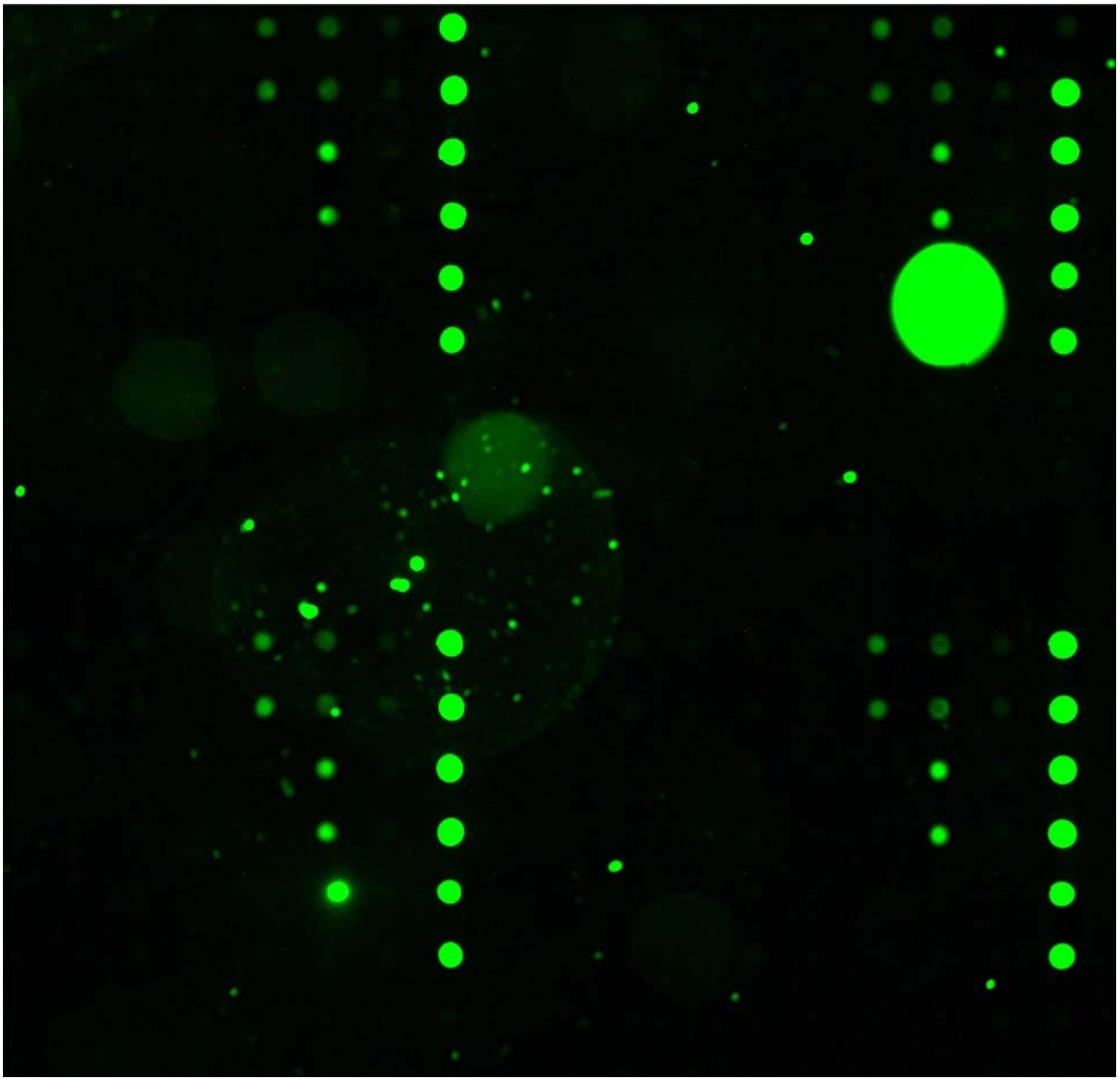


Figure 3.13 Scanned Image of Unknown F Hybridized to the AI Microarray. In a hybridization experiment with unknown F, 10 μg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix gene elements. The hybridization pattern is consistent with a type A influenza of undetermined subtype.

NDV N3 N2 N1 H9 H7 H5 M



hypothesis is that hybridization values associated with an H7 element belonging to the same phylogenetic clade as Ck/VIC/85 (H7N7) (none are present on the array) would have more closely approximated the matrix values and led to the HA subtype classification of unknown F. Also, because Ck/VIC/85 is an H7N7 and N7 elements are not represented on our array, no neuraminidase subtyping could be achieved.

3.2.7 Unknown G

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample G is shown in Figure 3.14. All 24 matrix gene elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. Similarly 100% of the N3 gene elements had positive signals (>2.5X mean background intensity) allowing it to be neuraminidase subtyped as N3. The only elements to pass the criteria for hybridization were those elements representing the H5 subtype. Consequently, microarray analysis indicated that the sample was an H5N3 Type A influenza.

This analysis was found to be correct as unknown sample A was found to be an H5N3 Type A influenza (Dk/Singapore/97). Further analysis utilizing average spot normalized intensity values, shown in Figure 3.15, reveals that unknown G hybridized most strongly with the H5 spots from PekingDuck/Singapore/645/97 (H5N3). Based on the fluorescent signal intensity values, it was determined that the hemagglutinin gene from unknown G was not only subtyped as H5, but was also most homologous to AIV isolates belonging to the Eurasia phylogenetic clade.

Figure 3.14 Scanned Image of Unknown G Hybridized to the AI Microarray. In a hybridization experiment with unknown G, 10 μg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix, NA3 and HA5 elements. The hybridization pattern is consistent with a type A influenza belonging to the H5N3 subtype and the Eurasian clade.

NDV N3 N2 N1 H9 H7 H5 M

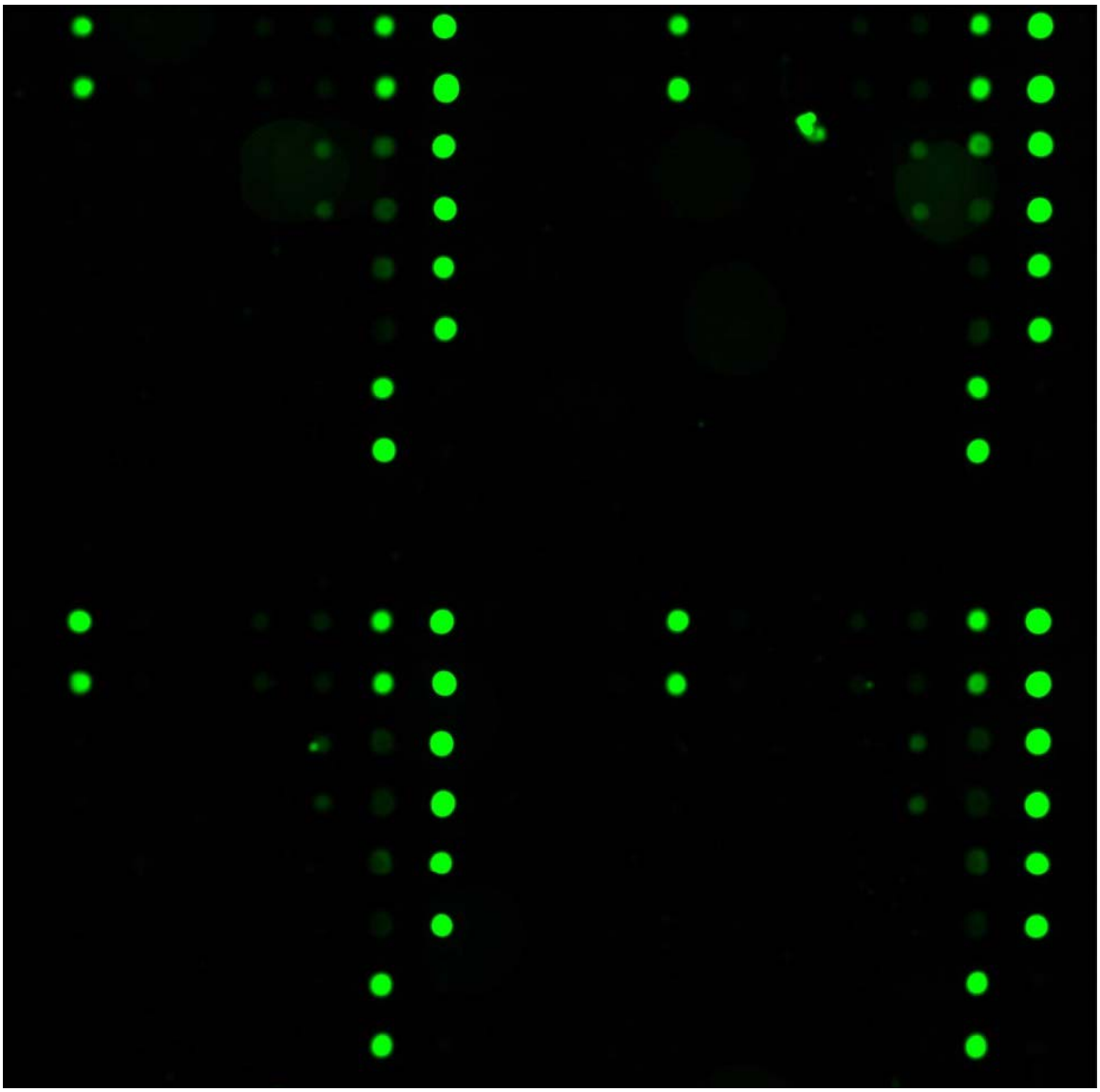
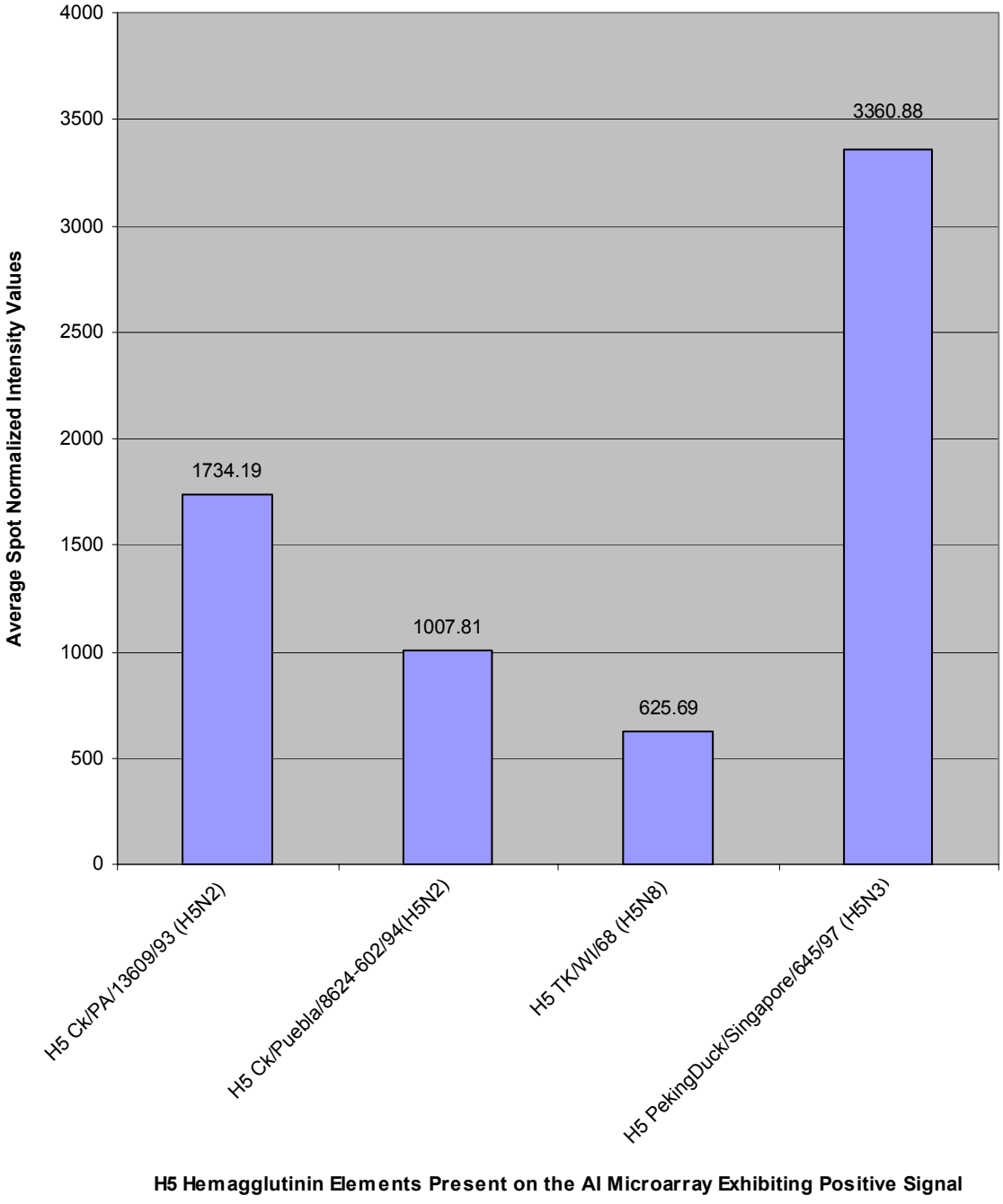


Figure 3.15 Unknown G Average Spot Normalized Intensity Values for the HA Elements on the Microarray Exhibiting Positive Signal. The four HA5 elements on the AI microarray exhibited positive signal. The signal intensity exhibited by the hybridization of unknown sample G to the HA5 element on the microarray, PekingDuck/Singapore/645/97 (H5N3), is illustrated and indicates that the sample is most homologous to strains from the Eurasia phylogenetic H5 HA clade.


4000
3500
3000
2500
2000
1500
1000
500
0
Average Spot Normalized Intensity Values
1734.19
1007.81
625.69
3360.88
H5 Ck/PA/13609/93 (H5N2)
H5 Ck/Puebla/8624-602/94(H5N2)
H5 TK/WI/68 (H5N8)
H5 PekingDuck/Singapore/645/97 (H5N3)
H5 Hemagglutinin Elements Present on the AI Microarray Exhibiting Positive Signal

3.2.8 Unknown H

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample H is shown in Figure 3.16. All 24 matrix gene elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. Similarly 100% of the N3 gene elements had positive signals (>2.5X mean background intensity) allowing it to be neuraminidase subtyped as N3. None of the hemagglutinin elements on the microarray had positive signals and therefore no hemagglutinin subtype was assigned. Consequently, microarray analysis indicated that the sample was an N3 Type A influenza of undetermined HA subtype.

This analysis was found to be partially correct as unknown sample G was found to be an H7N3 Type A influenza (Ck/PAK/1369-CR2/95). Although correctly identified as a type A influenza virus and subtyped as N3, the hemagglutinin subtype was undetermined. Our hypothesis is that hybridization values associated with an H7 element belonging to the same phylogenetic clade as Ck/VIC/85 (H7N7) (none are present on the array) would have more closely approximated the matrix values and led to the HA subtype classification of unknown G.

3.2.9 Unknown I

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample I is shown in Figure 3.17. All 24 matrix gene elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. Similarly 100% of the N1 gene elements had

Figure 3.16 Scanned Image of Unknown H Hybridized to the AI Microarray. In a hybridization experiment with unknown H, 10 μg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix and NA3 elements. The hybridization pattern is consistent with a type A influenza belonging to an undetermined HA subtype and N3 neuraminidase subtype.

NDV N3 N2 N1 H9 H7 H5 M

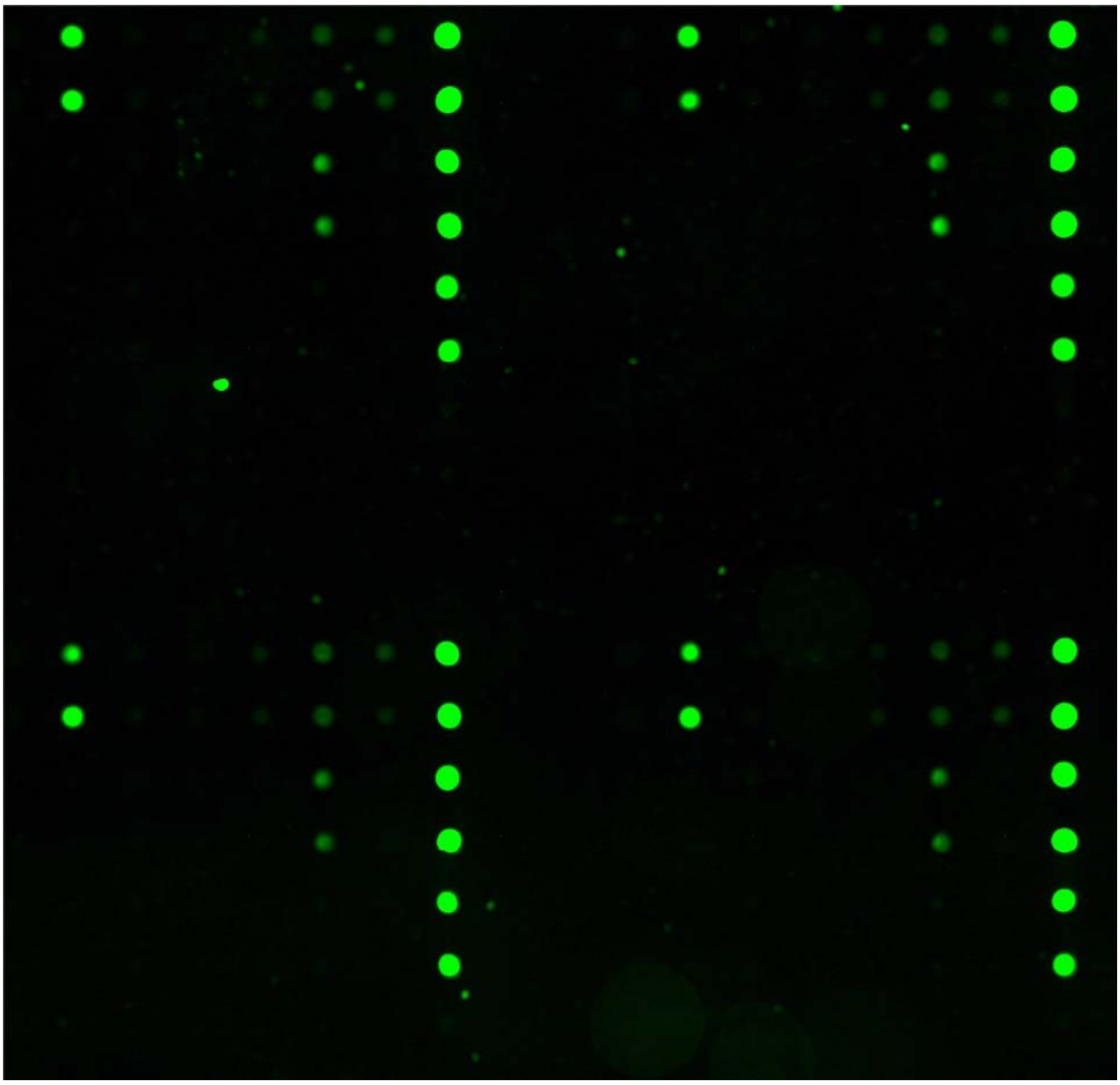
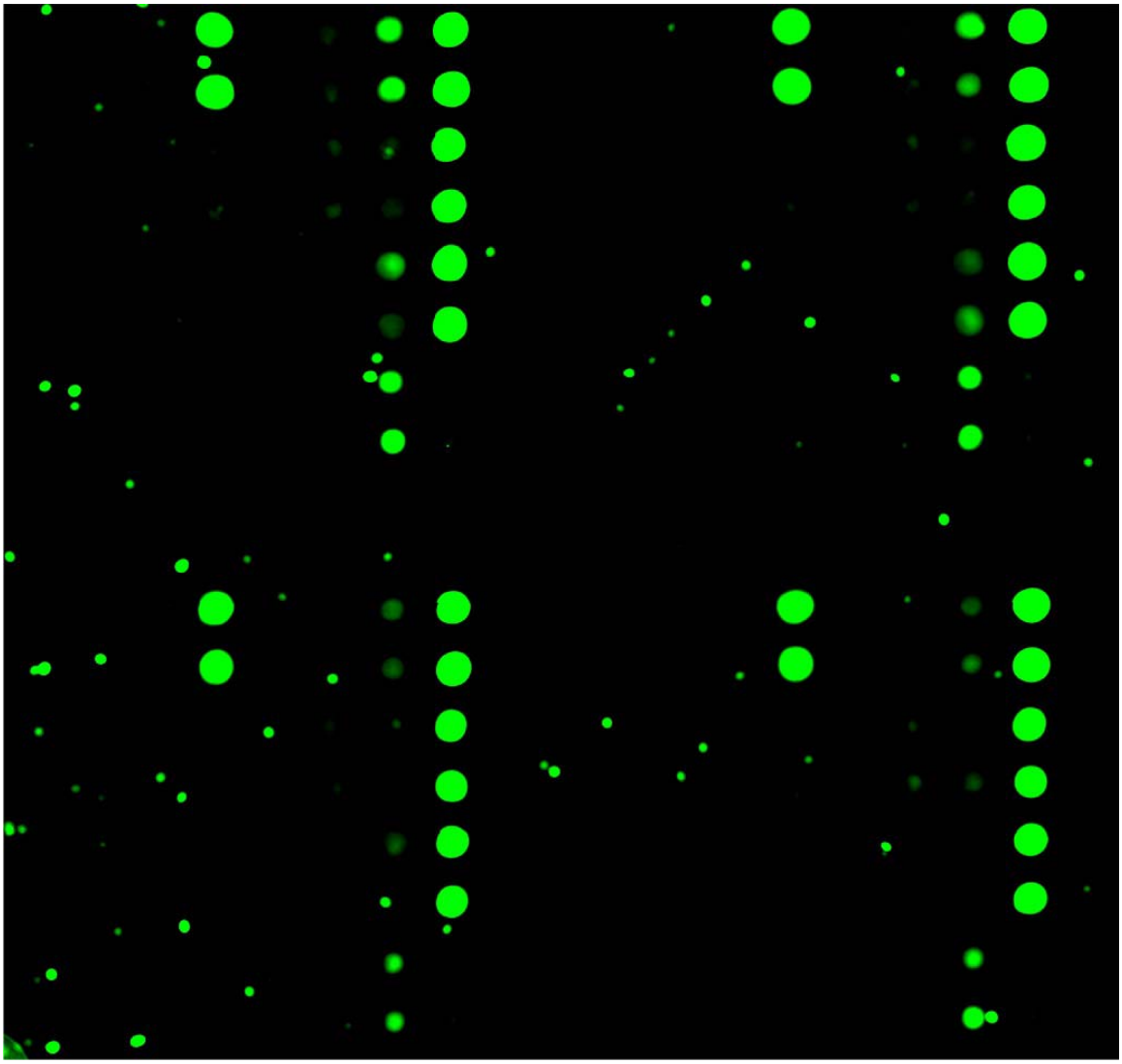


Figure 3.17 Scanned Image of Unknown I Hybridized to the AI Microarray. In a hybridization experiment with unknown I, 10 μg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix, NA1, and HA5 elements. The hybridization pattern is consistent with a type A influenza subtyped as H5N1 belonging to the Eurasian clade.

NDV N3 N2 N1 H9 H7 H5 M



positive signals (>2.5X mean background intensity) allowing it to be neuraminidase subtyped as N1. The only elements to pass the criteria for hybridization were those elements representing the H5 subtype. Consequently, microarray analysis indicated that the sample was an H5N1 Type A influenza.

This analysis was found to be correct as unknown sample I was found to be an H5N1 Type A influenza (Ck/Scotland/59). Further analysis utilizing average spot normalized intensity values, shown in Figure 3.18, reveals that unknown I hybridized most strongly with the H5 spots from PekingDuck/Singapore/645/97 (H5N3). Based on the fluorescent signal intensity values, it was determined that the hemagglutinin gene from unknown I was not only subtyped as H5, but was also most homologous to AIV isolates belonging to the Eurasia phylogenetic clade.

3.2.10 Unknown J

An image of the scanned AI array after hybridization to fluorescently-labeled aRNA derived from unknown sample J is shown in Figure 3.19. All 24 matrix gene elements (100%) had positive signals (>2.5X mean background intensity) indicating that the sample was a Type A influenza. Similarly 100% of the N2 gene elements had positive signals (>2.5X mean background intensity) allowing it to be neuraminidase subtyped as N2. The only elements to pass the criteria for hybridization were those elements representing the H7 subtype. Consequently, microarray analysis indicated that the sample was an H7N2 Type A influenza.

This analysis was found to be correct as unknown sample J was found to be an H7N2 Type A influenza (Ck/NJ/294508-12/04).

Figure 3.18 Unknown I Average Spot Normalized Intensity Values for the HA Elements on the Microarray Exhibiting Positive Signal. The four HA5 elements on the AI microarray exhibited positive signal. The signal intensity exhibited by the hybridization of unknown sample I to the HA5 element on the microarray, PekingDuck/Singapore/645/97 (H5N3), is illustrated and indicates that the sample is most homologous to strains from the Eurasia phylogenetic H5 HA clade.

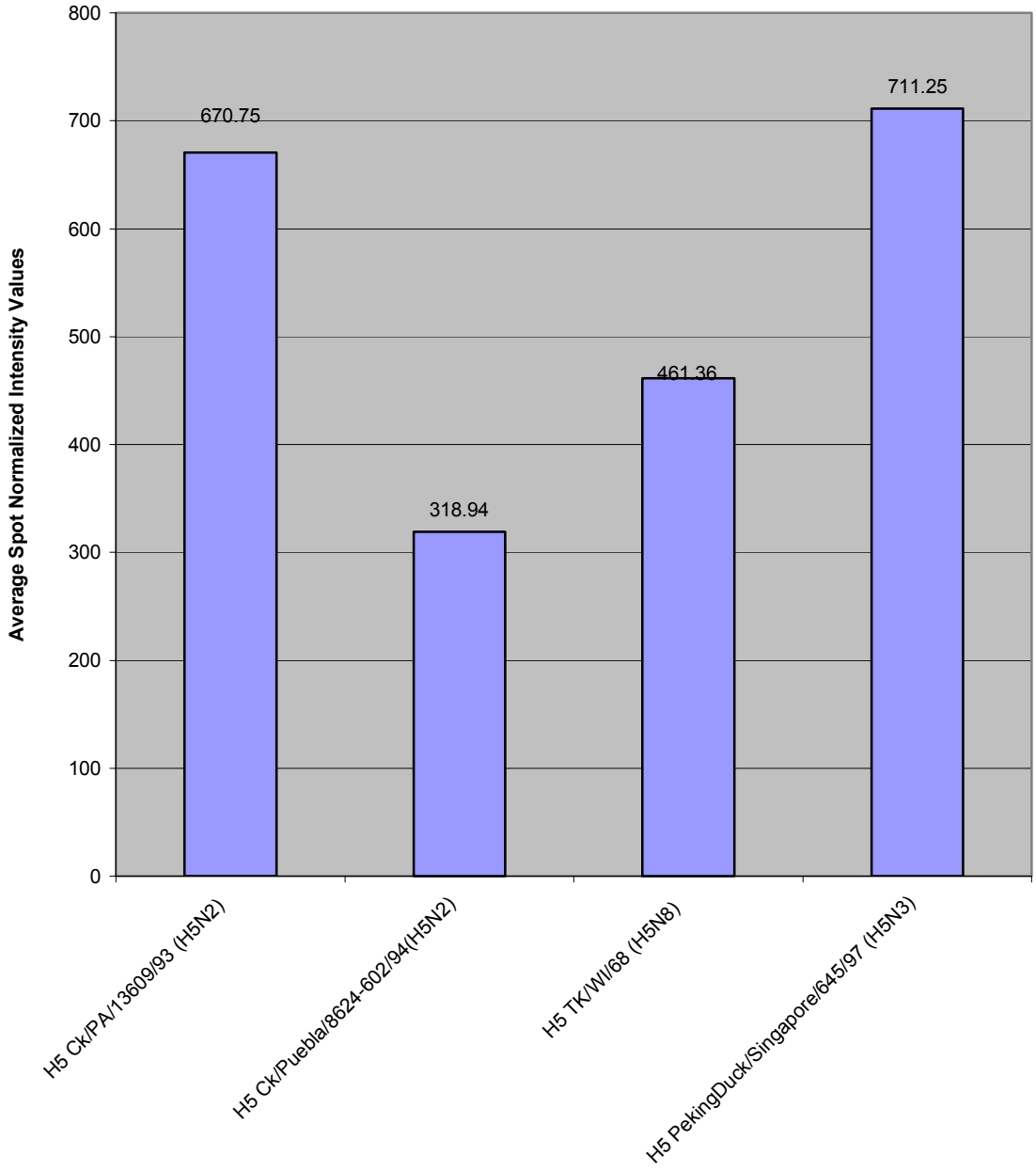
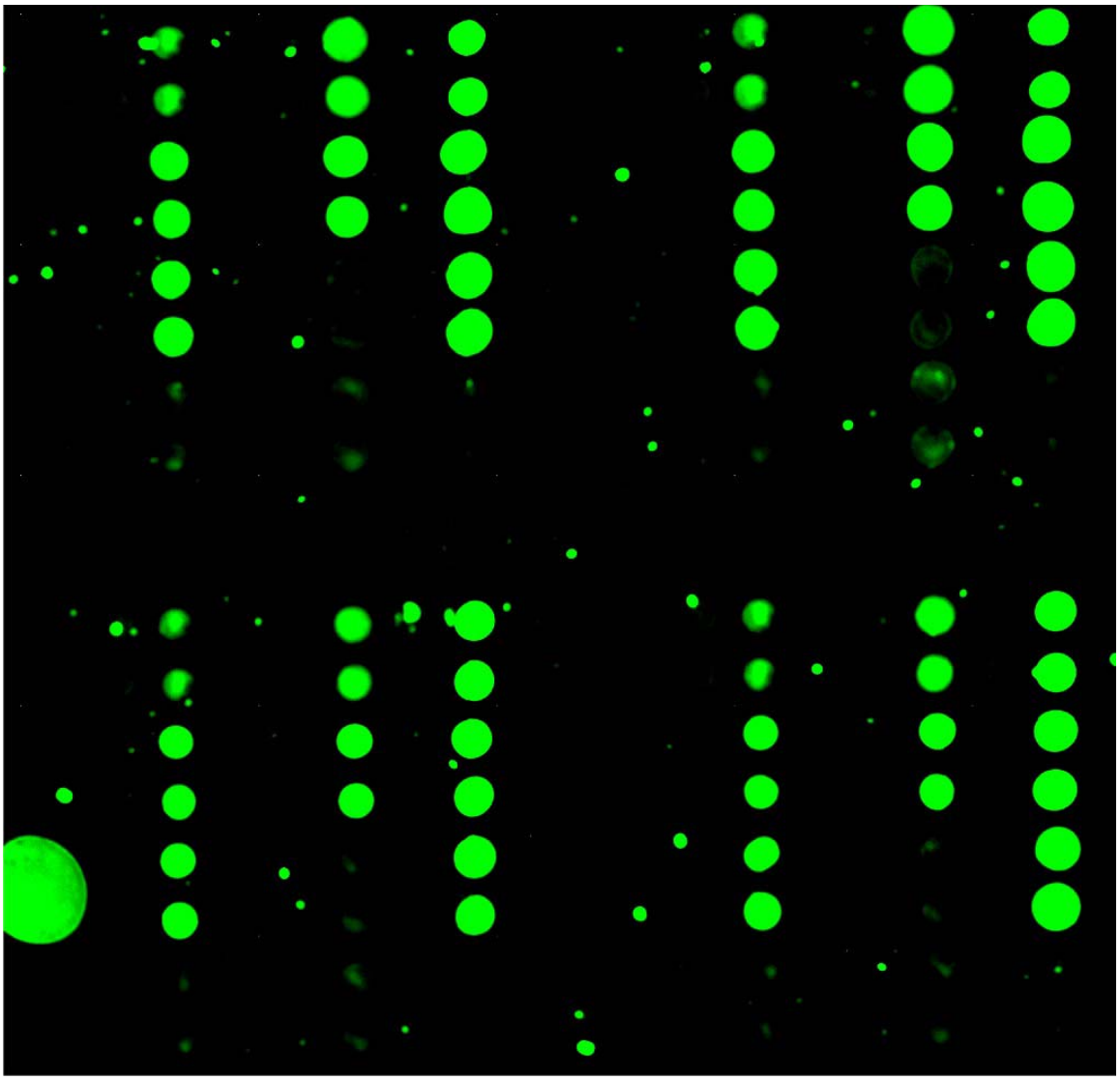

800
700
600
500
400
300
200
100
0
Average Spot Normalized Intensity Values
670.75
318.94
461.36
711.25
H5 Ck/PA/13609/93 (H5N2)
H5 Ck/Puebla/8624-602/94(H5N2)
H5 TK/WI/68 (H5N8)
H5 PekingDuck/Singapore/645/97 (H5N3)
H5 Hemagglutinin Elements Present on the AI Microarray Exhibiting Positive Signal

Figure 3.19 Scanned Image of Unknown J Hybridized to the AI Microarray. In a hybridization experiment with unknown J, 10 μg of Alexa555 labeled aRNA was hybridized to the array at 50°C for 3 hr. Fluorescent signals are observed for matrix, NA2, and HA7 elements. The hybridization pattern is consistent with a type A influenza subtyped as H7N2.

NDV N3 N2 N1 H9 H7 H5 M



3.2.11 Summary of Results

The summary of the microarray analysis of the unknown panel of AIV isolates is summarized in Table 3.3. The AI cDNA microarray identified 100% (10/10) of the unknown isolates correctly as type A influenza viruses. Complete HA and NA subtyping was achieved for 70% (7/10) of the isolates. In 7/10 and 9/10, only HA and/or only NA subtypes respectively were correctly identified. The microarray misidentified 1/10 HA subtypes and 0/10 NA subtypes. For the four H5 isolates evaluated, the microarray characterized the isolates correctly 100% of the time for their phylogenetic clade.

Upon analysis of the unknown panel, it was noticed that two of the unknowns, D and G, Ck/Puebla/8624-602/94 (H5N2) and Dk/Singapore/97 (H5N3) respectively, had homologous corresponding elements on the microarray. For this reason, Clustal W (version 1.83) analysis (Thomson *et al.*, 1994) was performed on each of the hemagglutinin gene sequences of the unknown panel isolates and compared to the hemagglutinin gene sequences present on the microarray to evaluate the ability of the microarray to correctly identify heterologous sequences. The comparisons are illustrated in Table 3.4. The analysis showed that of the unknown isolates correctly HA subtyped by our microarray, there was 78-100% homology between the unknown and the correct HA elements on the microarray. When sequence homology was < 78%, the microarray was unable to correctly identify the HA subtype. It should be noted that only partial sequence of the H5 HA elements on the microarray and some of the unknown isolates were available for analysis and this could potentially falsely alter the percent nucleotide homology.

Table 3.3 Results from the Unknown Panel of AIV Isolates. Subtype and strain designation as indicated in key after determination of microarray result (*).

Unknown Sample	Subtype*	Strain*	Microarray Result
A	H1N1	Dk/NJ/7717-70/95	H7 N1
B	H7N1	SB/IL/33445-136/92	H7 N1
C	H5N1	Ck/HongKong/220/97	H5 (Eurasia) N1
D	H5N2	Ck/Puebla/8624-602/94	H5 (Mexico) N2
E	H9N2	Ck/NJ/12220/97	H9N2
F	H7N7	Ck/VIC/85	Type A
G	H5N3	Dk/Singapore/97	H5 (Eurasia) N3
H	H7N3	Ck/PAK/1369-CR2/95	H_N3
I	H5N1	Ck/Scotland/59	H5 (Eurasia) N1
J	H7N2	Ck/NJ/294508-12/04	H7 N2

Table 3.4 Percent Homology Between Hemagglutinin Gene Elements on the Microarray and Hemagglutinin Gene Sequences from the Panel of Unknown Isolates. Clustal W (version 1.83) sequence analysis of unknown panel hemagglutinin gene sequences and hemagglutinin gene sequences present on the microarray. Only partial sequence (445 bp) was available and therefore analysis was unable to be acurately performed*.

	Unknown Panel									
	A	B	C	D	E	F	G	H	I	J
Microarray Element	Dk/NJ/7717-70/95 (H1N1)	SB/IL/33445-136/92 (H7N1) *	Ck/HongKong/220/97 (H5N1)	Ck/Puebla/8624-602/94 (H5N2)	Ck/NJ/12220/97 (H9N2)	Ck/VIC/85 (H7N7)	Dk/Singapore/97 (H7N3)	Ck/PAK/1369-CR2/95 (H7N3)	Ck/Scotland/59 (H5N1)	Ck/NJ/294508-12/04 (H7N2)
PekingDuck/Singapore/645/97 (H5N3)	62	-	93	80	57	35	100	54	88	10
Ck/PA/13609/93 (H5N2)	60	-	79	91	57	36	82	32	80	18
Ck/Puebla/8624-602/94 (H5N2)	60	-	78	100	57	36	80	32	78	27
Tk/WI/68 (H5N8)	61	-	79	88	57	32	80	32	82	28
Ck/NY/13142-5/94 (H7N2)	37	-	36	29	40	75	26	76	34	95
Tk/OR/71 (H7N3)	37	-	35	27	39	75	25	76	30	87
Ck/Korea/96006/96 (H9N2)	57	-	58	54	87	37	57	36	56	35

Chapter 4

DISCUSSION

In construction of an avian influenza cDNA microarray, our goals were to develop a novel and robust avian pathogen detection platform that could simultaneously HA and NA subtype AIV isolates. Microarray technology has already been utilized to detect over 140 sequenced viral genomes essentially representing all human respiratory tract viruses (8). Therefore we believe that this technology can also be developed to detect and diagnose avian influenza. This AI cDNA microarray is a "proof of concept" array demonstrating the ability of microarrays to detect avian viral pathogens.

This is the first report of an avian influenza cDNA microarray and it is the first influenza array to evaluate unknown isolates. Previous studies using microarrays as a method to detect and subtype human (Li *et al.*, 2001) and equine (Sengupta *et al.*, 2003) influenza isolates have been successful, and led to the recent production of an influenza integrated microfluidic device from CombiMatrix (Mukilteo, WA) that can identify type A influenza viruses and fully subtype them. However, this technology is, essentially, an advanced polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) on a glass-silicon wafer device, and not a microarray (Pal *et al.*, 2005). The most similar array to our AI cDNA microarray is the DNA microarray produced by Li *et al.* (2001), in which human influenza isolates were identified and subtyped using a cDNA

microarray. However, the human influenza cDNA microarray, as well as the equine influenza oligonucleotide microarray (Sengupta *et al.*, 2003), and the DNA Flow-Thru Chip (Kessler *et al.*, 2004) all validated their microarrays using known isolates of influenza with homologous elements on their microarray. This does not represent a "real world" situation in which isolates will be used on the microarray that do not have corresponding genes from the same isolate represented on the microarray.

Other unique attributes of our AI cDNA microarray are that it is exclusively avian, it has been tested with an unknown panel of AIV isolates, and our microarray also has the capability to determine the geographical origin/phylogenetic clade of the virus strain. Previous studies have used strains homologous to the probe spotted on the array in their hybridizations to validate their arrays.

The AI cDNA microarray was created using standard microarray protocols. Briefly, we spotted cDNA RT-PCR products from the hemagglutinin, neuraminidase, and matrix genes of various avian influenza isolates and subtypes onto a coated glass slide. In total, there are 16 elements on the microarray. Specifically, there are three matrix gene elements from three different isolates, three hemagglutinin subtypes represented by 7 isolates, three neuraminidase subtypes represented by 5 isolates, and finally one element, the NDV F gene, serving as a negative control. The hemagglutinin gene amplicons range from 920 to 1726 bp in size, the neuraminidase gene amplicons range from 1382 to 1385 bp, and the matrix gene amplicons are uniformly 849 bp long. These probes are fixed on silanated amine glass microarray slides in four subarrays containing duplicate spots yielding 8 replicate spots for each element, illustrated in Figure 3.1.

Once the microarray was constructed, each element on the array was evaluated for its overall functionality in yielding a fluorescent signal intensity. This was accomplished by first staining the slide with SYBR green to test for the presence and spot morphology of covalently bound cDNA to the silanated amine microarray slide. Following SYBR green staining, a hybridization of the sample from which the cDNA was amplified, was hybridized to the microarray in a homologous hybridization. Pending positive SYBR green staining and homologous hybridization results, the element was deemed fully functional and maintained on the microarray for evaluation of different AI isolate samples or unknown samples. In this manner, all 16 elements were confirmed.

To validate the microarray and evaluate its utility in avian influenza detection, an unknown panel of AIV isolates was tested. This unknown panel experiment differentiates our AI cDNA microarray from other microarrays. The unknown panel of ten avian influenza isolates, A through J, was provided by Dr. David L. Suarez of SEPRL ARS USDA (Athens, GA). This panel was tested against our array to validate its detection and subtyping ability. To do this, vRNA in DEPC-water was amplified and fluorescently labeled before being hybridized to the array. The results of the evaluation of the unknown samples by the AI cDNA microarray are illustrated in Table 3.3. Overall, the array was able to correctly identify 10/10 samples as type A influenza. Complete H and N subtyping was accomplished for 7/10 unknowns. Additionally, the array was able to correctly identify, fully subtype, and characterize, phylogenetically/geographically, 4/4 H5 subtypes present in the unknown panel.

It should be noted that the matrix gene elements on the microarray were RT-PCR amplified from three different HA subtypes (H5, H7 and H9) and that all three of these

matrix gene elements consistently provide high fluorescent signal intensity values, and consistently identified samples as type A influenza viruses. This result suggests that the microarray can identify type A influenza viruses regardless of their subtype designation.

Another strength of the microarray lies in its ability to further characterize HA genes by phylogenetic source. The microarray contains H5 HA elements representing four of the five phylogenetic clades for H5 HA subtypes (Figure 3.1 and 3.2). The microarray correctly identified all the H5 isolates present in the unknown panel. These isolates originated from chickens in Hong Kong, China; Puebla, Mexico; Scotland; and from ducks in Singapore. Having representative elements on the microarray from each phylogenetic clade allows us to further characterize the H5 isolates by geographical origin. The hybridization signal strength is not just observed qualitatively, but can be accurately quantified by data analysis of the average spot normalized intensity values using ANOVA.

The current microarray, however, has some limitations. In general, there are several subtypes which are currently not represented on the microarray, as only three of the 16 hemagglutinin subtypes and three of the nine neuraminidase subtypes are included on the present version. Without full HA and NA subtype representation on the microarray, we lack the ability to subtype certain isolates. This was demonstrated by unknown samples F and H. Specifically, the lack of representative H7 HA elements on the microarray from each of the four identified HA7 phylogenetic clades led to a failure to accurately subtype these two H7 isolates. Currently, the microarray has two H7 HA elements representing only one of the four avian H7 hemagglutinin clades. Figure 3.3 illustrates the H7 hemagglutinin phylogenetic tree. The two H7 HA elements on the

microarray are contained within the USA clade. Unknown F, CK/VIC/85 (H7N7) was only identified as a type A influenza virus and was not subtyped because there is not an H7 HA probe for the phylogenetic clade to which unknown F belongs (Africa, Eurasia & Australia). Unknown F was not neuraminidase subtyped due to the lack of NA N7 elements on the microarray. Unknown H, Ck/PAK/1369-CR2/95 (H7N3) was identified as a type A influenza and was correctly NA subtyped as an N3, but the array was unable to determine the HA subtype of the isolate even though it was an H7. This limitation was again due to the fact that there was not an H7 HA probe for the phylogenetic clade to which unknown H belongs (Africa & Eurasia).

The failure to accurately HA subtype unknown F and H is important because it exemplifies the importance of full phylogenetic clade representation on the microarray for each of the hemagglutinin subtypes. In homologous hybridizations, the fluorescent signal intensity within the hemagglutinin gene elements is comparable to the signal intensity of the neuraminidase and matrix gene elements. In these hybridizations, the average spot normalized intensity values for the corresponding HA element on the microarray either exceeds the average spot normalized intensity (SNI) values or fall within a 50% range of the average SNI values for the matrix or neuraminidase elements. This fact is supported quantitatively by Table 3.4, in which Clustal W analysis revealed the percent nucleotide identity (homology) between the HA sequences of the unknown panel and the HA sequences of the elements present on the microarray. Taken together, Tables 3.3 and 3.4 indicate that the microarray correctly identified HA sequences from the unknown panel that share ≥ 78% nucleotide identity with the HA sequences present on the microarray. In the case of the four H7 isolates in the unknown panel (B, J, F, and

H), only two of them were accurately subtyped. The explanation of full phylogenetic clade representation is important and supported quantitatively by Table 3.4 in which it can be seen (with the exception of unknown B due to the lack of available full sequence) that unknown J Ck/NJ/294508-12/04 (H7N2) had 95% and 87% homology to the H7 HA elements on the microarray, Ck/NY/13142-5/94 (H7N2) and Tk/OR/71 (H7N3), respectively. However, unknown F and H, which were unable to be HA subtyped as H7s only had 75% and 76% homology for both of the H7 HA elements on the microarray respectively. These findings illustrate the limit of the microarray, under current protocols, to identify HA sequences sharing less than 78% homology to the HA sequences present on the microarray.

Accordingly, the more heterologous the experimental isolate is from the element on the microarray, the less the fluorescent signal intensity. Therefore, if the isolate being hybridized to the microarray does not have a representative isolate from the same hemagglutinin phylogenetic clade, the fluorescent signal intensity decreases drastically (>80% on average) and does not yield positive signal. This problem is exacerbated when only some of the hemagglutinin subtypes are represented on the microarray because there can be considerable cross-hybridization to different hemagglutinin subtypes which leads to inaccurate HA subtyping.

Finally, unknown A was correctly identified as a type A influenza, belonging to the N1 NA subtype, but was incorrectly HA subtyped as an H7, when it was in fact an H1. This failure was a consequence of not having an H1 HA element on the array. Weak cross-hybridization to H7 HA elements led to the incorrect assignment of hemagglutinin subtype. Analysis of the nucleotide sequence homology, represented in Table 3.4,

illustrates that there was little homology of the HA1 sequence of unknown A (37-62%) and no correlation between homology of HA gene sequences between sample sequence and microarray sequences, and fluorescent signal intensity.

Our array was able to subtype isolates with as little as 78% sequence homology (within HA subtype) among hemagglutinin gene sequences within the same subtype. This represents the typical range of nucleotide differences within a hemagglutinin subtype (present in our DNASTAR MegAlign hemagglutinin multiple sequence alignments) and shows the ability of the array to recognize and hybridize diverse nucleotide compositions. Fluorescent signal intensity values measured for each element yield more specific information on each subtyped isolate and can be directly correlated to sequence homology of the probe and target, or element on the array and fluorescently labeled sample.

Issues of cost, time, and training of lab personnel still need to be evaluated with respect to use of microarrays in a clinical setting. The cost of performing the microarray experiment in our laboratory, from extracted vRNA to result, costs approximately $300 USD per sample. There are also indirect costs associated with the capital required to purchase and maintain the equipment needed to conduct microarray experiments. With regards to time, the microarray procedure takes approximately 42 hours total, and could potentially be completed in less time once hybridization duration is evaluated experimentally. Finally, microarrays require highly skilled lab personnel and a laboratory environment to conduct the RNA amplification, hybridization, and scanning of the microarray slide.

To evaluate the potential of microarrays to be used in a clinical setting, the microarray must be compared to current detection assays. Sensitivity experiments, using serial dilutions of titered virus stock and comparing microarray results to qPCR for validation and comparison, need to be conducted. To give the microarray more potential as a future diagnostic tool, starting material such as tracheal swabs or nasal aspirates should be evaluated to determine whether or not the virus sample must be cultivated in embryonated chicken eggs. If the step of growing virus in embryonated eggs could be avoided, this would save several days and considerable cost in the overall scheme of identifying avian influenza viruses.

In terms of applying microarray technology not only to clinical settings but also to point-of-care testing, the AI cDNA microarray could be adapted to a format similar to ELISA utilizing colorimetrics. This would decrease the expense involved in sample preparation and make the microarray capable of point-of-care testing in which the assay can be performed on-site instead of in a laboratory. Consequently, the time (reduced by shorter hybridization times) and need for skilled laboratory personnel would be greatly reduced, adding to the overall utility of a true microarray diagnostic.

Future experiments would evaluate the aforementioned issues such as sensitivity, cost, utility, and application of the microarray in diverse settings. The most imperative work would be adding the rest of the hemagglutinin (H1 through H16) and neuraminidase (N1 through N9) subtypes. This addition to the microarray would allow for complete H and N subtyping for all avian influenza isolates. Also, the addition of representative elements from each phylogenetic clade within hemagglutinin subtypes would result in further characterization of AIV isolates. If at all possible, we would like to conduct

phylogenetic analysis of all of the hemagglutinin gene subtypes and include representative HA elements on the microarray from each clade as we have done for the H5 HA elements on our microarray. Including other avian respiratory viral genes from infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), and Newcastle disease virus (NDV) would greatly expand the utility of the microarray because these avian respiratory diseases can often be confused with AI and must be ruled out in order to manage the flock appropriately. Bacterial respiratory pathogen genes from *Mycoplasma gallisepticum* and *Hemophilus paragallinarum* could also be added to the microarray to aid in differential diagnosis. The primers used in the aRNA amplification and indirect labeling protocol can be modified and other primer sets can be added to include IBV, ILTV, NDV, and various other avian respiratory pathogens of both vial and/or bacterial nature. Several modifications of the current protocols to accommodate pathogens containing DNA genomic material would have to take place before hybridization to a microarray, but such work has been completed for human respiratory pathogens (Lin *et al.*, 2006). The addition of these elements would greatly add to the diagnostic and surveillance power of the array as well as aid in differential diagnoses. Finally, using clinical material such as tracheal swabs as starting material for vRNA amplification would drastically reduce the amount of time involved in the processing of samples and obtaining results. Tracheal swabs are also more clinically relevant, as they are one of the standard samples procured during inspection of ill birds, and would also expand the utility of the microarray.

Another application of the AI microarray is to use the aRNA amplification and indirect labeling protocols described in Materials and Methods in hybridizations to an AI

oligonucleotide based array. An oligo array would be ideal for AIV diagnostics because it would have the ability to identify single nucleotide differences in sequence, track antigenic drift/shift, and can be directed toward the HA cleavage site, identifying the potential for a virus to mutate into a highly pathogenic strain. An oligonucleotide array could avoid some of the pitfalls of the cDNA array because signature oligonucleotides could be designed to hybridize to regions of high sequence similarity within an HA subtype or be designed specifically for particular clades or isolates allowing for more control over a microarray experiment. To develop an oligonucleotide array, signature oligonucleotides will need to be created representing the various elements present on the cDNA array.

The AI cDNA microarray has far-reaching applications. In our unknown panel, we were able to identify, subtype, and phylogenetically characterize Ck/HongKong/220/97 (H5N1). This virus is related to the H5N1 strain currently circulating in Southeast Asia and causes public health concerns. Recently, this strain has shown the ability to expand its host range (from avian to human), infecting human populations in Asian countries with over 50% mortality associated with infection (World Health Organization). Within 42 hours, our array can not only identify this virus as being a type A influenza virus, but it can subtype it as an H5N1 and determine that it is indeed from the Eurasian H5 clade and should be cause for concern.

In conclusion, the prototype avian influenza cDNA microarray has been proven effective at identifying type A avian influenza isolates, subtyping H5, H7, H9, N1, N2, and N3 hemagglutinin and neuraminidase subtypes, and charactering the H5 HA isolates based on their phylogenetic/geographical origin. The AI cDNA microarray currently

stands as a complementary tool in the detection and surveillance of avian influenza viruses.

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