ABSTRACT

Title of Document: EPIDEMIOLOGICAL ANALYSIS OF BIOSECURITY PRACTICES AND ASSOCIATED PREVALENCE OF DISEASES IN NON-COMMERCIAL POULTRY FLOCKS

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A cross-sectional study was conducted in backyard poultry flocks among nine counties of Maryland from May 2011 to August 2011. The objective of this study was to obtain baseline data from a survey on biosecurity practices and investigate risk factors associated with positive findings of avian influenza (AI), Newcastle disease (ND), infectious laryngotracheitis (ILT), *Mycoplasma gallisepticum* (MG), and *Salmonella* Enteritidis (SE). Serum, tracheal, and cloacal swabs were randomly collected from 262 birds among 39 registered premises. Analysis revealed flock prevalence and seroprevalence respectively for the following: AI (0%, 23%), ND (0%, 23%), ILT (26%, 49%), MG (3%, 13%), SE (0%, ND). Vaccine status could not be confirmed for ND, ILT, or MG. Premises positives were identified by partial nucleotide sequencing. No statistically significant associations were identified, however, AI seroprevalence was positively associated with exposure to waterfowl (Relative Risk [RR] = 3.14, 95% confidence interval [CI] 1.1-8.9) and absence of pest control (RR=2.5; 95% CI, 0.6-10.4).

EPIDEMIOLOGICAL ANALYSIS OF BIOSECURITY PRACTICES AND ASSOCIATED PREVALENCE OF DISEASES IN NON-COMMERCIAL POULTRY FLOCKS

By

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Thesis submitted to the Faculty of the Graduate School of the
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Advisory Committee:
Associate Professor Nathaniel Tablante, Chair
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Dedication

For my loving husband and family, who have been a source of encouragement and inspiration to me throughout my life.

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Other positive control samples were kindly provided by outside sources. The Frederick Animal Health Diagnostic Laboratory generously provided *Salmonella*

isolates and Dr. Naola Ferguson-Noel from the University of Georgia donated samples of *Mycoplasma gallisepticum* A5969 DNA. Ultimately this thesis would not have been possible without the collaboration from the Maryland Department of Agriculture (MDA) and Maryland backyard flock owners, who I would like to thank for their patience and assistance and a big thank you to all their birds who involuntarily participated in the study. Funding for this research was made possible through the Maryland Agricultural Experiment Station.

Finally, I would like to thank my family for all of their love and support, and teaching me to believe anything is possible. My husband, for all the places we've been, and for all the places we will go. Your love and encouragement is what kept me motivated.

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Chapter 1: Introduction

1.1 Rationale and Objectives

Poultry diseases can have major implications on a country's economy, food source, and public health. With recent concern over the highly pathogenic avian influenza outbreaks around the world, government agencies are carefully monitoring and inspecting for infectious poultry diseases and zoonotic pathogens. However, there remains no organized inspection or control of non-commercial poultry flocks which have served as the point source of several poultry disease outbreaks. At present, only a few studies have evaluated the prevalence of disease in backyard flocks. These factors among others contribute to the need for ongoing surveillance research to minimize the costs associated with quarantines, depopulation, and loss of production time which radiate beyond the affected premises. Even more significant are the impacts felt by immediate trade bans and export restrictions levied upon countries infected with diseases such as avian influenza or exotic Newcastle disease. The Delmarva region, a peninsula consisting of portions of Delaware, Maryland, and Virginia, is of particular concern due to its previous experiences with avian disease outbreaks. Delmarva has a densely populated poultry industry in close proximity to infamous live bird markets of the northeast, which have been susceptible to avian influenza in the past. This region is also located within the path of a major migratory flyway providing a means for disease reservoirs to travel across large geographical areas. Throughout their migration, infected birds may congregate with other wild and domestic species leading to further dissemination of diseases.

The ultimate goal of this study was to identify and understand potential transmission pathways of pathogens of economic and public health importance by conducting a cross-sectional molecular epidemiological study. Disease prevalence results were correlated with respect to biosecurity measures practiced among non-commercial poultry operations in Maryland. This research plan aimed to achieve seven major objectives.

1. Characterize Maryland non-commercial poultry flock owners and describe currently practiced biosecurity measures, obtained from self-administered questionnaire data.
2. Indentify seroprevalence and vaccination status of pathogens under investigation including avian influenza virus (AIV), Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILTV), and *Mycoplasma gallisepticum* (MG) using enzyme-linked immunosorbent assay (ELISA).
3. Determine prevalence of pathogens by flock status for avian influenza virus (AIV), Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILTV), *Mycoplasma gallisepticum* (MG), and *Salmonella* Enteritidis using Quantitative Polymerase Chain Reaction (q-PCR) and Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR).
4. Identify and differentiate strains from PCR positive flocks through nucleotide sequencing.
5. Test for the presence of antibodies to influenza A H5, H7, and H9 subtypes in a hemagglutinin inhibition (HI) assay.

6. Evaluate statistically significant biosecurity risk factors used in small flocks that may be associated with disease status.
7. Develop online eXtension educational programs on practical and effective methods of preventing and mitigating poultry disease outbreaks in non-commercial poultry flocks.

1.2 Literature Review

The poultry pathogens of particular concern in this study include avian influenza virus, Newcastle disease virus, infectious laryngotracheitis virus, *Mycoplasma gallisepticum*, and *Salmonella* Enteritidis. These pathogens are capable of causing substantial morbidity and mortality in poultry and/or humans and are reportable to national and/or international agencies (Table 1).

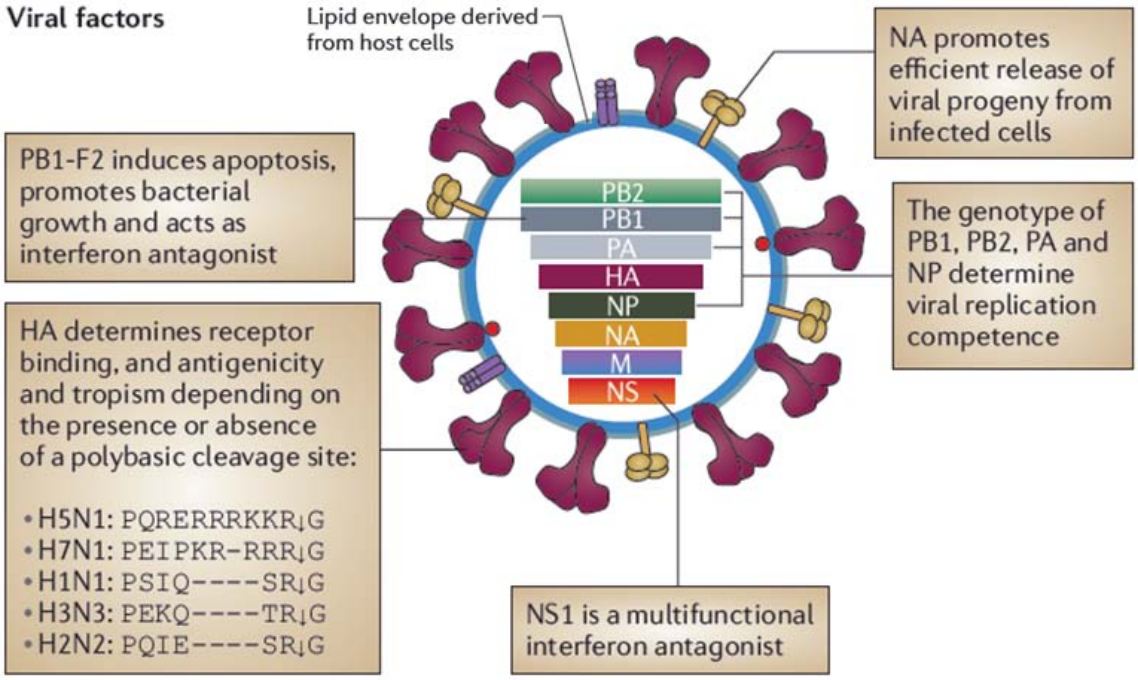
Table 1 Description of OIE reportable poultry diseases

Disease	***Type of Agent***	***Natural Hosts***	***Mortality Rate***	***Clinical Signs***
Avian Influenza (AI)	Type A influenza virus (family Orthomyxoviridae)	Most, if not all bird species	Highly pathogenic 90-100%	Severe respiratory disease, edema, cyanosis, decreased egg production
			Low pathogenic 10-20%	mild respiratory disease, ruffled feathers, decrease egg production
Newcastle Disease (ND)	Avian paramyxovirus type 1 (APMV-1)	Most, if not all bird species	Virulent Velogenic 100%	Dyspnea, edema, diarrhea, neurological signs: torticollis, paralysis, and opisthotonos
			Neurotropic Velogenic 50-100%	Severe respiratory disease and neurological signs, decreased egg production
			Mesogenic ND <10%	acute respiratory disease, decrease egg production, occasionally neurologic signs
			Lentogenic ND low	Mild coughing, gasping, sneezing, and rales
Infectious Laryngotracheitis (ILT)	Gallid herpesvirus 1 (family Herpesviridae)	Chicken and Pheasant	10-20%	Nasal discharge, rales, coughing, dyspnea, blood-stained mucus
***Mycoplasma gallisepticum* (MG)**	Gram negative, coccoid bacteria	Chicken, turkey, pigeon, peafowl, quail, passerine	Low in uncomplicated cases	Rales, coughing, nasal discharge, conjunctivitis, and in turkeys infraorbital sinusitis

1.2.1 Avian Influenza

Avian influenza (AI) is caused by type A influenza virus which has a segmented genome of eight negatively sensed, single stranded, RNA particles encoding 11 to 12 proteins, totaling 13.5 kb in length. Type A influenza viruses are categorized into serological subtypes based on surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Host tropism is highly dependent on HA as it binds to host cell receptors that contain terminal α-2,6 linked or α-2,3 linked sialic acid molecules. AI viruses preferentially bind to α-2,3 linked receptors of avian respiratory epithelium, while human influenza viruses have a higher affinity for α-2,6 linked receptors of the upper respiratory tract. Although human non-ciliated cuboidal bronchiolar and alveolar type II cells located in the lower respiratory tract contain α-2,3 linked receptors, infection with non-human adapted viruses is rare. HA also consist of a cleavage site with varying amino acid sequences that determine the tissue tropism and disease severity (Figure 1) (Medina and Garcia-Sastre, 2011). Currently, sixteen hemagglutinin and nine neuraminidase subtypes have been identified, with H5 and H7 often exhibiting the most virulence in poultry. Most laboratories initially rely on the matrix protein for detection of AI as it is the most abundant protein and highly conserved in all influenza A viruses (Spackman and Suarez, 2008).

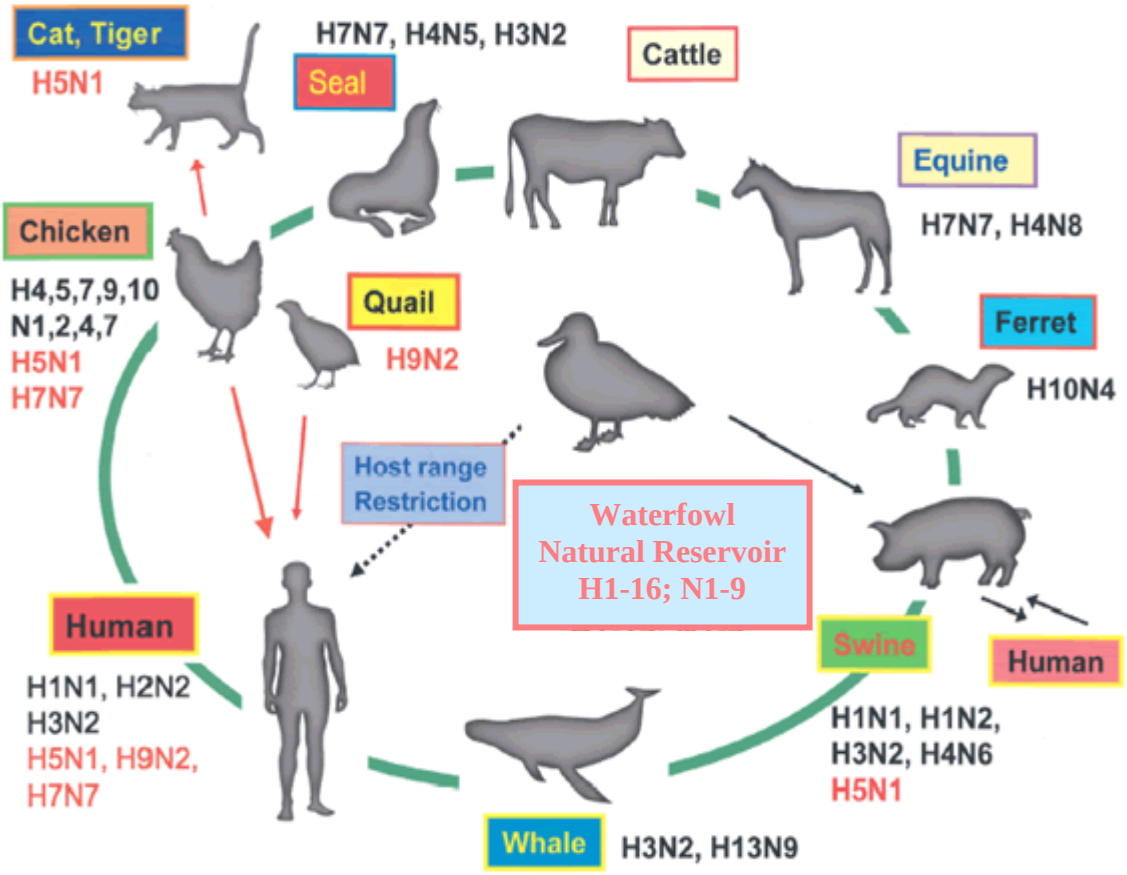
Figure 1 Avian Influenza virus genome and viral factors that influence pathogenesis (Medina and Garcia-Sastre, 2011)



Influenza type A viruses are zoonotic pathogens capable of infecting a wide range of species. Aquatic birds are the natural reservoir for influenza A viruses and can carry all 144 possible subtype combinations in their gastrointestinal tract, while human circulating strains affecting the respiratory tract are generally limited to H1N1, H2N2, and H3N2. Poultry may also carry a variety of HA and NA subtypes, including: (HA 4, 5, 7, 9, 10 and NA 1, 2, 4, 7), as well as H5N1 and H7N7 subtypes (Figure 2). Despite producing large quantities of virus, waterfowl generally present with no clinical signs of illness. However, infections in poultry and other incidental hosts may result in a wide variety of signs, further classifying the virus into highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI) (Causey and Edwards, 2008). HPAI viruses spread rapidly in poultry flocks, causing severe illness, and can kill 90 - 100% of

infected birds within 48 hours of exposure. However, most strains are LPAI with signs of disease ranging from none, to ruffled feathers, decreased egg production, and mild respiratory distress. Transmission occurs by direct contact with infected birds via their feces, saliva, or nasal secretions. Indirect transmission may occur through contact with contaminated equipment, clothing, litter, or drinking water. The primary route of infection is through oral ingestion, although conjunctival and respiratory routes are other potential means. AI viruses can persist for over a month in water and feces at 40°F and have an incubation period of 3-14 days (McMullin, 2004).

Figure 2 Ecology of avian influenza virus and species tropism (adapted from Medical Ecology)



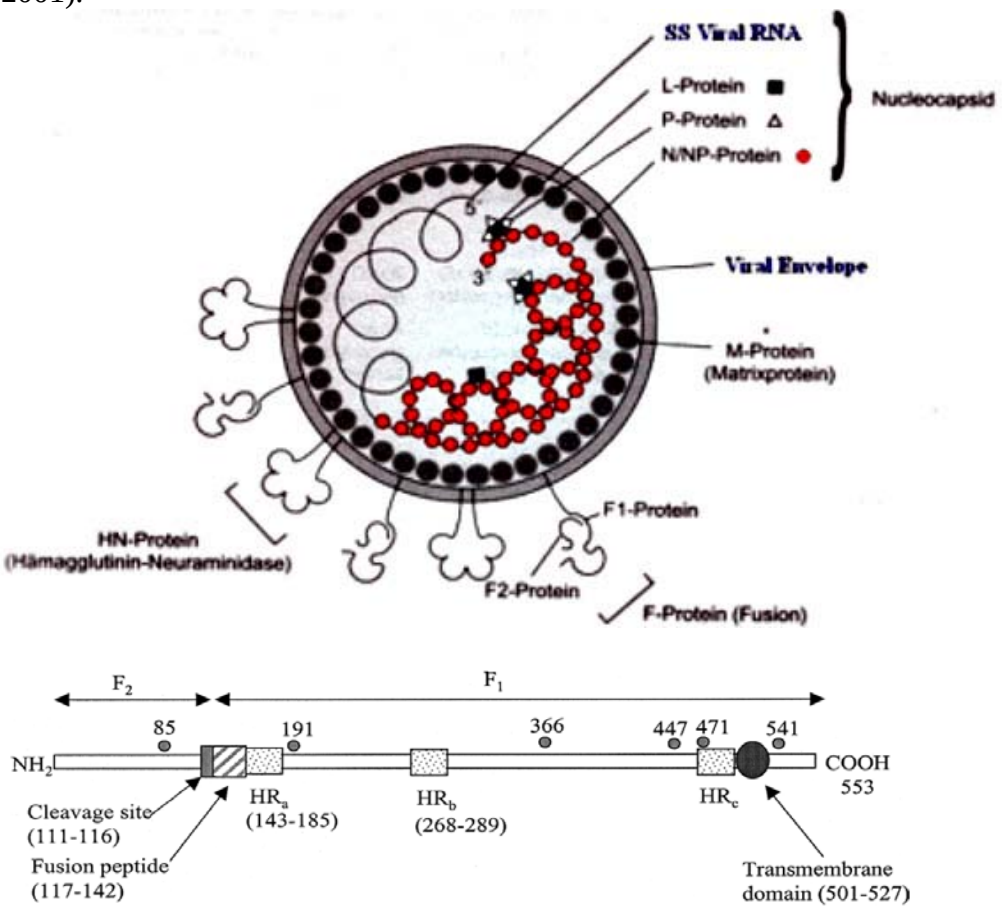
Of primary concern is HPAI H5N1, which has resulted in the death of millions of birds around the world. While endemic in some countries, it has not been detected in the U.S. and therefore commercial birds are generally not vaccinated. Another alarming characteristic of HPAI H5N1virus is its ability to cross the species barrier and infect humans. Influenza viruses are notorious for their ability to undergo antigenic drift and shift resulting in subtle or dramatic genotype changes respectively (Medina and Garcia-Sastre, 2011). As of June 2011, there have been 606 confirmed cases of human infection and 357 deaths, killing approximately 60% of those infected (WHO, 2011). While the virus is not easily transmitted from person to person, it results in an excessively high case fatality ratio making it a prime candidate for pandemic concern. Throughout the last century, three pandemics of novel influenza virus origins have erupted, placing mortalities in the millions worldwide. However, 2009 rang in the first influenza (H1N1) pandemic of the 21st century prompting animal and human health officials to remain attentive in monitoring and surveillance (WHO, 2009; CDC, 2005). All HPAI and LPAI H5/H7 subtypes are immediately reported to state and federal agencies as well as the World Organization for Animal Health (OIE) to help contain and eliminate the virus (USDA, 2007).

1.2.2 Newcastle Disease

Newcastle disease (ND) is caused by the avian paramyxovirus type I (APMV-1) serotype of the genus Avulavirus belonging to the Paramyxoviridae family and consists of 15,186 nucleotides. There are nine serotypes of avian paramyxoviruses, APMV-1 to APMV-9. Newcastle disease virus (NDV) is a nonsegmented, single-stranded, negative-

sense, enveloped RNA virus (Wakamatsu, 2007). The six open reading frames of NDV code for seven proteins (Figure 3). The hemagglutinin-neuraminidase glycoproteins bind to sialic acid cell surface receptors, triggering the fusion (F) protein to fuse the viral envelope to the host plasma membrane. Cleavage of the precursor glycoprotein F0 into F1 and F2 by host cell proteases is a requirement for viral infection. The fusion gene has been of particular interest as its diversity has allowed for genetic characterization of NDV isolates. The characterized amino acid sequence motifs at the F protein cleavage site are as follows: Lentogenic ^{112}G-R/K-Q-G-R↓L^{117}, Mesogenic/Velogenic ^{112}R/G/K-R-Q/K-K/R-R↓F^{117} (Dortmans et al., 2011).

Figure 3 Paramyxovirus genome structure and features of the F glycoprotein (Immunologisches Onkologisches Zentrum Köln www.ioz-cologne.de) (Yusoff and Tan, 2001).



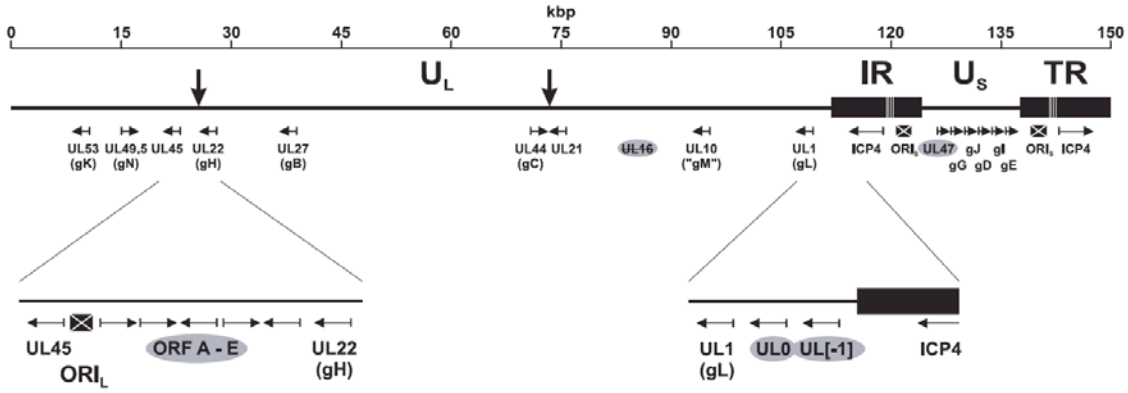
In poultry, NDV causes an array of clinical signs from subclinical to acute mortality. Signs vary depending on virus strain, host species, age of host, secondary infections, and stress. Associated signs include respiratory distress, diarrhea, cessation of egg production, inactivity, edema of the head, face, and wattles, nervous signs, and death. Strains of NDV have been grouped into five pathotypes on the basis of the clinical signs seen in infected chickens. Viscerotropic velogenic pathotype is a highly pathogenic form resulting in hemorrhagic intestinal lesions. Neurotropic velogenic form presents with high death rate subsequent to respiratory and nervous signs. Mesogenic pathotypes are characterized by respiratory and occasionally nervous signs and low mortality. Lentogenic forms present with mild or subclinical respiratory infection. The final pathotype is asymptomatic enteric consisting of subclinical infection. NDV is thought to primarily spread through inhalation of large droplets or via ingestion of infected feces which generally contain high viral loads. Incubation period, on average, is five to six days (Alexander, 2008). Prophylactic vaccination is practiced in all but a few of the countries that produce poultry on a commercial scale. The widespread presence of lentogenic strains in wild birds and the use of these viruses for live vaccines make diagnosis of disease difficult. Newcastle disease virus is also zoonotic and has been reported to cause eye infections in humans. Virulent NDV is considered an OIE listed notifiable disease (OIE, 2010).

1.2.3 Infectious Laryngotracheitis

Infectious laryngotracheitis (ILT) is a respiratory disease caused by Herpesviridae alphaherpesvirinae Gallid herpesvirus 1. ILT virus (ILTV) is a double stranded linear

DNA virus and its genome is 155 kb in size composed of an unique long (UL) and a unique short (US) region that is flanked by inverted repeats (IR). The ILTV genome has a total of 77 predicted open reading frames with 62 located in the UL region, nine in the US, and three in the IR (Ziemann et al., 1998) (Figure 4). Several studies have used glycoprotein C (gC), one of the major surface antigens of ILTV, for detection due to its conserved sequence, while the variable infected cell protein 4 (ICP4) has been used to differentiate between strains (Callison et al., 2006 and Chacon et al., 2009). Oldoni et al. have recently characterized circulating field strains by genotypes 1-6.

Figure 4 Map of double-stranded DNA genome of ILTV. (Fuchs et al., 2007)



As with other herpes viruses, ILTV has the ability to establish latent infections in the trigeminal ganglion, causing clinically inapparent infection which can persist in recovered birds for long periods with intermittent re-excretion of the virus. Incubation period is generally 6-12 days (Johnson et al., 2004). In areas with endemic disease, such as the U.S., ILT is controlled in layers with the use of modified-live virus vaccines such as chicken embryo origin (CEO) or tissue culture origin (TCO). However, studies have

shown that 63% of field isolates from commercial farms were similar to CEO vaccine strains. Providing further evidence of their ability to revert to virulence, non- attenuated CEO-related isolates can persist within naive backyard and fancier chicken flocks (Guy and Garcia, 2008).

ILT is primarily a disease of chickens; however, it may also affect pheasants, partridges, and peafowl. In chicken flocks, ILTV transmission occurs via respiratory and ocular routes. This virus presents clinically in three different forms: peracute, subacute, and chronic/mild. The peracute form produces the most sudden and severe cases of disease. The mortality rate may be over 50% with some deaths occurring prior to development of signs. Characteristics of the peracute form include anorexia, depression, and severe respiratory distress with coughing, gurgling, and rales. The neck is often extended upon inspiration as the trachea becomes partially occluded by bloody mucus exudate. In the subacute form, the onset of illness is slower and respiratory signs may be seen in the days prior to death. The mortality is lower than in the peracute form (between 10% and 30%), and signs of illness less severe, ranging from lacrimation, tracheitis, conjunctivitis, and mild rales. Chronic or mild ILT illness may involve spasms of coughing and gasping, nasal and oral discharge, and reduced egg production. ILT is notifiable to local, state, and federal agencies, as well as to OIE (OIE, 2010).

1.2.4 Mycoplasma Gallisepticum

Mycoplasma gallisepticum (MG) is a gram negative, coccoid, facultative anaerobe (0.25-0.5um) belonging to the family Mycoplasmataceae and is the most economically important of the avian mycoplasmas. Mycoplasmas are wall-less bacteria

and represent the smallest replicating organism. MG contains approximately 200 polypeptides in its plasma membrane which provide surface antigenic variation, adhesion, motility, nutrient transport and methods of immune evasion. MG targets sialic acid residues of the respiratory epithelium to initiate cytadherence and infection and has been known to survive intracellularly (Papazisis et al., 2000). PvpA, an integral membrane protein, has been used to identify sequence variations among strains as a result of its high frequency phase variation and size discrepancies ranging from 48 - 55kDa (Boguslavsky et al., 2000).

Mycoplasma gallisepticum is the etiological agent of chronic respiratory disease in chickens characterized by severe airsacculitis, coughing, rales, and poor growth. In turkeys and other game birds, swollen sinuses are commonly seen along with decreased meat and egg production. The severity of disease is greatly enhanced through stress and secondary respiratory pathogens (OIE, 2010). In poultry, the route of infection is via the conjunctiva or upper respiratory tract with an incubation period of 6-10 days. *Mycoplasma sp.* may be transmitted vertically through infected eggs, or by direct contact with birds, exudates, aerosols, airborne dust and feathers, and to a lesser extent, fomites. Spread is slow between houses and pens suggesting that aerosols are not normally a major route of transmission. However, fomites appear to be a significant factor in transmission between farms. Recovered birds remain infected for life and may experience recurrent disease (McMullin, 2004). While control of MG is widely maintained through biosecurity practices in breeding stock of turkey and chicken industries, U.S. layer flocks are considered endemic with disease occurring in over 50% of all egg laying facilities. Therefore, these facilities use live attenuated MG vaccines

such as the F strain, 6/85, and ts-11 (Evans et al., 2005). Mycoplasmosis is an OIE reportable disease (OIE, 2010).

1.2.5 *Salmonella* Enteritidis

Salmonella is the gram negative facultative anaerobe responsible for causing food-borne salmonellosis in humans (Pui et al., 2011). Previous epidemiological studies report that up to 3.7 million cases of samonellosis occur in the U.S. every year, with economic losses to poultry farmers ranging from $64-144 million annually. *Salmonella* serotype Enteritidis (SE) is one of the most prevalent serotypes of *Salmonella* bacteria reported globally. Using the Colindale phage-typing scheme, 16 phages have been used to identify 65 phages types for SE. Most types of *Salmonella* survive in the intestinal tracts of birds, but generally do not cause clinical illness (Omwandho and Kabota, 2010).

Although this serotype has been found in chicken meat, shell eggs are usually considered the most common vehicle for transmission of SE as human infection is typically acquired after consuming undercooked contaminated eggs. Signs and symptoms of salmonellosis include fever, abdominal cramps, and diarrhea lasting 4 to 7 days. Eggs become contaminated with SE by penetrating cracks in the shell. Vertical transmission has also been implicated as the bacterium can silently infect the ovaries of healthy appearing hens and enter the egg prior to shell formation. Despite the fact that birds may be originally purchased as culture-negative chicks, SE has been isolated from insect and animal hosts living in and around hen houses. It is estimated that one out of every 20,000 eggs is contaminated with SE, leaving a total of 2.2 million eggs contaminated in the market (CDC, 2010; Guard-Petter, 2001). In July 2010, a substantial

nationwide increase of *Salmonella* Enteritidis isolates with pulse field gel electrophoresis (PFGE) pattern JEGX01.0004 were noted on PulseNet, a national subtyping network that tracks molecular surveillance of food borne infections. In August 2010, 380 million eggs were recalled after being linked to the outbreak of *Salmonella* poisoning. The CDC states that from May 1 to November 30, 2010 approximately 2,000 cases of *Salmonella* related to contaminated shell eggs were reported (CDC, 2010).

1.3 Background

1.3.1 Surveillance and Prevention Programs

Monitoring and surveillance of poultry diseases are essential components to early detection, prevention, and mitigation of potential outbreaks in the United States and around the world. The diseases of interest within this study can cause significant consequences to trade markets, animal and public health, as well as food security and are therefore monitored through cooperative programs involving industry, state and federal agencies, as well as international organizations. Domestically, individual state Departments of Agriculture as well as the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) are responsible for conducting disease surveillance programs among distinct bird populations including live bird markets, commercial flocks, migratory birds, and to a lesser extent, backyard poultry. While USDA surveys for all of the diseases included in this study, additional authorities may be involved as well. In an effort to prevent the introduction of highly pathogenic avian influenza (HPAI) into the country, USDA, along with the Departments of Homeland Security (DHS), the Interior (DOI), and Health and Human Services (HHS)

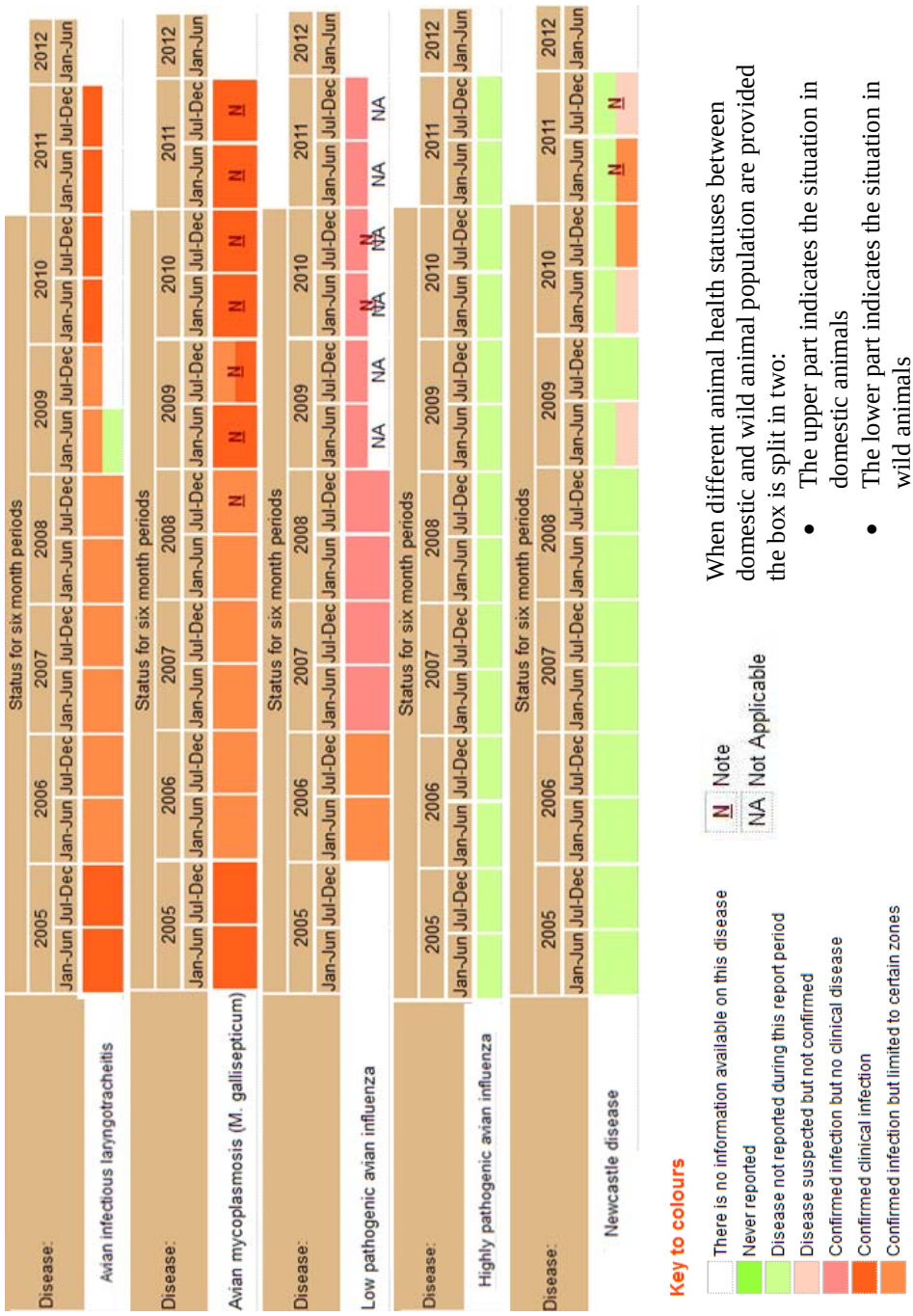
play important roles in surveillance, quarantine, enforcement of trade restrictions and interception of smuggled birds or bird products (USDA, 2007a). *Salmonella* on the other hand falls under the jurisdiction of USDA Food Safety and Inspection Service (FSIS) and Food and Drug Administration (FDA) surveillance. While USDA is responsible for the safety of meat, poultry, and processed poultry products, FDA conducts inspections of shelled eggs and production facilities (FDA, 2009).

There are also several international agencies that play a role in early warning and response to pathogens that could have considerable impact on animal and public health. The World Organization for Animal Health (OIE) provides global oversight and protection from the spread of reportable animal diseases by integrating animal health information from countries worldwide. By declaring disease status to OIE, a country gains the trust of neighboring and international trade partners. OIE's World Animal Health Information Database (WAHID) also provides online access to comprehensive information of disease status by country (www.oie.int/wahis/public.php). All of the diseases, except for *Salmonella* Enteritidis, are considered OIE reportable diseases. The United States has been no exception to OIE reportable diseases and has experienced several positive cases within the last decade (Table 2).

There are four methods of surveillance employed during domestic poultry monitoring including passive, active observational, active serologic, and active antigen surveillance, each targeting various flock subpopulations. While passive surveillance relies on voluntary reporting of clinical disease, active observational surveillance keeps a cognizant watch for morbidity and mortality. Serologic surveillance involves collecting blood samples and analyzing for the presence of antibodies indicating recent exposure to

disease. However, measurable antibody levels may take a week or more to develop after exposure. Antigen detection is also used to determine infection status, but is limited to pathogen shedding time points (USDA, 2007b).

Table 2 Disease timeline of OIE reportable diseases in the United States (2005 - 2012) (Source: www.oie.int/wahis/public.php)



Currently in the U.S., much of the poultry disease surveillance is initiated at the industry level, where poultry companies often employ their own veterinary staff and in-house diagnostic laboratory. Commercial poultry surveillance methods are generally considered sensitive and thorough. For example, using only an active observational surveillance approach, it has been predicted with 95% probability that large-volume industries would identify an outbreak of highly pathogenic avian influenza within two weeks from the flock's first exposure. While commercial industries undergo stringent biosecurity regulations and routine inspections, non-commercial flocks do not and may therefore be more vulnerable to pathogen exposure from wild animals and lax biosecurity practices. Other studies, such as the USDA National Avian Influenza Surveillance Plan, have reported a similar conclusion, signifying the need for non-commercial surveillance (USDA, 2007b).

Ownership of backyard poultry is also becoming a fast growing trend for many Americans as more and more people prefer fresh eggs and meat. However, these small poultry flocks present a unique challenge to surveillance as they are a diverse community with varying education and management practices. While USDA provides biosecurity education, it does not regulate private small flocks and some states do not require poultry registration. Historically, monitoring of backyard flocks has relied on passive surveillance from voluntary reporting of dead birds to Extension agents, private veterinarians, or state animal health diagnostic laboratories. While some owners would certainly notify authorities in the event of increased mortalities, others may dismiss the occurrence and dispose of carcasses without further action, thus allowing the disease to incubate for several weeks within the flock. In recent years some states, including

Maryland, have begun taking an active approach to surveillance by requiring registration and testing of flocks prior to participating in fair or auction markets, operating hatcheries, or selling local eggs or poultry. However, this population only accounts for a small percentage of owners. In 2005, USDA surveyed the reasons for having birds among backyard flock owners and reported that only 11.2% of owners ranked these combined categories (extra income and clubs/social interactions e.g. 4H/avian organizations) as high and very high importance (USDA, 2005).

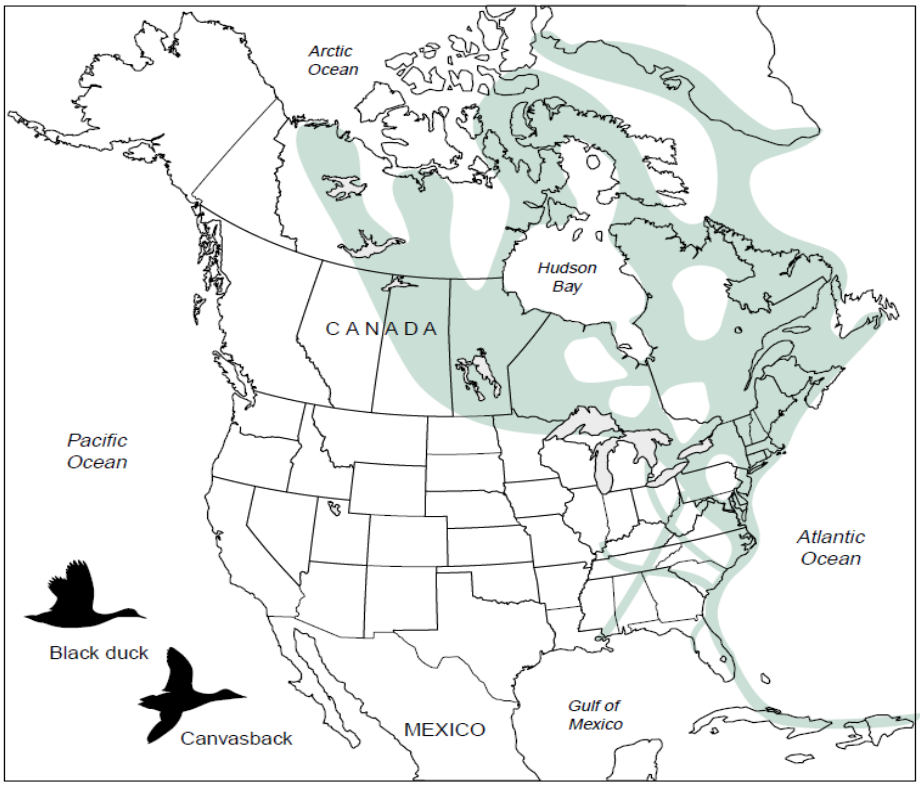
1.3.2 Poultry Industry

The United States is the world's largest producer and exporter of poultry meat and the second largest egg producer with almost 18 percent of total poultry production being exported. According to 2011 USDA statistics, U.S. broiler and turkey meat production totaled over 43 billion pounds while egg operations produced over 91.9 billion eggs annually, valuing the whole U.S. poultry industry at over $35.6 billion a year (USDA, 2012). Majority of the revenue stems from broiler operations concentrated in the mid to southeastern corner of the U.S. including the Delmarva region with over 1,500 registered broiler producers. In 2011, Maryland ranked eighth in the nation for state broiler production, valued at over $690 million with 5 counties leading in U.S. broiler production. According to 2010 Delmarva Poultry Industry data, Somerset ranked 25th, Worcester 26th, Wicomico 36th, Caroline 40th, and Dorchester 63rd among all counties in the nation (DPI, 2010). Therefore, prevention measures and surveillance are necessary to protect the health of U.S. poultry flocks, minimize economic effects of the disease, and greatly reduce the health risks to the U.S. public.

1.3.3 Delmarva

Delmarva and the Chesapeake Bay area are zones of interest when it comes to bird surveillance due to its high prevalence of migratory birds and poultry operations. The Delmarva Peninsula is known to coincide with the last major convergence point of the Atlantic Migratory Flyway serving waterfowl from the far reaches of the Arctic Ocean, Northwest Territory of Canada, and Greenland (Friend et al., 1999) (Figure 5). Other non-waterfowl species may share the same migratory pathways to and from their wintering grounds, increasing the potential for disease transmission and dissemination among the 1,500 plus domestic poultry operations in the Delmarva vicinity.

Figure 5 Atlantic Migratory Flyway followed by North American Waterfowl. (Friend et al., 1999)



The potential for future outbreaks can be seen by examining Delmarva and bordering states' avian disease history within the last 30 years. From 1998 - 2000, Delmarva poultry experienced an outbreak of infectious laryngotracheitis resulting in $4 million in losses due to response and recovery efforts as well as lost production time (Ritter, 2000). The 1999 outbreak of *Mycoplasma gallisepticum* in North Carolina also affected Delmarva broiler producers and a layer facility with one million birds as it was discovered that MG infected eggs from a North Carolina broiler breeder flock were shipped, hatched and grown in Delmarva (Dohms, 2000). Even more alarming are the previous reports of avian influenza in and around Delmarva. During 1983, commercial poultry operations in Pennsylvania, Virginia, Maryland and New Jersey experienced an outbreak of HPAI H5N2. As a result, 17 million birds were euthanized and damages totaled $65 million (Panigrahy et al., 2002). Had the Delmarva industry as a whole been affected, the predicted economic impact would have cost over $360 million accounting for the number of birds destroyed and equivalent production time lost (Musser and Mallinson, 1996). In 2004, Maryland, Delaware, and Pennsylvania were found to have flocks positive for LPAI. Delaware and Maryland poultry cases were identified as H7N2, while the Pennsylvania layer flock subtype was H2N2. This event led to the culling of 400,000 chickens (CEI VS, 2004). In 1998, a survey of free flying resident ducks on the Eastern Shore of Maryland revealed that almost 14% of the sampled population was positive for AI, representing nine different HA-NA combinations (Slemons, 2003). Another study reported that shorebirds migrating through the Delaware Bay had the highest prevalence of AI virus compared with similar populations along the Atlantic

flyway (Hanson, 2003). This further establishes the fact that the Delmarva region should maintain vigilant observation of surrounding non-commercial poultry.

1.3.4 History of Backyard Poultry Outbreaks

Epidemiological investigations have found several devastating poultry disease outbreaks originating from backyard flocks. In 1983, the outbreak of highly pathogenic H5N2 avian influenza (AI) in commercial poultry industries of Pennsylvania, Virginia, and New Jersey was attributed to traffic of contaminated workers, vehicles, and equipment associated with live bird markets in New York, which usually have an affiliation with backyard flocks (Beard, 2000). Exotic Newcastle Disease has been diagnosed in U.S. backyard game fowl on three occasions in 1975, 1998, and again in 2002-2003. The 2002 outbreak emerged in southern California where more than 149,000 birds in 2,671 premises were culled to stamp out the disease. Despite the efforts, the disease spread to 21 commercial egg laying flocks containing over 3 million birds. The virus continued to spread to Nevada and Arizona and was suspected to be the result of the mobility of backyard flocks and the lack of reporting for fear of quarantine or destruction (Kinde et al., 2003). Johnson et al. also determined that in the 1998 Delmarva outbreak of infectious laryngotracheitis, case flocks were 36 times more likely to be stationed within a one mile radius of backyard flocks compared to controls, implicating backyard poultry as a potential source of transmission. Other studies have found evidence that asymptomatic backyard flocks may be sources of *Mycoplasma gallisepticum* infection in commercial flocks (McBride et al., 1991).

1.3.5 Biosecurity

Biosecurity consists of the cumulative measures used to prevent the introduction of disease-causing organisms into a flock and to prevent the transmission of diseases within an infected area to nearby locations. Biosecurity can reduce the risk of introducing disease and serves as an important determinant for the health of a flock (Shane, 1993; Nespeca et al., 1997). Transmission of avian influenza (AI) viruses through trade of infected poultry and contaminated equipment is considered one of the main means of spread. Exposure to migratory birds has also been implicated as vectors for transmitting highly pathogenic H5N1 AI viruses (Sims et al., 2005). The USDA recommends the following practices to prevent disease in their *Biosecurity for the Birds* program: keep your distance, keep it clean, don't haul disease home, don't borrow disease from your neighbor, know the warning signs of infectious bird diseases, and report sick birds.

Chapter 2: Materials and Methods

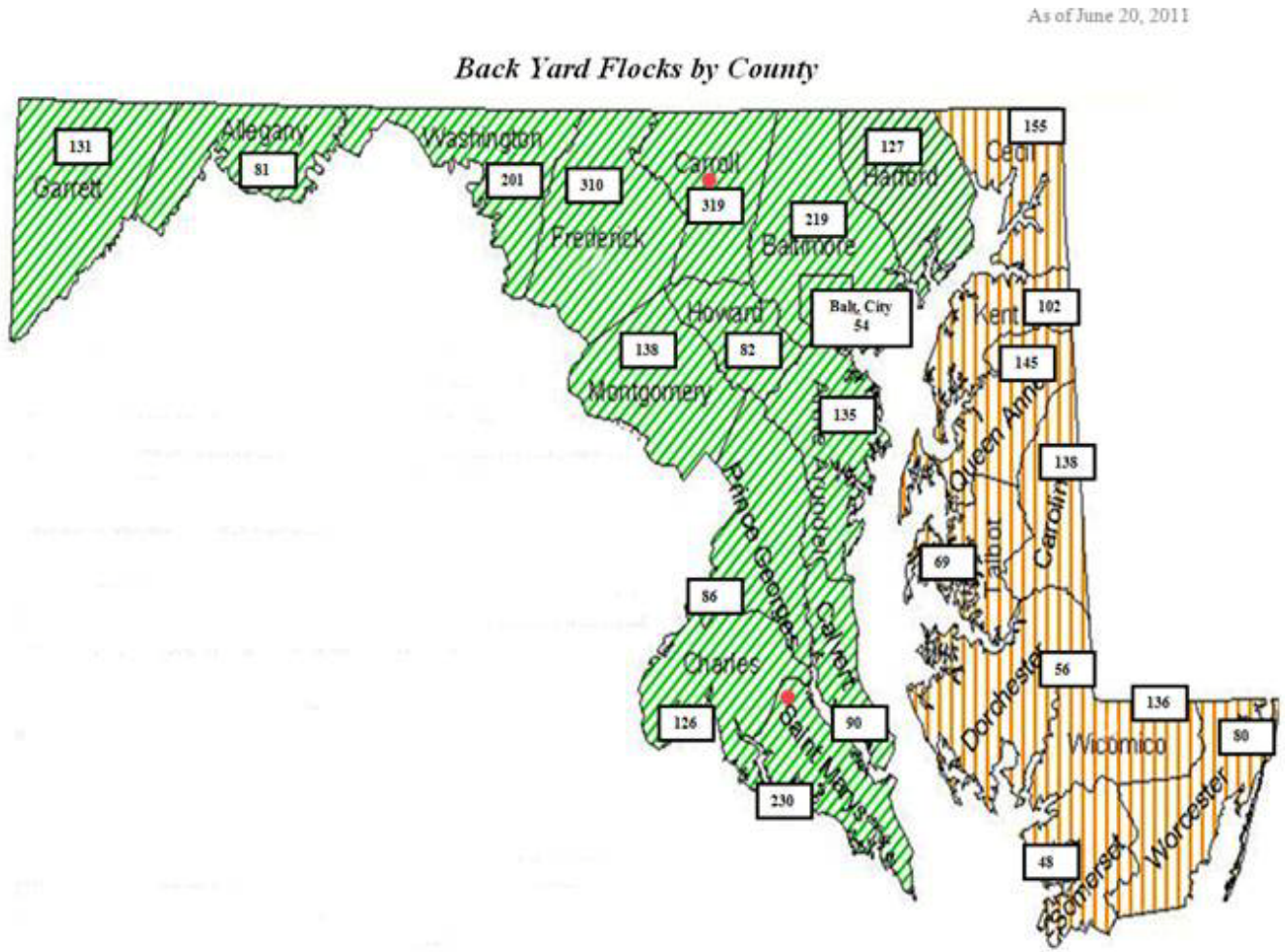
2.1 Study Design and Population

This study used a cross-sectional survey design and convenience sampling method to determine disease prevalence and associated biosecurity risk factors among Maryland non-commercial poultry operations. Surveillance included active observational, active serologic, and active antigen methods. In an effort to control the transmission of poultry diseases, some states in the U.S. require poultry owners to register their flocks with their state's department of agriculture. In 2005, the Maryland General Assembly passed this legislation into law. As of June, 2011, there have been 3,258 backyard flocks registered with the Maryland Department of Agriculture (MDA) (Figure 6). In May 2011, the MDA confidentially mailed 1,000 informational letters and return postcards to poultry owners enrolled in the Maryland Poultry Registration Program. Backyard flocks were defined using the NAHEMS "Poultry '04" guidelines as residences with fewer than 1,000 birds (other than pet birds or in addition to pet birds).

Study sites were designated by counties within three regions of Maryland: Northern (Frederick & Carroll), Southern (St. Mary's & Charles), and Eastern Shore (Caroline, Dorchester, Talbot, Wicomico, & Worcester). County selection was based on their high proportion of registered backyard flock owners as well as their relative location to commercial industries and auction markets. Approximately 50% of registered owners were contacted in Northern and Southern counties, while 100% of selected Eastern Shore counties were contacted. If poultry owners wished to participate, they were asked to contact the University of Maryland via email or prepaid return postcards. Participants

were eligible for the study if they lived within Maryland, owned domesticated fowl, and had a flock size fewer than 1,000. Respondents were offered a free diagnostic health status report of their flock as an incentive to participate in the study. Upon agreement to participate, owners were sent an information sheet and consent form providing further details of the study including the risks and benefits of participation (Appendix A). It was made clear that disease positive/seropositive birds were to be reported by law to the MDA who would conduct an internal follow up investigation and respond appropriately. Written informed consent was obtained from all participants prior to sample collection. This study was approved in accordance with the University of Maryland's Institutional Review Board (IRB #11-0335), Federal Policy for the Protection of Human Subjects (45 CFR 46), and Institutional Animal Care and Use Committee (IACUC # R-11-27).

Figure 6 Maryland registered backyard flocks by county, 2011. Live bird markets indicated by red dots (Source: MDA).



2.2 Biosecurity Questionnaire

A four page questionnaire, adapted from USDA Poultry 2010 and previous publication by Tablante et al. was created in order to determine biosecurity factors that may be associated with the health status of small flocks (USDA, 2010; Tablante et al., 2002). Prior to implementation, the survey was reviewed by the thesis committee and the MDA for feedback and final modifications. Each backyard flock owner, following initial correspondence, was sent a copy of the questionnaire as well as a link to an online version of the survey. Participants were asked to self-report information on the number and species of poultry reared, presence of other animals, animal husbandry, opportunities for interaction between wild birds and poultry, farm biosecurity measures, and health status of poultry. A copy of the survey can be found in Appendix B.

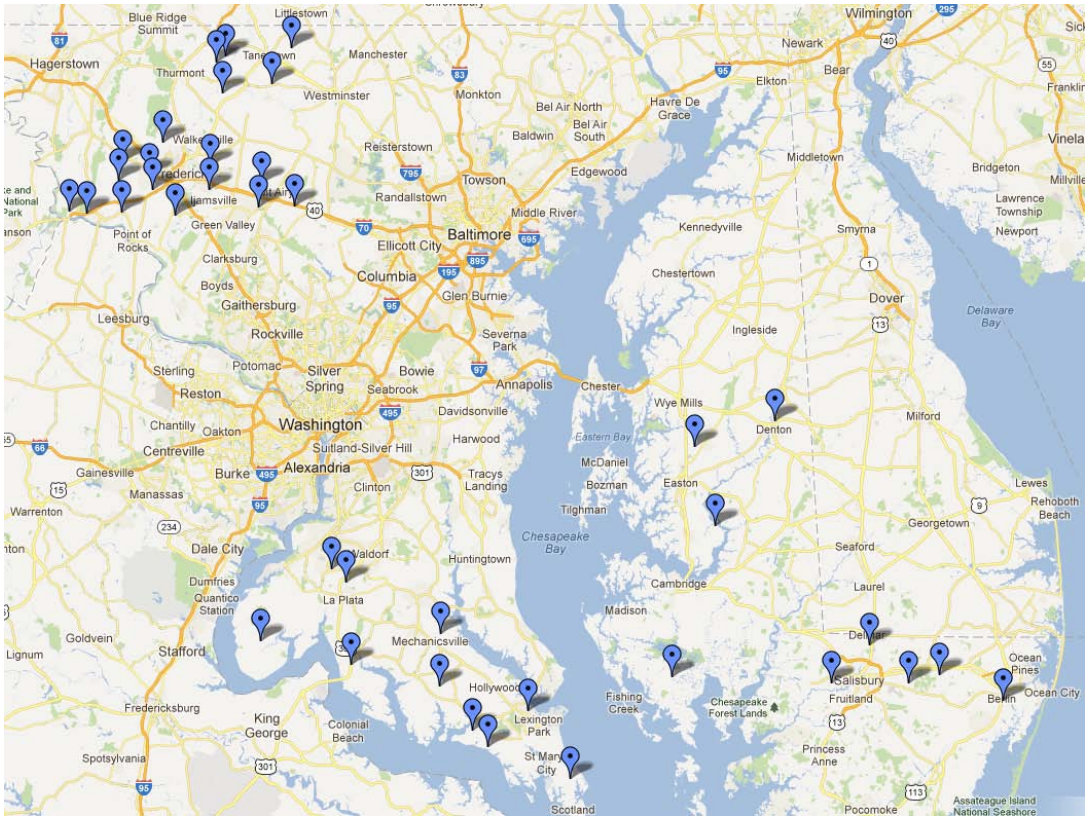
2.3 Sample Sites

Sites were sampled once from June 15th – August 25th, 2011 corresponding with USDA surveillance recommendations for highest seasonal AI prevalence in migrating shorebirds (USDA DOI, 2006). A sample size of 246 birds was sufficient to obtain a confidence interval of 95% with a desired precision of $^{+}/_{-}$5% when the estimated prevalence of diseases was 20%. A total of 262 birds were sampled from 39 backyard premises (Table 3) (Figure 7). Sampled poultry were various ages and species including 227 chickens (*Gallus domesticus*), 16 turkeys (*Meleagris gallopavo*), 15 ducks (*Anas platyrhynochos, Cairina moschata*), two guinea fowl (*Numida meleagris*), and two pheasants (*Phasianus colchicus*).

Table 3 Distribution of birds and sampling premises by Maryland counties.

County	*Birds*	*Premises*
Northern MD	**151**	**20**
Frederick	135	17
Carroll	16	3
Southern MD	**61**	**10**
St. Mary's	33	6
Charles	28	4
Eastern Shore	**50**	**9**
Caroline	6	1
Dorchester	4	1
Talbot	12	2
Wicomico	25	4
Worcester	3	1
Total	**262**	**39**

Figure 7 Map of 39 backyard flock sample sites throughout Maryland.



2.4 Sample Collection

Proper biosecurity measures were taken during sample collection to ensure disease transmission was minimized. Personal protective equipment (PPE) comprised of gloves, boot covers, and coveralls were donned and doffed before and after sample collection from each backyard flock premises. Equipment was cleaned with RelyOn® disinfectant or Clorox® wipes after sampling and used materials/PPE were bagged and left with the flock owners for proper disposal. During sampling procedures, birds were gently restrained and samples obtained appropriately and quickly. Specimen collection was performed once on each bird. Swab samples collected from birds included two tracheal and one cloacal swab to determine current infection of screened diseases. Gallinaceous birds (chickens, turkeys, quail) had both tracheal and cloacal swabs taken, while Anseriformes (ducks) only had cloacal specimens collected as the upper respiratory pathogens (ILTV and MG are not commonly isolated in these species). Blood was also drawn from each bird and serum processed for the presence of antibodies to establish previous exposure. Environmental swabs were collected for the prevalence of *Salmonella* Enteritidis.

Polyester swabs with plastic handles were used for the collection of swab specimens as swabs with wooden handles may be treated with substances (formalin) that can inactivate live virus and inhibit the isolation of viral nucleic acid (Johnson, 1990). Different sized swabs (adult and pediatric) were used for large and small bird species respectively, to avoid injuries. Tracheal swabbing was performed by gently securing the bird and opening the beak. Upon viewing the epiglottis and trachea, the swab was inserted once the cartilage protecting the trachea opened to allow the passage of air. The

trachea was swabbed along the back and sides. Cloacal swabs were pre-wetted in transport media and collected by gently securing the bird and inserting the entire tip of a suitably sized swab into the cloaca. The walls of the cloaca were swabbed in a circular motion until the swab was visibly coated with fecal material.

Tracheal and cloacal swabs were placed separately into vials containing 2.5ml of protein based brain-heart infusion (BHI) transport media. The presence of protein in the transport media helps prevent the degradation of live virus during handling and transport. Antibiotics were not added as samples were processed for bacteria as well. Samples from birds of the same species raised on the same farm were pooled together. Pooled samples consisted of a maximum of either two tracheal swabs or two cloacal swabs. The second tracheal swab was placed in Frey's medium for *Mycoplasma* culturing. All tubes were labeled with date, species, sample type, and location. Once samples were collected, they were stored in a cooler and chilled to 4°C for transport back to Avrum Gudelsky Veterinary Center, University of Maryland College Park (UMCP) for diagnostic analysis.

Blood was collected by sterile technique from the brachial (wing) vein of each bird using a 1-3 ml sterile disposable syringe with 23 gauge and 1 in. needle length. Care was taken to minimize vein damage and blood loss. Approximately 1-3 ml of blood were immediately transferred to a serum separator vacutainer and allowed to clot at environmental temperatures. Samples were then placed in a cooler with ice packs for transport to UMCP.

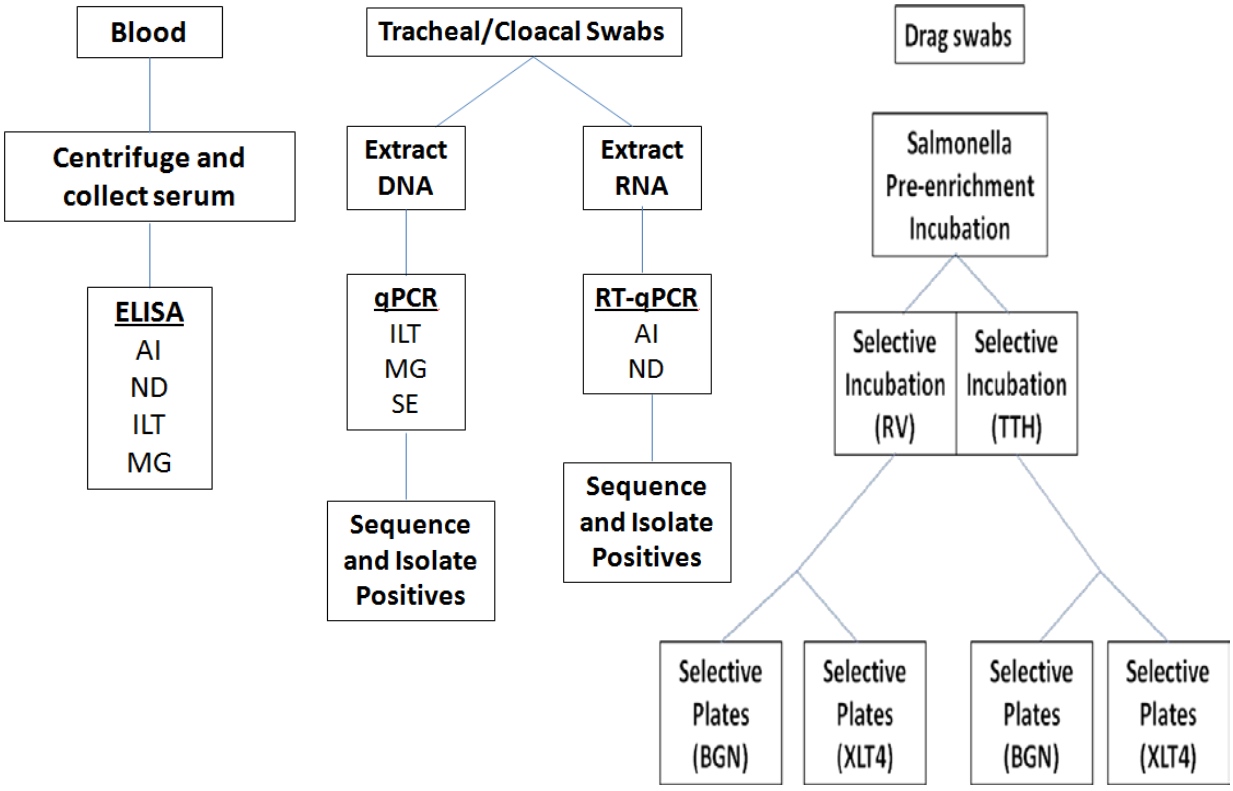
Environmental sampling for *Salmonella* subsp. Enteritidis was performed using sterile drag swabs stored in double-strength skim milk. Sterile gauze pads (3x3) were fastened to twine, autoclaved, and transferred to a sterile Whirl-Pak® bag (Nasco, Fort

Atkinson, WI) with 15 ml of double strength skim milk. Poultry housing and pen areas were sampled and pads returned to the storage bag. Each bag was sealed and promptly stored at 4°C prior to culturing.

2.5 Sample Processing.

Upon returning to UMCP, samples for bacterial culturing were placed on a shaker at 37°C. Swab samples were immediately processed or kept at 4°C for no longer than 48 hours before nucleic acid extraction. Figure 8 illustrates the processing procedure for each sample type.

Figure 8 Flowchart of sampling processing.



2.5.1 ELISA

Serum was separated from the clot by centrifugation at 1,000 to 1,300 x g for 10 minutes in a swinging bucket centrifuge. Serum was then pipetted into a sterile 1.5ml tube, labeled with an identification number, and stored at -20°C prior to ELISA testing. The Synbiotics Corporation generously provided the following USDA-licensed screening kits: ProFLOK® AIV *Plus*, *Flu* DETECT® BE, ProFLOK® *Plus* Newcastle Disease Virus, ProFLOK® Laryngotracheitis (LT) and ProFLOK® Mg. Turkey and duck conjugates were also provided. For the ProFLOK® ELISA kits, chicken serum and reagents were brought to room temperature prior to setting the dilution plate. Dilution plates were prepared by adding 300 ul of dilution buffer to each well of the 96 well plates, except for the positive and negative control wells. Serum samples were vortexed and 6 ul added to each well to create a 1:50 dilution. Positive and negative control serum were also prepared and added to plate. Then 50ul of dilution buffer and 50ul of serum were added to the corresponding wells and incubated for 30 minutes at room temperature. Plates were then washed three times with 1x wash solution for three minutes. After washing, diluted conjugate (1:100) was added to each well and incubated for 30 minutes at room temperature. Test plates were then washed as previously described and then filled with 100ul of substrate solution for 15 minutes. After 15 minutes, 100ul of 1x stop solution was added to each well. Serum from turkey and duck were used following the same procedure with the respective species conjugate. Plates were read using the ELX800 microplate reader (BIO-TEK instruments, INC., Winooski, VT) and ProFILE3 software (Synbiotics Corp., Kansas City, MO)

The *Flu* DETECT® BE kit is a sensitive and specific blocking ELISA designed to aid in the detection of Type A antibody to AIV in serum from multiple species. Serum samples and reagents were brought to room temperature prior to conducting the assay. 75ul of BE Dilution Buffer was added to each sample test well, followed by 25ul of serum sample for a dilution of (1:4). Next, 100ul of BE Positive Control and 100ul of Negative Control were added to designated wells. Plates were mixed by gently tapping the side and incubated for 60 minutes at room temperature. After incubation, the fluid was discarded and 100ul of diluted BE HRP Conjugate was added to all wells, mixed and incubated for 30 minutes. Then fluid was discarded and wells filled with 350ul of diluted Wash Solution. Plates were washed four more times. After all residual liquid was removed, 100ul of ABTS Substrate was added to each well and incubated at room temperature. After 15 minutes, 100ul of diluted Stop Solution was added to each well. Plates were read using the ELX800 microplate reader (BIO-TEK instruments, INC. Winooski, VT) and ProFILE3 software (Synbiotics Corp, Kansas City, MO).

2.5.2 Hemagglutination Inhibition

Hemagglutination Inhibition (HI) assays were conducted on AI positive serum to determine previous exposure to influenza subtypes H5, H7, and H9 as they are known to infect both humans and birds. The World Health Organization (WHO) also believes that they have the greatest pandemic potential (Sorrell, 2009). Prior to conducting a HI assay, the following viruses had to be propagated (H5: A/Mallard/PA/10218/84/H5N2) (H7: H7N3 WT P1 in MDCK supernatant) (H9: A/Quail/Arkansas/20209-1/93/H9N2). Section 2.5.7 contains the virus isolation procedures. After virus propagation, serum

samples were pretreated with Receptor Destroying Enzyme (RDE) in a 1:3 dilution and incubated overnight in a 37°C water bath. After 16 hours, RDE activity was stopped by heating the samples to 53°C for 45 minutes in the water bath. Tubes were then brought to room temperature and made to a 1:10 dilution by adding 600ul of saline solution. Next, 96 well plates were labeled for serum samples across A1-A12 and 50ul of corresponding serum was added. A plate for each hemagglutinin subtype was prepared. Wells B1 -H12 were filled with 25ul of Phosphate Buffered Saline (PBS). Performing a serial 2x dilution, 25ul of serum from A1-A12 was mixed into the wells B through H, discarding 25ul from the last mixed well. Then 25ul of virus dilution containing 4 HAU/25ul was added to each well. Plates were mixed by gently shaking and incubated for 15 minutes at room temperature, allowing antibodies (if present) to neutralize the virus. Finally, 50ul of 0.5% chicken red blood cells (RBC) were added to each well and left for 30-45 minutes. Each plate contained positive and negative antibody wells and a RBC control. Wells were read HI negative if a diffuse sheet of agglutinated RBCs covered the bottom, indicating no antibody was present to prevent hemagglutination. HI positive wells were considered positive if they produced a dot, indicating complete hemagglutination and therefore the presence of H5, H7, or H9 antibodies, respectively.

2.5.3 DNA/RNA Purification

Tracheal and cloacal swab specimens were purified for DNA and RNA material. The swabs were removed from the BHI transport media by squeezing out the remaining sample fluid using forceps that were disinfected with 70% ethanol after each vial. Sample fluid was vortexed for 3-5 seconds and centrifuged for 5 minutes at 5,000 x g to

pellet large debris and potential PCR inhibitors. Supernatant was then withdrawn and placed into a clean tube. Samples not used in the DNA/RNA purification procedure were placed in cryovials and stored at -80°C. Freeze thaw cycles were avoided as much as possible to prevent degradation of viral or bacterial samples and their genomic material.

DNA samples were extracted using the Qiagen (Valencia, CA) QIAamp DNA Mini Kit following the protocols for DNA Purification from Blood or Body Fluids (Spin Protocol). In a 1.5ml microcentrifuge tube, 20ul of proteinase K was added followed by 200ul of sample. Two hundred ul of Buffer AL was added to the sample, mixed, and incubated at 56°C for 10 minutes. Then 200ul of 100% ethanol was added to the sample, mixed, and briefly centrifuged. The mixture was transferred to a QIAamp Mini spin column and spun at 6,000 x g for one minute. The spin column was placed in a clean 2ml collection tube and 500ul of Buffer AW1 was added. The column was centrifuged for one minute at 6,000 x g and filtrate discarded. Then 500ul of Buffer AW2 was added and centrifuged at full speed (20,000 x g) for three minutes. The spin column was placed in a clean 1.5ml tube and 200ul of Buffer AE was added. The column was incubated at room temperature for one minute and then spun at 6,000 x g for one minute. The column was discarded and the tubes were labeled and kept at -20 °C awaiting qPCR analysis.

The organic method was chosen for RNA extraction as it leads to high yields of RNA and is the preferred method for cloacal swab samples. Two hundred and fifty ul of field sample supernatant was added to a 1.5ml tube. Then, 750ul of TRIzol® (Invitrogen, Carlsbad, CA) was added and vortexed to deactivate RNases, while maintaining the stability of RNA. After mixing, 200ul of chloroform was added and the tubes were mixed for 15 seconds and left to incubate at room temperature for seven minutes.

Following incubation, the tubes were centrifuged for 15 minutes at 12,000 x g. The upper aqueous phase containing RNA was transferred to a new tube while avoiding the interphase and organic phases of the mixture as they contain DNA and proteins that can decrease RNA yield and inhibit RT-qPCR reactions. Then 500ul of isopropanol was added along with 1 ul of 5mg/ml stock glycogen to aid RNA precipitation. Tubes were inverted several times and incubated at room temperature for 10 minutes. Samples were spun at 12,000 x g for 10 minutes and fluid decanted. One ml of 75% ethanol was added and gently mixed. Mixtures were centrifuged at 10,000 x g for five minutes and ethanol decanted. Tubes were inverted on paper towels to dry for 10-15 minutes. After all ethanol had evaporated, 40ul of RNase free water was added. RNA samples were stored at -80°C awaiting qRT-PCR analysis.

2.5.4 qPCR and RT-qPCR

Quantitative PCR (qPCR) and Reverse Transcription Quantitative PCR (RT-qPCR) were conducted on the Bio-Rad (Hercules, CA) CFX96 Real-Time thermal cycler and analyzed with CFX Manager Software. Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, California) was used in qPCR reactions to detect the presence of DNA from ILTV, MG, and SE in samples. QuantiTect SYBR® green RT-PCR kit (Qiagen, Valencia, CA) was used for one step RT-qPCR to ascertain positive complimentary DNA (cDNA) from AIV and NDV. An internal control was included in each method to ensure extraction quality and confirm the fidelity of negative samples. All primers were taken from previous publications or designed using SeqMan and EditSeq (DNASTAR; Lasergene, Inc. Madison, WI) and optimized by performing

standard curve and thermal gradient analysis. Each plate contained samples in duplicate as well as positive and negative controls. All plate runs were followed with a melt curve analysis to confirm the absence of nonspecific amplification such as primer-dimer formation. PCR positives were replicated twice.

Tracheal DNA swab samples were used for ILTV and MG qPCR analysis, while cloacal DNA swab samples were tested for SE. In preparation for amplification, a master mix containing 10ul of 1x Power SYBR Green PCR Master Mix, 0.5ul each of respective forward and reverse primers of 10uM concentration (IDT) (Table 4) were added to appropriate wells of a 96 well plate. Nine ul of sample DNA was then added for a total 20ul PCR reaction. Plates were sealed, gently mixed by rubbing the well bottoms, and spun at 1,000 rpm for 1 minute. Once in the thermal cycler, samples were incubated at 95°C for 10 minutes to activate the AmpliTaq Gold polymerase, followed by 40 cycles 95°C at 15 seconds for denaturation and 60°C at one minute for annealing and extension. Samples were held at 4°C.

For gallinaceous poultry (chickens, turkey, quail, pheasant) tracheal RNA swab samples were used for AIV and NDV RT-qPCR analysis as these viruses primarily replicate in the respiratory tract. For waterfowl, cloacal RNA swab samples were used as AI virus primarily replicates in the intestinal tract and NDV has been isolated most consistently from cloacal swabs (Spackman and Suarez, 2008;Alexander, 2008). Samples and reagents were kept on ice while preparing the reaction master mix. A master mix was prepared in a sterile 1.5ml tube containing 10ul of 1x QuantiTect SYBR Green RT-PCR Master Mix, 0.5ul each of forward and reverse primers of 10uM concentration (IDT) (Table 5), 0.2ul of QuantiTect RT mix, 2.3ul of nuclease free water, and 0.5ul of

RNase inhibitor (13Units/ul) (RNasin, Promega). After adding the master mix to the 96 well plates, 6ul of sample RNA was added for a total reaction volume of 20ul. Plates were sealed, gently mixed by rubbing the well bottoms, and spun at 1,000 rpm for one minute. Once in the thermal cycler, samples were incubated at 50°C for 30 minutes for reverse transcription, 95°C for 15 minutes to activate the HotStarTaq DNA polymerase followed by 40 cycles 94°C at 15 seconds for denaturation, 60°C at 30 seconds for annealing, and 72°C at 30 seconds for extension. Samples were held at 4°C.

Table 4 DNA qPCR primers for ILTV, MG, SE, and internal control

Target	Gene of Interest	Amplicon Size	Nucleotide Position	Sequence (5' - 3')
ILTV	Glycoprotein C (gC) *(Callison et al., 2006)*	103 bp	60	CCTTGCGTTTGAATTTTTCTGT
			163	TTCGTGGGTTAGAGGTCTGT
MG	16S rRNA *(Lauerman et al., 1998)*	183 bp	1,242	GAGCTAATCTGTAAAGTTGGTC
			1,425	GCTTCCTTGCGGTTAGCAAC
SE	invA *(Rahn et al., 1992)*	284 bp	1,488	TCATCGCACCGTCAAAGGAACC
			1,772	GTGAAATTATCGCCACGTTCGGGCAA
Internal control	chicken alpha2 (VI) collagen *(Islam et al., 2004)*	75bp	2,360	GGGAACTGGAGAACCCAATTTT
			2,435	CGTGCCGCTGTCTCTACCAT

Table 5 RNA RT- qPCR primers for AIV, NDV, and internal control

Target	Gene of Interest	Amplicon Size	Nucleotide Position	Sequence (5' - 3')
AIV	Matrix (M) *(Spackman and Suarez 2008)*	99 bp	25	AGATGAGTCTTCTAACCGAGGTCG
			124	TGCAAAAACATCTTCAAGTCTCTG
NDV	Fusion (F)	158 bp	240	CTTGGATGCATACAACAG
			398	GCGGCCGCTGTTATTTGTG
Internal control	Chicken GAPDH *(Lee et al., 2010)*	98 bp	223	GGCACTGTCAAGGCTGAGAA
			321	TGCATCTGCCCATTTGATGT

2.5.5 Sequencing and Phylogenetic Analysis

Once quantitative PCR positives were confirmed, gene fragments were sequenced, aligned, and strain differentiated. In order to differentiate between pathogen strains, a gene of lower conservation was selected. Gene fragments were amplified using Platinum PCR SuperMix High Fidelity (Invitrogen, Grand Island, NY) on the Eppendorf Mastercycler (Hamberg, Germany). In a 0.5ml tube, 38ul of SuperMix, 1ul of each forward and reverse primer of 10uM concentration (IDT) (Table 6), and 10ul of template DNA was added for a 50ul reaction. Thermal cycler settings were 94°C at two minutes for enzyme activation, followed by 40 cycles of 94°C at 30 seconds for denaturation, 55°C at 30 seconds for annealing, and 68°C at one minute per kilo-basepairs (kb) for extension. Samples were held at 4°C until processed by gel electrophoresis on a 1% agarose gel at 100 volts for 40 minutes. Appropriately sized bands of DNA were cut from the gel and purified using the QIAquick Gel Extraction Kit and protocol (Qiagen, Valencia, CA).

DNA then underwent modification sequence amplification using the previously described thermal cycler. In a 0.5ml tube, 8ul of PCR product DNA, 2ul of BigDye, 4ul of 5x Buffer, 1ul of forward or reverse primer, and 5ul of distilled water was added. Thermal cycler settings were 96°C at four minutes, followed by 25 cycles of 96°C for 10 seconds, 50°C for five seconds, and 60°C for four minutes. Samples then underwent ethanol precipitation. Two ul of 125mM EDTA and 60ul of absolute ethanol were added to the sample tubes and vortexed. Samples were then centrifuged for 15 minutes at 13,700 rpm and supernatant decanted. Then 60ul of 70% ethanol was added, mixed, and centrifuged for 10 minutes at 13,700 rpm. Sample supernatant was decanted and left to

completely dry. Once moisture had evaporated, 10ul of Hidi Formamide (Applied Biosystems, Carlsbad, California) was added, and samples were loaded onto a 96 well sequencer plate. Amplification product sequencing was done using the 3130 XL Genetic Analyzer (Applied Biosystems, Carlsbad, California). Each amplification product was sequenced in both directions with overlapping forward and reverse primers (Table 6).

Table 6 Sequencing primers for ILTV and MG PCR positives.

Target	Gene of Interest	Amplicon Size	Nucleotide Position	Sequence (5' - 3')
ILTV	ICP4	688 bp *(Chacon et al., 2009)*	181	ACTGATAGCTTTTCGTACAGCACG
			869	CATCGGGACATTCTCCAGGTAGCA
		595 bp	640	CAGAGGACCAGCAAAGAC
			1325	CTAACTGTTCCACTGGCATC
MG	pvpA	702 bp *(Ferguson et al., 2005)*	545	GCCAMTCCAACTCAACAAGCTGA
			1247	GGACGTSGTCCTGGCTGGTTAGC

Assembly of sequence "contigs" and initial multiple-sequence alignments were performed with sequencing project management (SeqMan) and multiple-sequence alignment (MegAlign) programs, respectively (DNASTAR; Lasergene, Inc. Madison, WI). Sequences were subjected to a Basic Local Alignment Search Tool (BLAST) database to find similar sequence matches using the Megablast nucleotide collection. Homologous sequences, including vaccine strains, were compared in a multiple alignment by ClustalW and single nucleotide polymorphisms (SNPs) were used to differentiate positive field samples. Alignment of protein sequences were compared for amino acid alterations which could potentially lead to changes in pathogen protein function. Phylogenetic analysis was based on the neighbor-joining method using MEGA4 software (Tamura et al., 2007). Bootstrap values in the phylogenetic tree were calculated using 1000 replicates. The following accession numbers were used to design

the tree: USDA reference strain (JN542534.1), LT Blen® (JQ083493.2), Laryngo Vac. (JQ083494.2), 25/H/88/BCK (EU104899.1), Brazil/2008/USP/07 (GQ499345.1), Brazil/2008/USP/62 (FJ477377.1), Brazil/2003/USP/15 (GQ499348.1), Brazil/2008/USP/80 (FJ794468.1), Brazil/2008/USP/74 (FJ794467.1), Jiang-2011-3 (JN969100.1), 501/C/06/BR (EU104920.1), 7/B/99/BR (EU104919.1), 205/J/06/BR (EU104916.1), 63140/C/08/BR (JN542536.1), 288269/2007 (HM230781.1), 305/K/05/BR (EU104913.1), 2/A/04/BR (EU104912.1), 417/A.06/BR (EU104914.1), 20/F/04/BR (EU104915.1), Peru/2008/USP-81 (FJ794469.1), WangGang (DQ995291.1), 24/H/91/BCK (EU104910.1), 12/D/02/BCK (EU104911.1).

2.5.6 Bacterial Propagation

National Poultry Improvement Plan (NPIP) and Food and Drug Administration (FDA) guidelines were followed for MG and SE isolation respectively. *Mycoplasma sp.* isolated from tracheal swabs that had been kept in Frey's media with penicillin was incubated at 37°C for 4-5 days. Cultures were then inoculated on a Frey's agar plate using a wire loop sterilized with a Bunsen burner. Plates were incubated for 3-5 days at 37°C in a high humidity incubator with 5% carbon dioxide. Positive plates had tiny circular and translucent colonies 0.2-0.3 mm in diameter (Yoder, H 1980). Viewing with a microscope showed the classic "fried egg" appearance with a central dense mass (CFSPH, 2007).

Salmonella sp. isolation was done from environmental drag swab samples. One hundred ml of buffered peptone water was added aseptically to the Whirl-Pak® bag

containing the environmental gauze pad and shaken vigorously for 30 seconds. Bags containing the pre-enrichment media were kept in an open glass container and incubated for 24 hours at 37°C. Afterwards, 1ml of incubated pre-enrichment fluid was transferred into 10ml of tetrathionate (TTH) broth and 0.1 ml of pre-enrichment fluid was transferred to 10ml of Rappaport-Vassilliadis (RV) medium. TTH and RV broth were incubated at 42.5°C in a water bath for 24 hours. After incubation, a loopful of TTH was streaked onto brilliant green with novobiocin (BGN) and xylose lysine tergitol 4 (XLT4) agar plates. This procedure was repeated for RV broth as well. Plates were incubated for 24 hours at 35°C. Positive BGN plates had pink/white opaque colonies surrounded by red zones. XLT4 positive plates had black colonies with a yellow periphery after 18-24 hours of incubation (FDA, 2008).

2.5.7 Virus Propagation

Virus propagation was only attempted after obtaining positive PCR results as a form of secondary confirmation. Positive AI controls were also propagated. All viral propagating procedures were conducted in a biosafety level 2 (BSL-2) hood. All viruses were thawed on ice from -80°C storage. Inoculum was prepared using 100ul of sample and a 1:10 dilution of antibiotics (100x Pen-Strep -Amp). For ILT sample propagation, USDA ILTV reference strain and Trachivax® were also included as positive controls. Using nine day old eggs, chicken embryos were inoculated via chorioallantoic membrane (CAM) and top route methods after candling to ensure embryo viability and location. CAM route procedure was conducted by disinfecting the egg and punching a hole on the center of the lateral side away from the embryo, taking care not to tear the shell

membrane. Then a hole was punched all the way through the shell to the air cell on top of the egg. The egg was placed horizontally on a tray with the first hole facing up. A three ml syringe and 18 gauge needle were inserted into the air cell to drop the CAM by gently pulling on the plunger. After the CAM dropped, the inoculum in a one ml syringe and 25 gauge 5/8" needle was injected into the newly formed air cell. Both holes were sealed with Duco® cement (ITW Devcon, Danvers, MA). Top route methods were performed in a similar manner, however, only one hole was punched a few cm above the air cell line and the inoculum was injected into the allantoic fluid (AF). CAM route eggs were left on their side for 24 hours in a humidified incubator at 37°C. Eggs were examined daily for dead embryos or until seven days post inoculation. At this point, eggs were placed in 4°C for 3-4 hours prior to CAM extraction and visualization. Viable virus produces white opaque plaques on the CAM.

Influenza virus positive controls were propagated via the top route method as previously described. However, eggs were placed in 4°C after 48 hours. While eggs were in the refrigerator, a 96-well round bottom plate was prepared for the hemagglutinin assay (HA) by adding 50ul of PBS to each well. After 3-4 hours, eggs were disinfected with 70% ethanol and the shell over the air cell was cracked and air cell membrane was removed. The AF was aspirated and placed in a 15ml tube. Then 50 ul of AF from each sample is added to the first well. After mixing, 50ul was placed into the second well, continuing the process of a twofold serial dilution down the column of the plate and discarding 50ul from the last well. Fifty ul of 0.5% chicken RBC was added to all wells. A positive, negative, and RBC control was used on each plate. The plate was then incubated for 45 minutes at room temperature. Positive wells appear as "mat," indicating

virus prevented RBC agglutination. Negative wells appear as a "dot," indicating virus was not present.

2.6 Statistical Analysis

Data collected from the survey was entered into a Microsoft Excel® database. Exposures were defined as lack of biosecurity practices listed throughout the survey and disease outcomes were assessed by ELISA and PCR screening tests. The flock was the unit of interest for PCR and ELISA outcome analysis. A flock positive status only required one bird to test positive. After descriptive data analysis (mean, median, and range), univariate and multivariate statistical analyses were carried out. The association of the independent variables elucidated from the questionnaire, such as biosecurity practices and the dependent variables (bird or flock disease positive) were analyzed using Fisher's exact test, (right sided) for the categorical variables (Table 7). Disease status and independent variables of each flock were coded into a binary outcome (Disease=1, No disease=0) and (Exposed=1, Not exposed=0). Strengths of associations were reported as relative risks or odds ratios. Mean titers of normal distribution and equal variance were compared using two sample t-test. Other continuous variables were analyzed by simple logistic regression (Table 8). A p-value ≤ 0.25 was set as the inclusion threshold for categorical and continuous variables into multivariate analysis. Multiple logistic regression containing all continuous and categorical variables with a p-value ≤ 0.25 was executed for selection into a final stepwise backward elimination regression model. Variables with a p-value ≤ 0.05 were considered statistically significant for association with the outcome. Data were analyzed using Statistical Analysis System (SAS) software

for Windows v9.2 (SAS Institute, Cary, NC) and Statistix9 for Windows (Analytical Software, Tallahassee, FL).

Table 7 Categorical variables examined for association with AI seropositive flocks.

Biosecurity risk factor	Description
Mixed species (MIXSPCS)	Chicken vs. other species
Housing (HOUSING)	Free range vs. coop
Species Separate (SPECSEP)	Together vs. separate
Owner exp wild waterfowl (OWNWFOWL)	Exposed vs. not exposed
Owner exp wild birds (OWNWDBRD)	Exposed vs. not exposed
Owner exp neighbor birds (OWNNEBRD)	Exposed vs. not exposed
Owner exp rodents (OWNRODNT)	Exposed vs. not exposed
Owner exp wild carnivore (OWNCARN)	Exposed vs. not exposed
Owner exp livestock (OWNLVSTK)	Exposed vs. not exposed
Bird exp wild waterfowl (BRDWFOWL)	Exposed vs. not exposed
Bird exp wild birds (BRDWDBRD)	Exposed vs. not exposed
Bird exp pets (BRDPETS)	Exposed vs. not exposed
Bird exp rodents (BRDRODNT)	Exposed vs. not exposed
Bird exp wild carnivore (BRDCARN)	Exposed vs. not exposed
Bird exp livestock (BRDLVSTK)	Exposed vs. not exposed
Allow visitors (ALLVIS)	Allow visitors vs. no visitors
Isolate new birds (ISONWBRD)	No isolation vs. isolation
Disease mortality (DIESICK)	Deaths vs. no deaths
Diarrhea (DIARRHEA)	Sick vs. not sick
Respiratory disease (RESPDIS)	Sick vs. not sick
Neurologic disease (NEURODIS)	Sick vs. not sick
Weight loss (WGTLOSS)	Sick vs. not sick
Footbath/footwear (FOOTBATH)	No footbath vs. footbath
Clean and disinfect (CLEAN)	Don't clean vs. do clean
Pest control (PESTCON)	No pest control vs. pest control
Region (REGION)	North vs. other regions

Table 8 Continuous variables examined for association with AI seropositive flocks.

Biosecurity risk factor	Description
Commercial farms (COMMFARM)	Number of farms within 1/4 mile
Backyard farms (BACKFLCK)	Number of backyard flocks within 1/4 mile
Years of ownership (YEAROWN)	Number of years kept poultry
Flock size (FLCKSZE)	Number of birds in flock
Visit commercial (VISCOMM)	Number of times visit commercial farm (1yr)
Visit backyard flocks (VISBKYD)	Number of times visit backyard flock (1yr)

Chapter 3: Results

3.1 Questionnaire

Of the 1,000 mailed questionnaires, 41 (4.1%) were returned completed. From the returned surveys it was determined that just over half (51.2%) of farms had chickens as the only species of domestic poultry. The remaining farms had a mix of chickens and other domestic bird species, however, chickens accounted for 86.5% of the reported birds overall. Surveyed flock size ranged from 3 to 901 birds with the median of 38 birds per flock (Table 9). Fortyfour percent of surveyed owners have kept backyard poultry between 13 months and 5 years, while only 25.9% have had poultry over six years. Of that population, only 1.5% have kept poultry over 10 years (Figure 9). The predominant reason for keeping poultry was personal consumption of eggs (56.1%), followed by selling eggs (29.3%). Other owners kept poultry for live sale (4.9%), as pets (4.9%), home consumption (2.4%), or to sell as meat (2.4%) (Figure 10).

Housing management for poultry was recorded as 39% cooped, fenced-in and covered, 17% cooped, fenced-in but not covered, 29.3% free-range, and 14.7% free-range near a water source such as ponds or streams. Of the 20 backyard flocks with multiple species, only six owners (30%) keep them separate. Owner and poultry exposure to other animals was variable. Overall, 53.7% of poultry were reported accessible to wild birds while only 9.8% are exposed to waterfowl. Personal interaction with livestock such as pigs, sheep, cattle, etc. was reported by 41.5% of owners while 31.7% of the poultry population was exposed to livestock (Figure 11). Only five out of 41 (12.2%) farms reported being within a quarter mile of a commercial flock while 68% of owners reported

being within a quarter mile of other backyard flocks. In the average year, 10 owners visited commercial poultry sites 1 – 12 times (median = 7), with 3 coming in direct contact with birds.

In the average year, 10 owners also visited other backyard flocks 1 – 78 times (median = 7.5), with nine coming in direct contact with birds. Only one owner did not allow visitors and of those who permitted guests, 73% allowed direct contact with birds. Only 5% (2/41) of owners sold birds at a market and 7.3% (3/41) bought birds from markets. Within the last year, 83% of owners purchased new birds. Of those who purchased birds, 17.6% did not isolate them before introduction into the flock. Those who did isolate, on average, separated their new birds for 9-10 weeks with three owners keeping new birds separate indefinitely.

Table 9 Backyard poultry species distribution and median flock size among the surveyed regions of Maryland

	Northern Maryland	***Southern Maryland***	***Eastern Shore***	***Overall***	***Range*** [a]
Number of Farms	20	12	9	41	
Median Flock Size	40	32	42	38	
Species:					
Chicken	2174	328	1173	3675	3- 901
Turkey	216	8	35	259	0-210
Duck	38	107	12	157	0-65
Guinea Fowl	50	33	0	83	0-25
Pheasants	0	33	0	33	0-30
Geese	13	2	9	24	0-8
Peacocks	4	6	0	10	0-6
Chuckers	0	3	0	3	0-3
Doves	3	0	0	3	0-3
Total	2498	520	1229	4247	

[a] Number of birds per farm

Figure 9 Distribution of ownership time among backyard flock owners

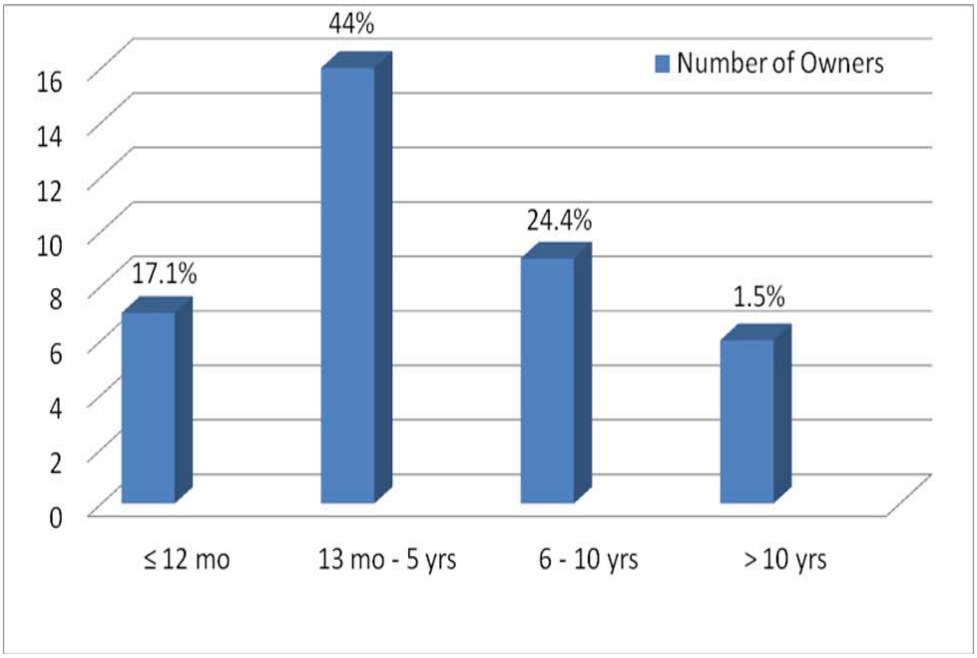


Figure 10 Primary reasons for keeping backyard poultry.

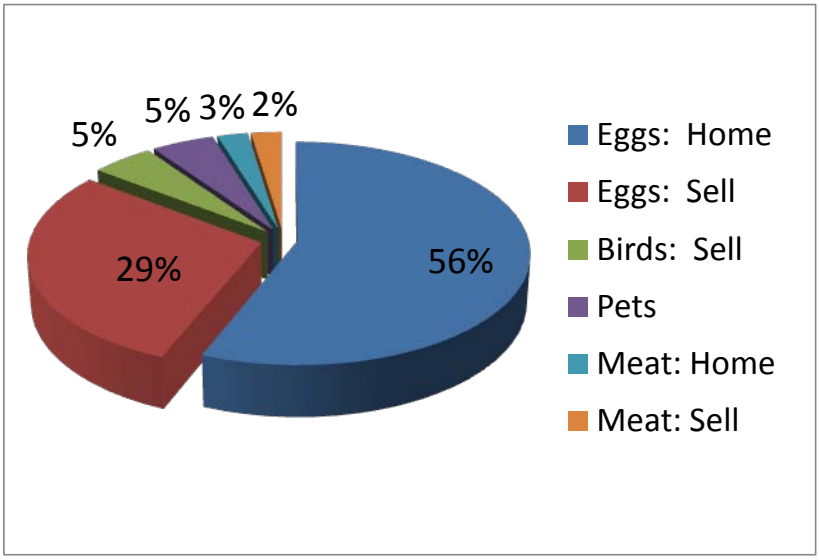
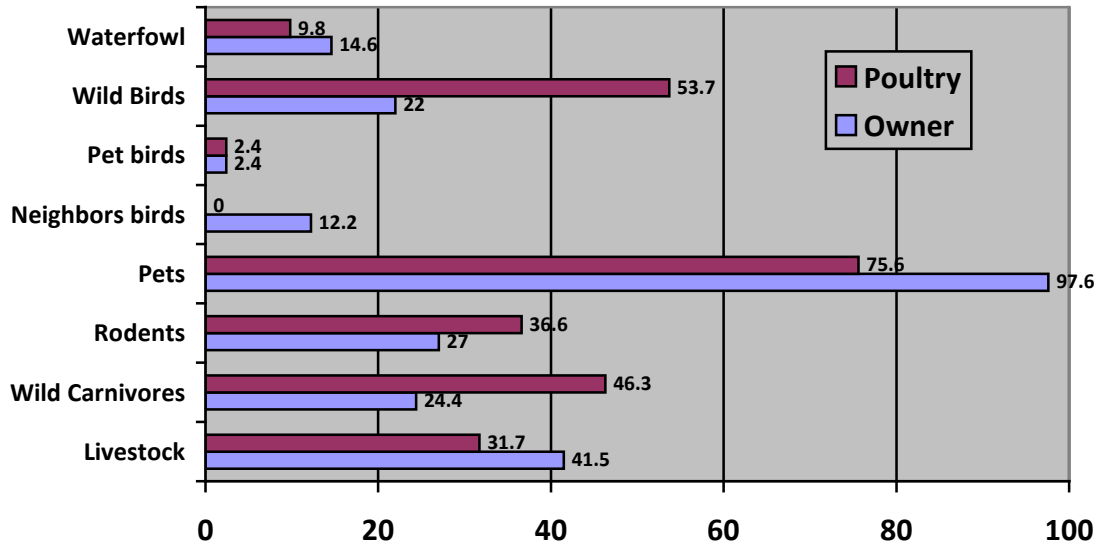


Figure 11 Percentage of owner and poultry exposure to other animals.



In regards to poultry health, only one owner out of 41 vaccinated their birds once they were on the premises. In the previous two years, owners reported predation (57.1%) as having the highest cause-specific mortality rate followed by disease (30.2%), unknown (8.7%), and injury (4%). Just over half (56%) of owners also reported observing signs of disease in their flock within the last six months. Almost one third of all owners recalled seeing birds with diarrhea (29.2%), while another 29.2% reported a decrease in egg production or soft/misshapen eggs. A few reported respiratory disease (9.7%) such as coughing, sneezing, nasal secretions or swollen sinuses and 7.3% observed weight loss or decreased appetite in their flock. Two owners reported neurological signs (4.87%) such as lack of coordination or weakness while no owners had birds with swelling of the head, comb, wattles, or hocks. Six backyard flock owners (14.6%) reported that they have previously sent an ill bird to a diagnostic laboratory.

For sanitation and decontamination, only three of the owners (7.3%) routinely use a footbath at the entrance to their poultry premises and two of the three also use dedicated footwear. Of the remaining owners, 11 wear a different pair of shoes when entering their poultry area. While the majority of owners (65.8%) always wash their hands before and after handling their birds, many (63.4%) never wear dedicated clothing (Table 10). Over two thirds of owners clean and disinfect housing and equipment (68.3%) and all but one disposes of litter by means of composting (47.4%), setting in a manure pile (31.6%), spreading on field (18.4%), or having it hauled away (2.6%). Dead birds were usually buried on site (43.6%), composted (20.5%), thrown in trash (15.4%), hauled away (10.25%), or fed to other animals (10.25%). Pest control such as mouse traps or insecticides were used in 39% of backyard flock premises.

Table 10 Frequency of biosecurity practices among flock owners

Biosecurity Factor	***Always***	***Sometimes***	***Never***
Hand washing	27/41	9/41	5/41
Dedicated clothes	3/41	12/41	26/41
Visitor wash hands	20/32	5/32	7/32
Visitor clothes	4/32	1/32	27/32

3.2 Seroprevalence of Avian Diseases

Thirty nine backyard poultry flocks were examined for previous exposure to NDV, ILTV, MG, and AIV (Table 11). All serum samples, except those from pheasants and guinea fowl (n=4) were tested with the ND, ILT, and MG Enzyme-Linked Immunosorbent Assay (ELISA). The AI blocking ELISA was capable of testing all

species. The determination of positive serum is interpreted from the serum sample to positive (SP) or negative (SN) (AI blocking ELISA) control ratio designated by the Synbiotics kit. Of the 258 serum samples tested, 11.63% of birds were positive for antibodies to ND based on the SP value threshold of 0.151 or greater. The seropositive chickens and turkeys originated from nine farms in three counties. As no owners declared vaccinating their flocks for ND, hatcheries of origin were traced back and questioned. Of the nine positive flocks, two owners obtained birds privately and no vaccination history could be obtained. The remaining flocks originated from three out of state hatcheries, which vaccinate the breeder flock and started pullets for ND, however, chicks were only vaccinated for Marek's disease. For the ILT ELISA, all duck and turkey serum tested negative and since chickens are the primary natural host, the ILT seroprevalence was calculated for chickens only. Seroprevalence in this species was found to be 16.3% (37/227). For greater specificity, the SP threshold was set to 0.276, compared to the kit standard of 0.151. Seropositive chickens were located on 19 farms in six counties. Only one backyard flock had been vaccinated for ILT. Two owners received their poultry from commercial farms and three obtained their birds privately. The vaccination status of flocks originating from hatcheries is unknown, but is primarily done only when requested.

The MG ELISA kit divides antibody status into Negative (SP $\leq$ 0.199), Probable (SP 0.2 -0.599), or Positive (SP $\geq$ 0.6). Of the 258 samples, 32 (12.4%) were considered probable, while 18 (6.98%) were positive. A total of five farms from four counties had MG positive chickens and/or turkeys. No owners vaccinated for MG and hatcheries generally vaccinate only on request. One owner obtained his birds from a commercial

farm. Based on the AI ELISA (SP $\geq$ 0.35) and AI blocking ELISA (SN< 0.6), all 262 samples were tested for antibodies to AI. Vaccination for avian influenza in the U.S. requires USDA licensure and approval from both state and federal governments prior to field deployment and therefore is rarely practiced (Swayne and Akey, 2005). Seroprevalence of AI in backyard flock birds was 4.2% (11/262). Chickens were the only species found positive from nine premises of four counties. One bird from each premises was seropositive except one Frederick and one St. Mary's flock which had two seropositive birds. HI tests did not detect H5, H7, or H9 subtype-specific antibodies among AI ELISA-positive sera.

Table 11 ELISA antibody titers for ND, ILT, AI, and MG from backyard flocks in Maryland.

	Number of birds with titer				***Total no. tested***	***Positive threshold SP (titer)***
Disease	<1000	1000-1999	2000-3999	$\geq$4000		
AI	7	2	2	0	262	SP $\geq$ 0.35 (338^{+}) Blocking SN < 0.6
ND	5	1	6	18	258	SP $\geq$ 0.151 (345^{+})
ILT	75	21	6	12	258	SP $\geq$ 0.276 ($1{,}000^{+}$)
MG	31	5	4	4	258	SP $\geq$ 0.6 (744^{+})

3.3 Prevalence of Avian Diseases

Flock status for the presence of AIV, NDV, SE, ILTV, and MG was determined from swab samples using q-PCR. Neither Influenza A nor Newcastle disease virus RNA was detected through RT-qPCR among the 262 paired tracheal/oropharyngeal or cloacal swabs collected from domestic poultry and waterfowl. *Salmonella* Enteritidis DNA was also not observed among collected cloacal swab specimens. All tracheal swabs (n=247)

of bird species, excluding ducks, were analyzed for ILTV and MG by qPCR. Based on the paired swab results, 10 premises (25.6%) were found positive for ILT among three counties with all swab positives emerging from chickens (Figure 12). One Frederick county flock with chickens and turkeys was positive for MG and was also one of the ILT positive flocks (Figure 13). This flock of young poults presented with clinical respiratory disease as seen in the captured video footage (Madsen, 2011).

Figure 12 Agarose gel electrophoresis of the ILT PCR positive flock isolates using the ICP4 primers. Some flocks were positive by qPCR but not visible on gel. Sequencing required a greater volume reaction not shown here. Lanes are as follows: (1) 1 Kb Ladder, (2) ILT positive Trachivax®, (3-12) field isolates from each flock by bird ID.

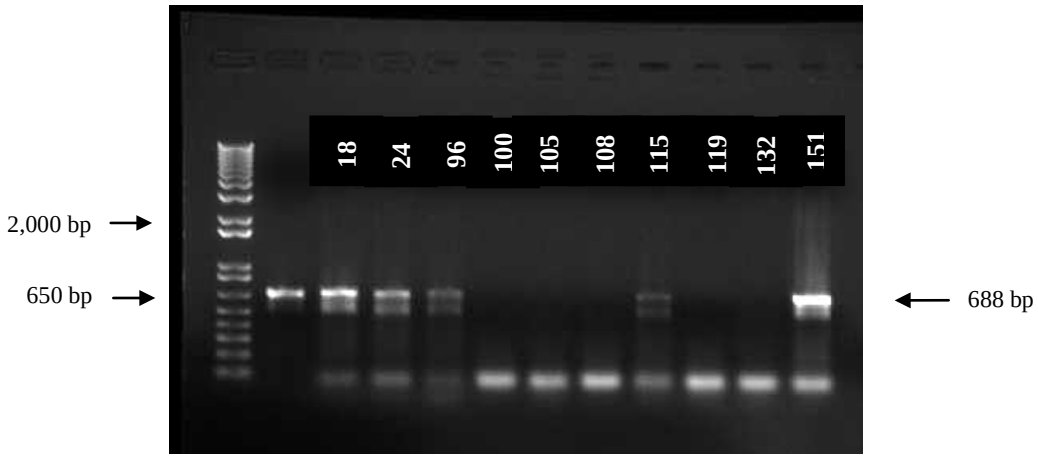


Figure 13 Agarose gel electrophoresis of the MG S6 Flock PCR isolates using the pvpA primers. Lanes are as follows: (1) 1Kb ladder, (2) MG positive strain A5969, and (3-8) field isolates.

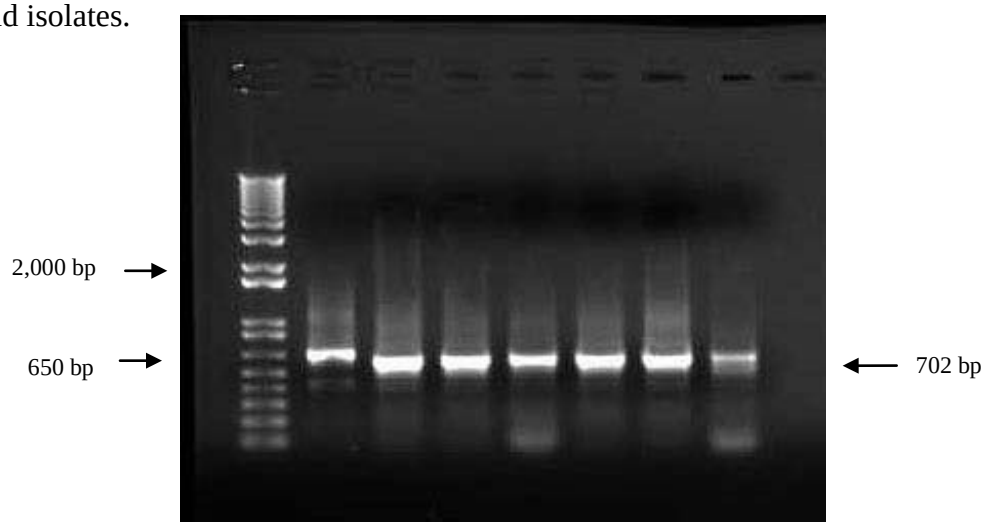


Table 12 Results of serological screening of 262 blood samples from 39 backyard flocks

Disease	*Seropositive birds / total birds*	*Seropositive flocks / total flocks*	*Positive Flocks / total flocks*
AI	11/262 (4.2%)	9/39 (23.1%)	0
ND [a]	30/258 (11.6%)	9/39 (23.1%)	0
ILT [a]	37/227 (16.3%)	19/39 (48.7%)	10/39 (25.6%)
MG [a]	18/258 (6.98%)	5/39 (12.8%)	1/39 (2.6%)
SE	N/D	N/D	0

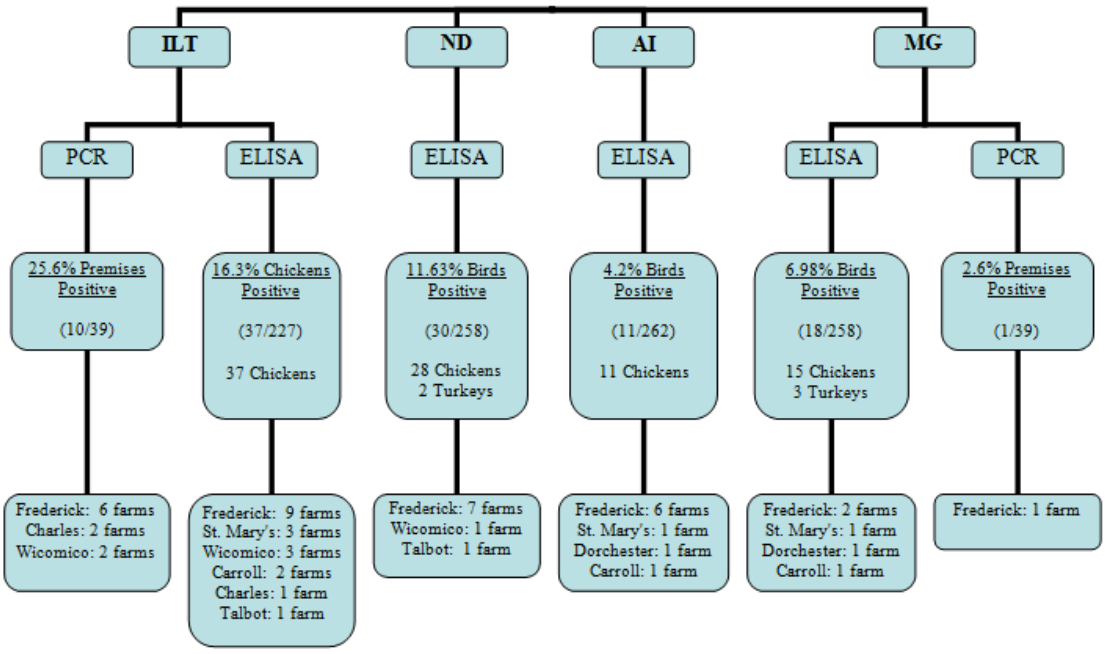
[a] *Flock vaccination history could not be determined.*
N/D Not done

Table 13 Flock seroprevalence and prevalence of diseases among the sampled counties in Maryland

	Northern MD (n = 20)		***Southern MD (n = 10)***		***Eastern Shore (n = 9)***				
	Frederick	Carroll	St. Mary's	Charles	Wicomico	Talbot	Caroline	Dorchester	Worcester
Seroprevalence									
ND[a]	7/17	-	-	-	1/4	1/2	-	-	-
ILT[a]	9[b]/17	2/3	3/6	1/4	3/4	1/2	-	-	-
MG[a]	2/17	1/3	1/6	-	-	-	-	1/1	-
AI	6/17	1/3	1/6	-	-	-	-	1/1	-
Prevalence									
ND	-	-	-	-	-	-	-	-	-
ILT	6/17	-	-	2/4	2/4	-	-	-	-
MG	1/17	-	-	-	-	-	-	-	-
AI	-	-	-	-	-	-	-	-	-
SE	-	-	-	-	-	-	-	-	-

[a] *Flock vaccination history could not be determined.*
[b] *One flock vaccinated by owner*

Figure 14 Flowchart of ELISA and PCR positives for NDV, ILT, MG, and AI according to species, number of premises, and county location.



3.4 Phylogenetic Analysis

A total of ten ILTV field isolates representative of each positive premises underwent partial nucleotide sequencing of the ICP4 gene for comparative genomic analysis. The amplification product size ranged from consensus nucleotide positions 240bp - 832bp. Field sequences were compared to the genomic sequences of Gallid herpesvirus 1 (NC_006623.1), USDA reference strain (JN542534.1), CEO LT BLEN® vaccine (JQ083493.2), CEO Trachivax® vaccine (Schering-Plough Animal Health, Millsboro, DE,USA), and TCO LT IVAX® vaccine (Schering-Plough, Animal Health, Millsboro, DE, USA) to identify single nucleotide polymorphisms and establish differentiation. While accession numbers were identified in GenBank for the first three sequences, the genome fragments of Trachivax® and LT IVAX® vaccines were not

available and therefore sequenced and assembled with the field isolates. Results showed a single flock having an identical match to the CEO LT BLEN® vaccine while eight flocks had a 100% match to the TCO LT IVAX® vaccine. The remaining flock amplification product MD/ch151/11 (state/species/field#/year) is 98.5% identical to the LT BLEN® vaccine (Table 14). At consensus nucleotide position 717bp the field isolate had nine sequential nucleotide insertions resulting in the addition of three serine amino acids (Figure14). No identical matches could be located in published articles or in the BLAST database of GenBank. To assess the integrity of the amplification product, additional overlapping primers were used to sequence the isolate. The product revealed a consistent sequence from consensus nucleotide positions 697bp -1192bp. According to Oldoni et al. (2008) genotype groupings, MD/ch151/11 would be classified as group V which is distinguished by CEO-related isolates. Phylogenetic analysis compared the genomic similarity among vaccine and other ILTV strains isolated from the U.S., Italy, China, Peru, and Brazil published in GenBank (Figure 16).

The MD/ch151/11 ILT flock was also MG positive and again underwent sequence analysis using the pvpA gene to differentiate MG strains. The amplification product size ranged from consensus nucleotide positions 586bp - 1041bp. Field sequences were compared to the genomic sequences of strain $R_{(high)}$ (NC_017502.1), Strain F (NC_017502.1), ts-11 vaccine (AY556382.1), 6/85 vaccine (AY556308.1), S6 strain (AY556307.1), and strain A5969 (AY556305.1). Evaluation of single nucleotide polymorphisms indicate that the MG positive flock was infected with the S6 strain with an identical match among the partial sequences (Table 15).

Table 14 Comparison of nucleotide sequence polymorphisms among ILT reference (USDA), TCO (LT IVAX) and CEO (LT BLEN®, Trachivax®) vaccines, and field strains. The dashes (-) indicate regions where the sequences are identical to those of Gallid Herpes virus GaHV-1 and deletions within the sequences are shown with asterisks (*). Field isolates are identified by bird ID number.

	Position in Gene																												
Strain	272	273	274	274	275	276	277	278	279	280	281	282	283	438	456	594	598	612	745	746	747	748	749	750	751	752	753	805	821
GaHV-1	C	G	G	C	C	C	A	A	G	A	C	G	G	G	G	*	G	G	*	*	*	*	*	*	*	*	*	A	A
USDA Ref.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
LT BLEN	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	C	A	*	*	*	*	*	*	*	*	*	*	G	G
Trachivax	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	C	A	*	*	*	*	*	*	*	*	*	*	G	G
LT IVAX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
24	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	C	A	*	*	*	*	*	*	*	*	*	*	G	G
96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
103	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
106	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
115	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
132	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	*	*	*	*	*	*	*	*	*	*	G	G
151	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	C	A	*	C	T	C	T	T	C	C	T	C	G	G

Figure 15 Comparison of ILT protein sequences and amino acid changes in MegAlign by ClustalW. Each positive farm is represented by bird ID number. Sequences are compared to the USDA reference strain, GaHV-1, CEO vaccines LT BLEN® and Trachivax, ® and TCO vaccine LT IVAX®.

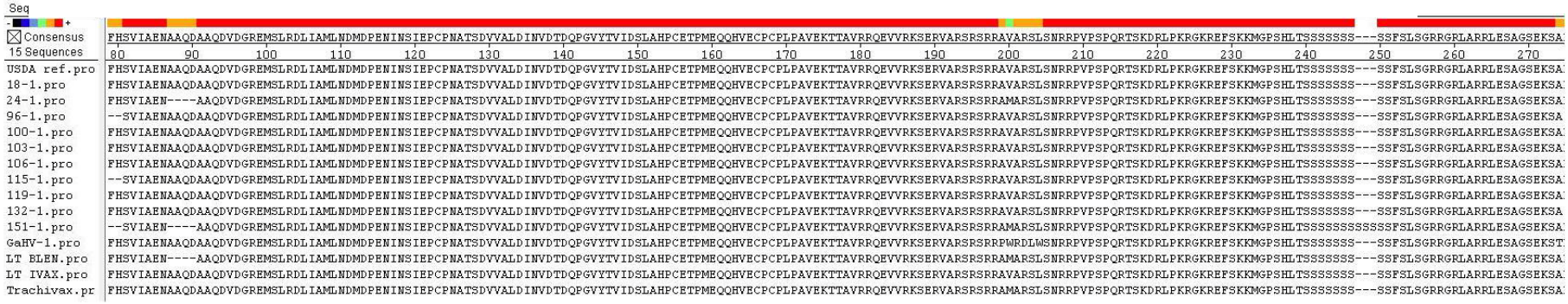


Figure 16 Phylogenetic relations among ILTV strains including MD-ch151-11. Analysis was based on nucleotides 238-844 (606bp) of the ICP4 gene. Bootstrap values from 1,000 replicates with neighbor-joining search method are indicated adjacent to the branches. MD/ch151/11 isolate is underlined.

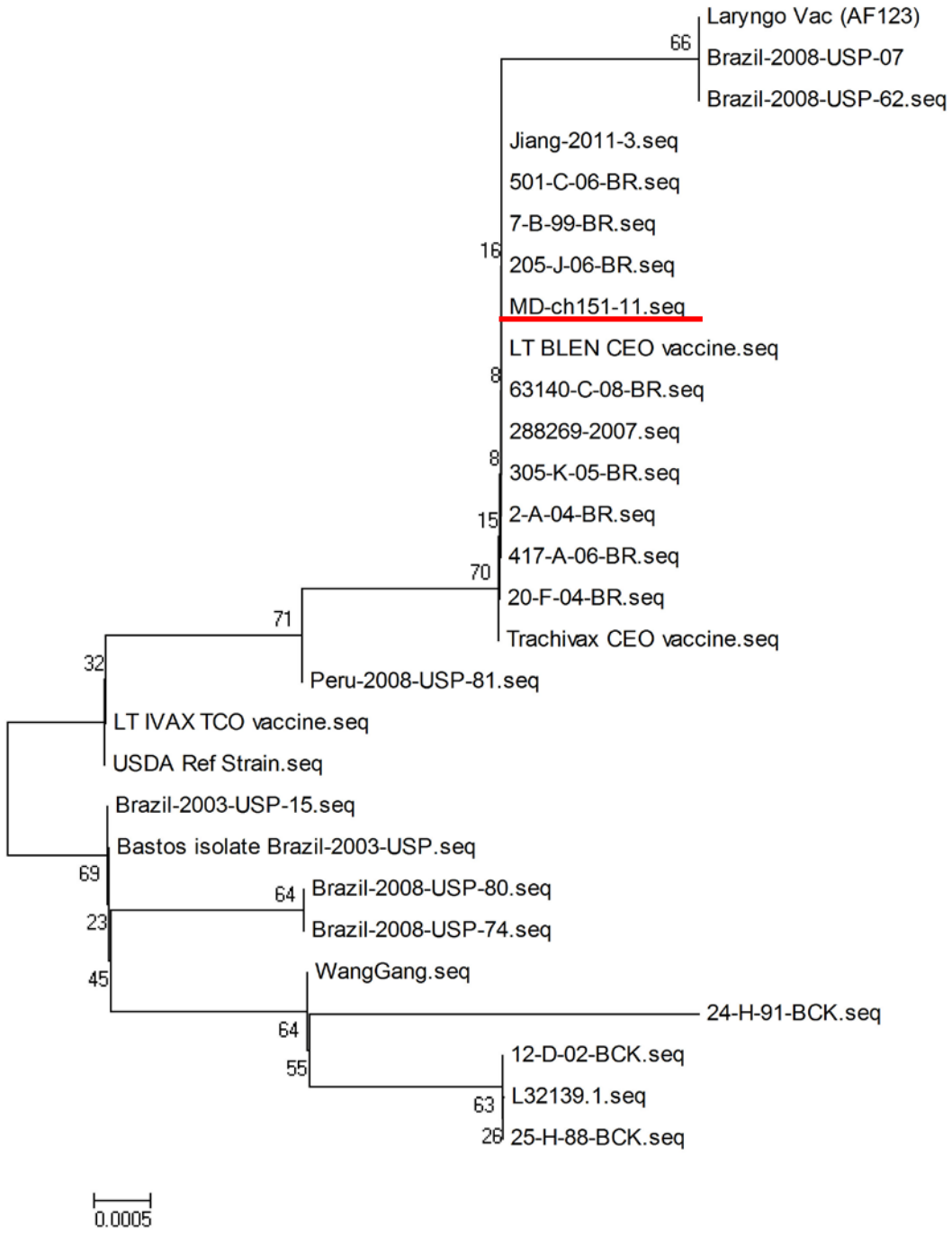


Table 15 Comparison of nucleotide sequence polymorphisms among MG reference strains (R_{High}, A5969), vaccines (F, TS-11, 6/85), and field strain. The dashes (-) indicate regions where the sequences are identical to those of strain $R_{(high)}$ and deletions within the sequences are shown with asterisks (*). Field isolate is identified by bird ID number.

	Position in Gene																																
Strain	620	625	644	668	672	711	729	743	750	752	763	784	796	797	802	806	807	808	809	810	811	820	843	846 - 905		911	935	940	970	974	983	994	1015
$R_{(High)}$	G	C	C	C	T	A	A	T	C	C	C	G	C	A	G	G	T	G	T	C	A	A	C	A	A	T	G	G	T	C	C	G	C
F	-	T	-	-	C	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	C	-	-	-	*
TS 11	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	C	C	-	-	-	-
A5969	A	-	T	-	C	G	-	-	A	A	A	A	T	-	C	T	C	C	C	A	C	G	-	-	-	-	-	C	C	-	-	-	-
6/85	-	T	-	T	-	-	C	C	-	A	A	A	G	-	-	-	C	C	C	A	C	G	T	*	C	C	A	-	C	A	A	A	*
S6	-	-	-	-	-	-	C	C	-	A	A	A	G	G	-	-	C	C	C	A	C	G	T	*	C	C	A	-	C	A	A	A	*
144	-	-	-	-	-	-	C	C	-	A	A	A	G	G	-	-	C	C	C	A	C	G	T	*	C	C	A	-	C	A	A	A	*

Figure 17 Map of Maryland flock distribution with swab positive backyard flocks. Poultry industries were grouped based on a 15km radius.

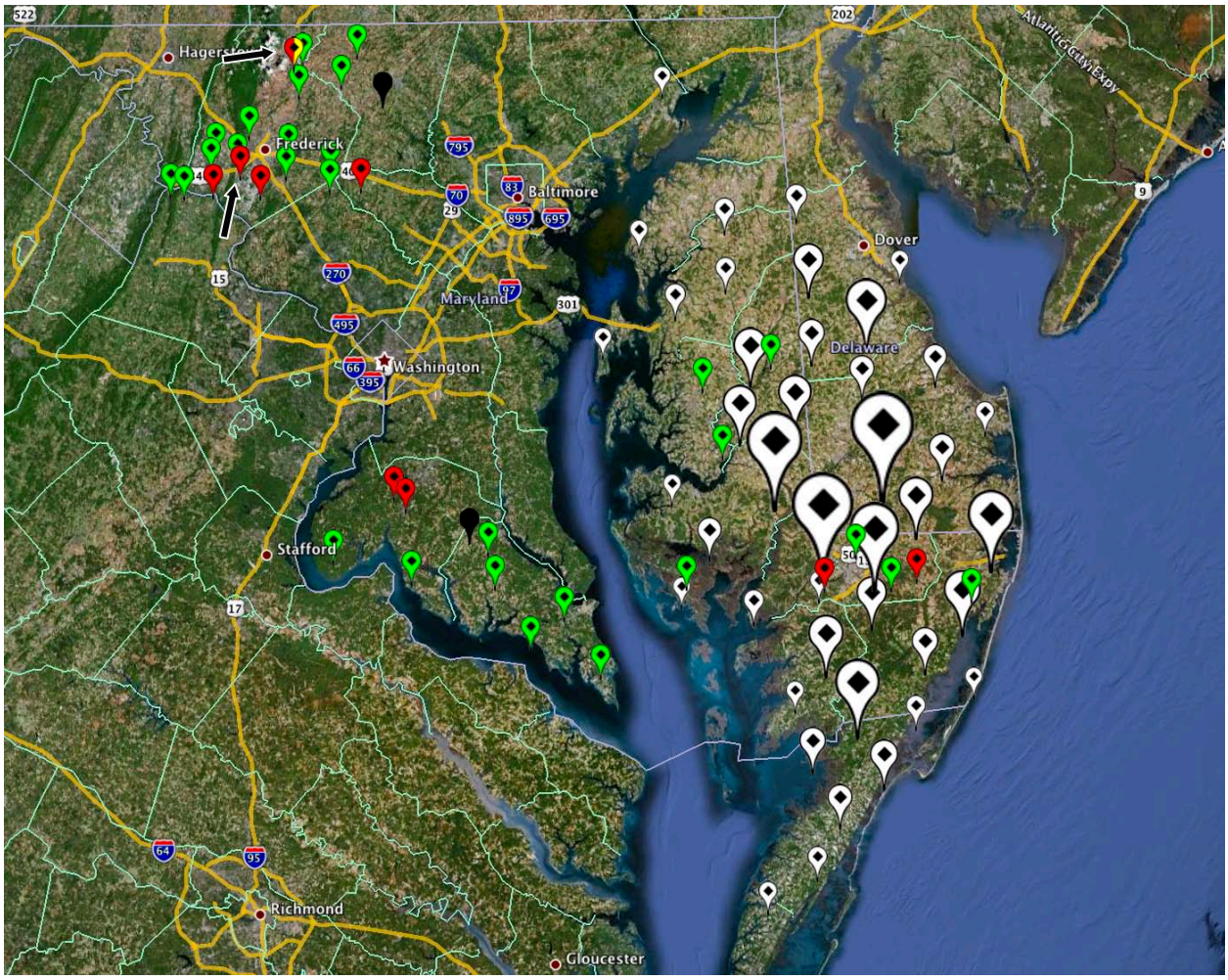
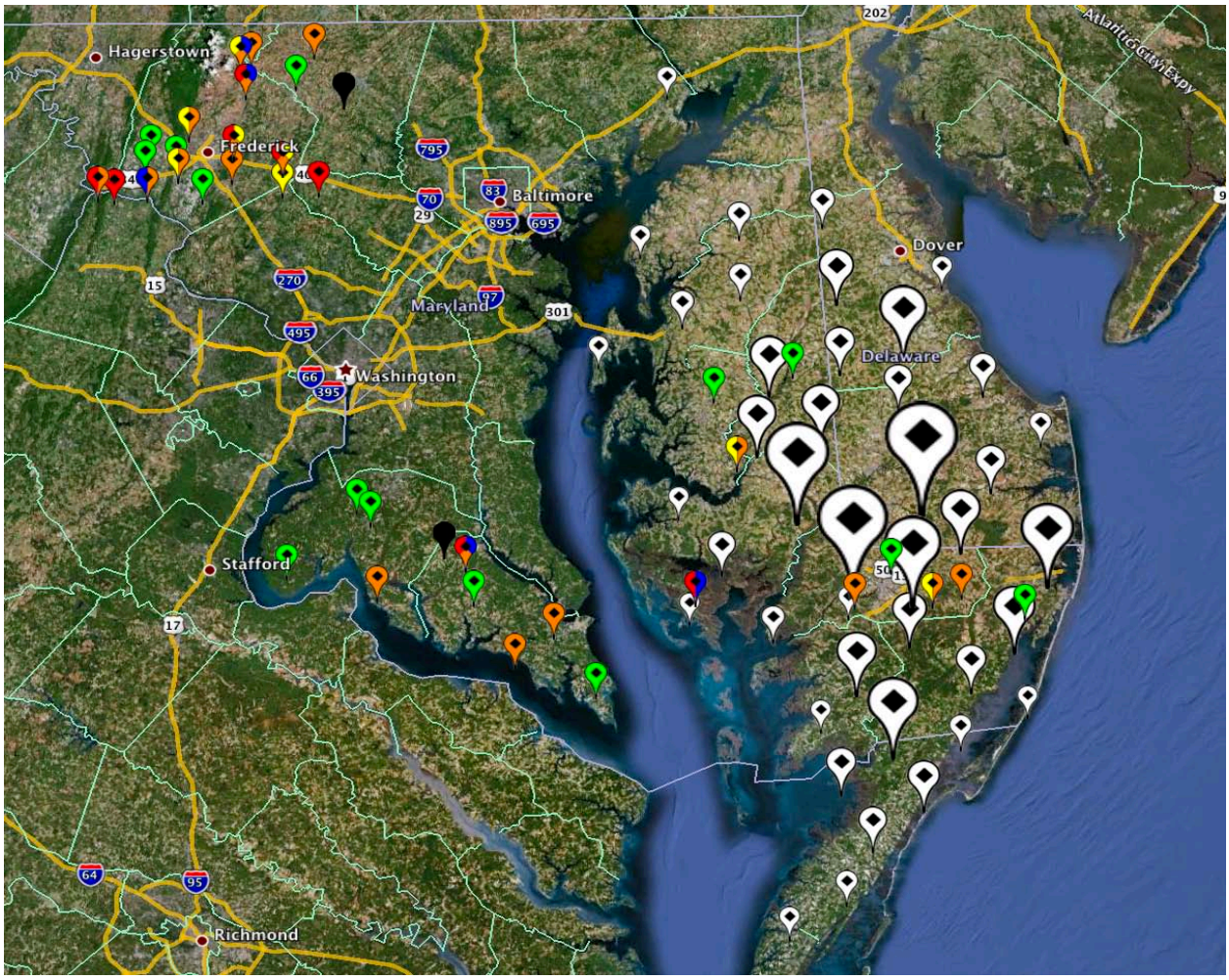


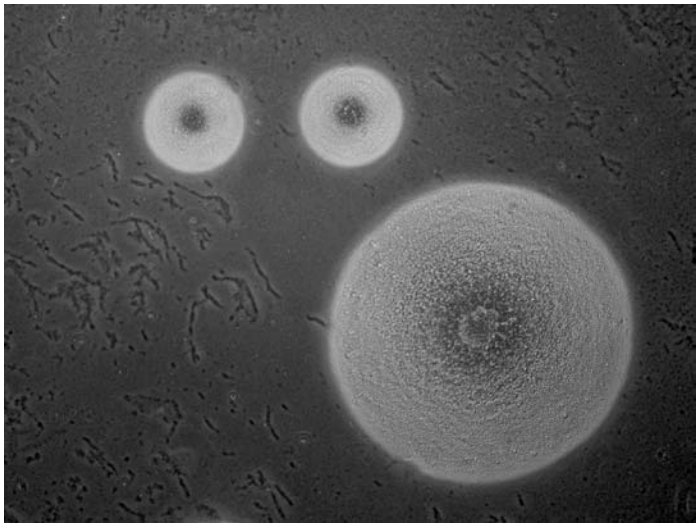
Figure 18 Map of Maryland flock distribution with seropositive birds. Poultry industries were grouped based on a 15km radius.



3.5 Bacterial and Viral Propagation

SE was not isolated from any of the environmental drag swabs; however, MG colonies did grow on Frey agar plate from the MG qPCR positive flock. Colonies were evaluated with the Zeiss HAL 100 refracted light microscope using a 10x objective lens and AxioVision v4.8.2 software (Figure 19). Attempts were made to isolate the unidentified ILTV strain after qPCR analysis, however, no plaques were observed on the sample's CAM. The CAM was homogenized, extracted for DNA and evaluated by qPCR which also tested negative. These outcomes are believed to be the result of viral and/or DNA degradation over time as sequencing and initial qPCR both confirm the presence of ILT virus. Earlier isolation attempts may have increased the potential to detect positive results.

Figure 19 MG colonies grown on a Frey's medium agar from a qPCR MG positive flock. Image taken with refracted light microscope with a 10x objective.



3.6 Cross-sectional Associations

Of the 39 flocks sampled, 36 also completed the survey and were analyzed for statistically significant associations. As disease prevalence was low and vaccination status could not be accurately verified among several flocks, associations between dependent and independent variables were analyzed for AI seroprevalent farms only (9/36). Farms that kept chickens as the only species were found to be significantly associated (p=0.041) with AI seroprevalent birds under univariate analysis, however, the association was no longer deemed significant after multivariate logistic regression when other variables were taken into consideration. No other significant associations were identified by univariate or multivariate analysis. However, some risk factors showed a positive association after relative risk calculations. Relative risk measures the strength of an association and is the ratio of the probability of disease occurring in the exposed group versus a non-exposed group. 67% (2/3) of seropositive flocks were exposed to waterfowl compared to 21% (7/33) that were not exposed. Seropositive flocks exposed to waterfowl were 3.14 times as likely to be AI seropositive than those not exposed to waterfowl (p=0.15). 33% (7/21) of seropositive flocks did not use pest control compared to 13% (2/15) that did (p=0.17). Seropositive flocks that did not use pest control were 2.5 times as likely to be AI seropositive than those that did. 35% (7/20) of seropositive flocks were from Northern Maryland while 13% (2/16) were from other regions (p=0.12). Seropositive birds from Northern Maryland were 3.8 times as likely to be AI seropositive than those from Southern or Eastern Maryland. Five out of 11 flocks (46%) that were AI seropositive had also experienced diarrhea in the past 6 months compared to 16% (4/25)

of AI seropositive flocks without diarrhea (p=0.08). Results from statistical analysis may be found in Tables 16, 17, 18, and 19.

Table 16 Univariate analysis of categorical biosecurity variables ($P \leq 0.25$).

Variable	Description	Prevalence Ratio	95% Confidence Interval	P-value
Diarrhea	Reported within past 6 mo.	2.84	0.939-8.596	0.075
Location	North vs. other regions	2.80	0.672-11.670	0.122
Pest control	Implemented pest control	2.5	0.601-10.394	0.165
Waterfowl	Exposed to wild waterfowl	3.14	1.116-8.853	0.148
Species	Chicken only vs. mix species	3.9	0.937-16.326	0.041

Table 17 Simple logistic regression of continuous biosecurity variables ($P \leq 0.25$)

Variable	Description	Odds Ratio	95% Confidence Interval	P-value
Time owned	How many years kept poultry	1.04	0.98-1.10	0.236
Visit comm.	How often visit commercial sites	1.08	0.88-1.32	0.223

Table 18 Multivariate logistic regression ($P \leq 0.25$)

Variable	Description	Coefficient	P-value
Time owned	How many years kept poultry	0.613	0.133
Visit comm.	How often visit commercial sites	2.701	0.104
Diarrhea	Reported within past 6 mo.	-1.314	0.380
Location	North vs. other regions	2.500	0.204
Pest control	Implemented pest control	-0.107	0.942
Waterfowl	Exposed to wild waterfowl	18.377	0.736
Species	Chicken only vs. mix species	29.275	0.112

Table 19 Backward selection stepwise logistic regression model to examine association between biosecurity risk factors and flock positives. ($P \leq 0.05$)

Variable	Description	Coefficient	P-value
Time owned	How many years kept poultry	0.154	0.127
Visit comm.	How often visit commercial sites	0.713	0.080
Location	North vs. other regions	2.379	0.102
Species	Chicken only vs. mix species	6.628	0.094

Chapter 4: Discussion

4.1 Principal Findings and Interpretations

This study confirmed that backyard flocks are no exceptions to infectious and zoonotic diseases and that Maryland flocks may have been introduced to AI from exposure to waterfowl. Similar findings were also reported in a 2006 New Zealand study that identified five out of 24 flocks to be seropositive for AI (Zheng et al., 2006). Nearly half of the surveyed premises kept their birds on free range, with some accessible to water sources such as ponds or rivers. Over half of farms reported that their flocks could be exposed to wild birds, while less than half practice pest control management. The lack of a secure housing environment and location near a congregation source for wild birds increases the likelihood of disease transmission from exposure to wild birds, waterfowl, and pests. In this study, while not statistically significant, a higher proportion of AI seropositive flocks were found to be exposed to waterfowl and did not use pest control. Over half of the flocks were also exposed to wild birds.

These potential risk associations with disease reservoirs and vectors are consistent with findings from other studies. For example, wild birds most frequently reported visiting poultry enclosures where sparrows and European starlings, both of which are susceptible to experimental highly pathogenic H5N1 infection and excrete high titers of virus (Boon et al., 2007). Another study conducted in an artificial barnyard setting found that mallards recently infected with H5N2 and H7N3 could transmit the influenza A viruses to chickens, blackbirds, rats and pigeons making laboratory science applicable to real world scenarios (Achenbach and Bowen, 2011). Other avian

pathogens, such as MG, have also been isolated from free ranging house finches and multiple other songbird species in the eastern U.S. and Canada. These house finch MG isolates have been shown to cause clinical infections in chickens and turkeys (O'Connor et al., 1999). One study found that darkling beetles collected from commercial broiler flocks contained live ILT virus while a rodent had ILT DNA in its lungs, further indicating that pests may not only serve as fomites, externally carrying the viruses, but as vectors harboring the virus internally as well (Ou et al., 2012). Pigeons and doves, along with waterfowl, have played a major role in the panzoonotic spread of Newcastle disease. Newcastle disease virus isolates, identical to strains identified in cormorant outbreaks, were discovered in free range turkeys from North Dakota (USAHA, 1993). These cases support the importance of isolating backyard flocks from other birds and pests that could be infected or carry diseases, especially during the summer months of migration when birds return to breeding grounds.

Of the 41 flocks surveyed, all but one allowed visitors onto their poultry premises, with almost 75% permitting direct contact with their flock. This increase in flock traffic potentially increases the risk of introducing disease via fomites as visitors' vehicles, boots, and clothing may carry pathogens. Several outbreak investigations have linked fomites in connection with disease spread, such as the 1983 HPAI outbreak in commercial poultry which was associated with human and equipment traffic from New York live bird markets (Beard, 2000). Another study found an association between farms of the 1999 MG outbreak in North Carolina as a poultry grower returned to his flock after visiting an MG positive farm without changing his contaminated clothing or footwear (Vaillancourt et al., 2000). While many owners reported always washing their hands

before and after handling their birds (66%) admittedly never wore designated clothing. Majority of backyard flocks (68%) also reported being within a quarter mile (0.4km) of other backyard flocks. One study designed a model to predict the potential wind-borne transmission of HPAI virus particles between farms given the conditions of the Dutch 2003 HPAI epidemic. These researchers estimated that 24% of the observed infections could be explained by wind-borne transmission up to 25km (15.5mi) (Ssematimba, 2012). Another case-control study determined that ILT case flocks were 9.9 times more likely to be located within the wind vector of a clinical ILT flock during the 14 day incubation period compared to control flocks (Johnson et al., 2005). While dependent on variable conditions, these findings indicate that backyard flocks could potentially bridge the gaps between farms during an outbreak, especially in a poultry-dense location.

Case analysis of the single ILT/MG positive flock revealed that the owner had kept poultry for 15 years, primarily to sell meat. Flock size was 857 birds with a mix of chickens, turkeys, ducks, guinea fowl, and geese which were not separated by species or age. MG was isolated from young turkeys and the ILT DNA was sequenced from a mature hen. Birds were free range and exposed to wild birds, pets, rodents, and livestock. The owner visited commercial poultry locations while coming into direct contact with birds and allowed guests onto the premises, but restricted direct contact. The owner had purchased new birds within the last year and did not isolate new birds from the flock. Within the last year, the owner reported 200 deaths (23%) from predation and 100 deaths (11%) from disease. Only respiratory signs of disease were observed in the last six months. The owner never washes hands before or after handling birds, never wears dedicated clothing or footwear, and does not use a footbath. However, the owner does

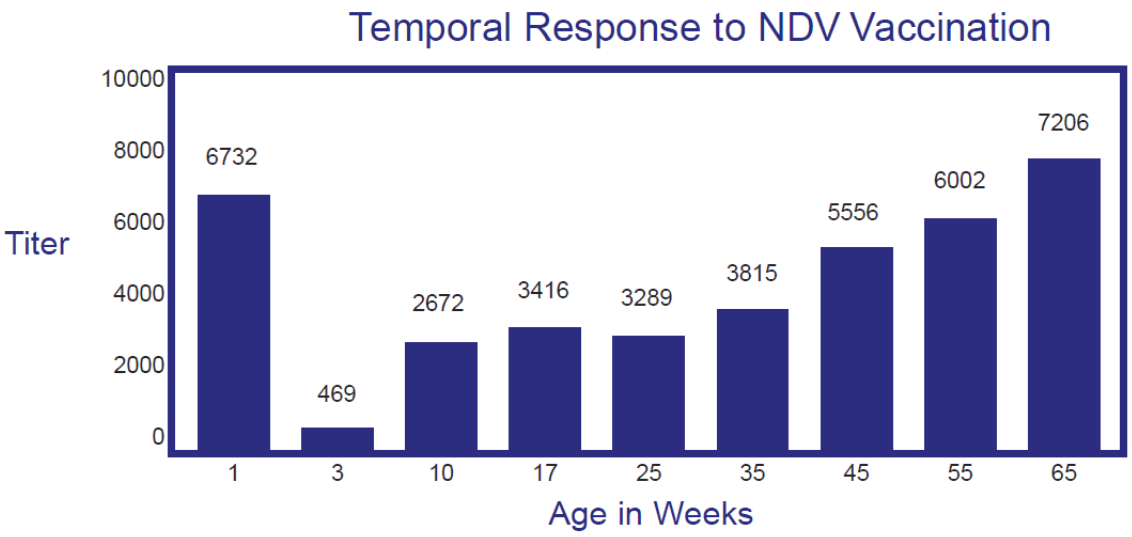
clean and disinfect equipment and practices pest control. A Google® maps search for the term "poultry farm" was conducted and revealed one turkey farm with over 100,000 birds within five miles (8km) of this flock.

Despite low to non-existent prevalence rates for the various pathogens of interest, serum analysis provided a further look into the history of flock exposure to diseases. While seroprevalence for ND could not be accurately differentiated between vaccination or field exposure status, some of the ILT and MG seropositives were distinguishable. It is strongly believed that MG seropositives are a result of field strain challenges, as most hatcheries generally do not vaccinate flocks. Instead, MG is controlled and eradicated through routine testing and biosecurity to maintain a NPIP MG clean status (USDA NPIP, 2006). While only one flock owner reported vaccinating for ILT, no other backyard flock owners vaccinated birds once on their premises. Owners often obtained birds from various sources and vaccination practices among hatcheries and growers vary on several factors including state regulations, personal preference, or age of the bird being purchased, therefore details for individual flocks could not be accurately determined. Beyond these issues, antibody titers also depend on a variety of other factors such as dose of infection/vaccination, length of infection, virulence, age of bird, and route of infection/administration to name a few. It is also possible that poultry with a serological response may no longer be shedding the organism, and equally so, seronegative poultry may be infected but with insufficient immunoglobulin concentrations.

The ProFLOK PLUS ELISA kit used in this study presents a titer profile response to a typical NDV vaccination program over time to maintain protective immunity in a

flock (Figure 20). This vaccination program is generally used when disease is mild and sporadic in a region and follows a protocol similar to the OIE recommended guidelines: live Hitchner-B1 by conjunctival or spray administration at day one of age; live Hitchner-B1 or La Sota at 18-21 days of age in drinking water; live La Sota in drinking water at 10 weeks of age, and an inactivated oil emulsion vaccine at point of lay (OIE, 2009). The mean titer from one NDV seropositive flock (7767, range: 3610-12738) was significantly higher ($p=0.0025$) than the mean titer of all other NDV seropositive flocks (4086, range: 522-7890). However, it cannot be determined whether this represents a vaccine or field challenge response.

Figure 20 Typical antibody profile in response to standard NDV vaccination program of approximately 350 flocks. Measured by Synbiotics NDV+ ELISA test.



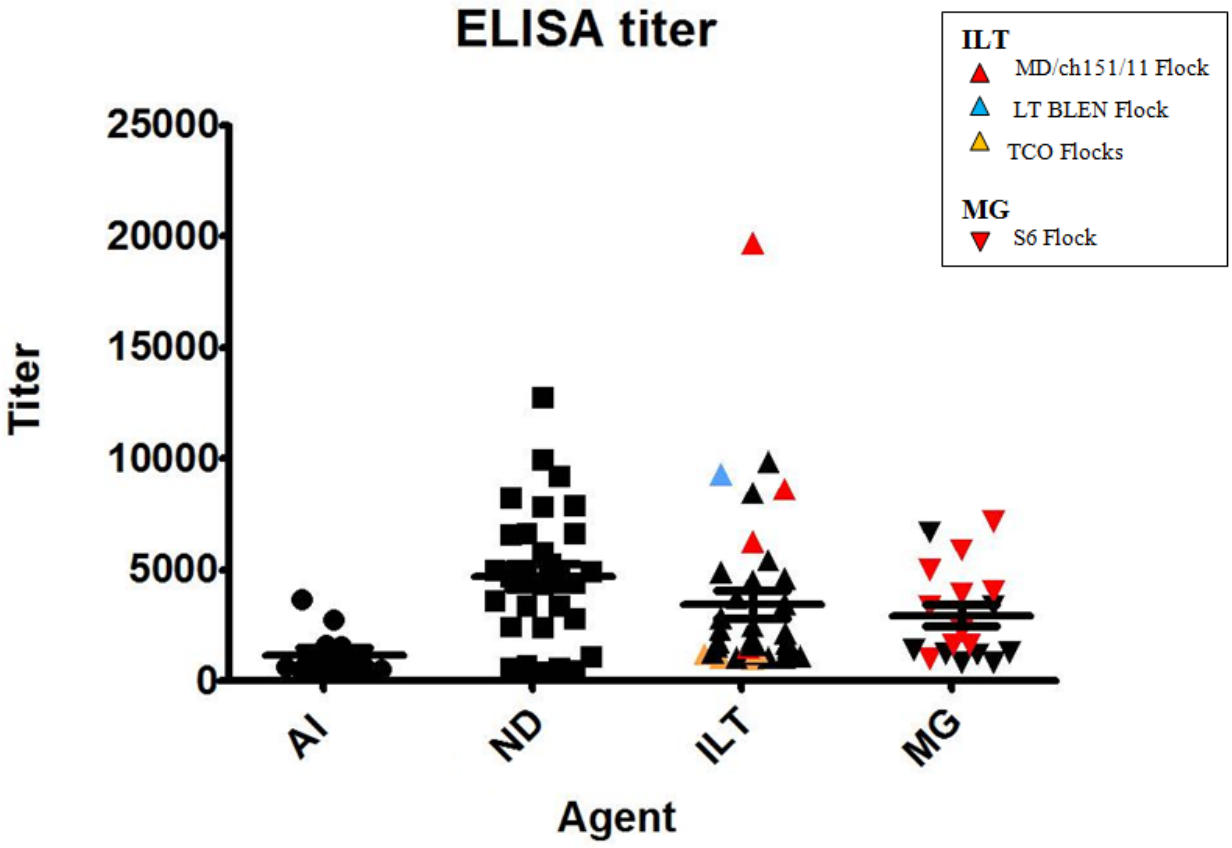
It is believed that all of the AI seropositive flocks identified in this study were exposed to LPAI viruses as the birds survived the infection and owners did not report any significant mortalities in their flock from disease. As previously discussed, LPAI viruses

may produce subtle to no signs of clinical infection. Almost one third of owners who were tested observed diarrhea in their flock within the past six months, which includes 46% of AI exposed flocks. One third of owners also reported a decrease in egg production or soft/misshapen eggs. Of the AI seropositive flocks, only 33% reported this observation. While 11% of sampled backyard flock owners recalled respiratory disease within the last six months, only one AI seropositive flock exhibited coughing, sneezing, nasal secretions, or swollen sinuses. Weight loss or decreased appetite was also observed in 8% of tested flocks but none of the AI seropositive flocks. Another indication that flocks were exposed to LPAI viruses was in the negative results of the HI assay for H5, H7, or H9 influenza subtypes. Previous influenza surveillance studies conducted in wild Maryland waterfowl have reported the presence of hemagglutinin subtypes H2, H3, H6, H9, H11, and H12, while the majority of North American HA subtypes consist of H3, H4, and H6 (Slemons et al., 2003; Pascua et al., 2006; Hanson et al., 2003). Figure 21 displays a scatter plot of all the ELISA positive titers. Bird titers from PCR positive flocks were identified to evaluate the potential titer distribution throughout a flock in response to an exposure.

A handful of studies from around the world have evaluated the seroprevalence of disease in non-vaccinated backyard flocks. While one study in New Zealand found 20.8% (5/24) AI flock seroprevalence, comparable with this study (23%), a Minnesota team only detected one flock out of 150 (0.66%) for AI antibodies (Zheng et al., 2010; Yendell et al., 2012). In Switzerland, researchers reported 37.5% (15/40) seroprevalence of AI in fancy breeding flocks. This same study also determined seroprevalence for SE (3/40, 7.5%), ILT (18/28, 64.3%), ND (6/34, 17.6%, and MG/MS (33/40, 92.5%), reporting

higher prevalence rates overall comparatively to this study's findings (Wunderwald and Hoop, 2002). California backyard flocks were found to have a MG seroprevalence of 38% (12/32) (McBride et al., 1991), and another study in Argentina reported a MG seroprevalence of 33% (22/67) (Xavier et al., 2010).

Figure 21 Scatter plot of ELISA titers for AI, ND, ILT, and MG.



4.2 Implications

ILT has been controlled in the U.S. historically by two live attenuated vaccines, those attenuated by multiple passages in embryonated eggs (CEO) and those passaged in tissue cultures (TCO). However, isolates from ILT outbreaks demonstrate that attenuated vaccines, especially those of CEO, are capable of reverting to virulence and causing disease in naive birds. In an effort to mitigate the spread of ILT in non-commercial poultry, the MDA in 2009 mandated new policies restricting the use of CEO vaccine in exhibition poultry, with the recommendation of TCO vaccines. This study showed that while CEO vaccines are still circulating in backyard flocks, TCO vaccines were the predominant (8/10) ILT isolates detected from poultry flocks. Of the 19 ILT seropositive flocks, three can be attributed to TCO vaccine, and two to CEO vaccines. Reemergence of MG has been a concern over recent years thought to be a result of locating large poultry populations within a limited area and under poor biosecurity. However, this study reported a much lower seroprevalence in backyard poultry compared to other serological surveys. Of the five seropositive premises, one flock was positive for current infection with the S6 strain of MG, an infectious strain first isolated in 1954 from the brain of a turkey in a California flock (Zander, 1961).

Concern remains with the fact that almost a quarter of backyard flocks were seropositive for AI, however, some reassurance follows with the absence of H5, H7, and H9 subtypes. It is evident based on the findings of this study that biosecurity education in non-commercial flocks should be promoted and is a necessary step towards disease prevention as many of the owners are relatively new to backyard poultry.

4.3 Strengths and Limitations

This is the first known study to report associations between biosecurity management practices and disease prevalence/seroprevalence of these five major pathogens of interest among backyard flocks located within close proximity to the Delmarva commercial poultry region. Flock positives were also sequenced and strain differentiated. However, this study was subject to some limitations. The overall response rate of this study (4.1%) was relatively poor, but believed to stem from the concern over the mandatory reporting of flock positives to the State Veterinarian and potential repercussions, such as "Hold Orders" that restrict the movement of birds onto or off the premises, as well as the stigma attached to having an infectious disease. A larger sample size may have also increased the ability of this study to detect significant associations between biosecurity risk factors and disease prevalence. While association could be hypothesized based on proportional analysis, wide confidence intervals indicate that these estimates have low precision from an inadequate sample size. While methods of convenience sampling are often assumed to be representative of a population, sampling biases (most notably selection bias) do occur, making it difficult to develop statistically valid estimates of disease prevalence, regardless of how many birds are sampled. This study was also limited to a population of backyard flock owners that had registered with the MDA. It is believed that disease prevalence estimates are lower than those occurring in the true population as most owners with clinically ill birds would be hesitant to participate.

Chapter 5: Summary and Conclusion

5.1 Meeting the Objectives

Surveillance is a dynamic process that requires the continuous observation, collection, and analysis of data in order to identify the presence of a disease and contain its spread. While wild waterfowl has been the main target of disease investigations, domesticated poultry warrant consideration as well, particularly with its growing trend. This study aimed to capture a snap shot of pathogen prevalence during an outbreak-free period in order to provide information on the background level of disease in this potentially vulnerable population and to develop a better understanding of its molecular epidemiology. Maryland small flock exposure history was also analyzed and interpreted to the best of the researcher's ability to evaluate a broader range of exposure and transmission pathways.

The goals of this study were successfully implemented and completed as surveillance data from this project provided a better understanding of biosecurity and pathogen transmission relationships within backyard flocks, particularly of those exposed to avian influenza. The first objective led to the characterization of Maryland backyard flock biosecurity practices, identifying the strengths and weaknesses among flock owners. Sampling, laboratory analysis, and partial genome sequencing resulted in the identification and differentiation of positive flocks. Ten flocks were found to be positive for ILTV. Eight were identified as TCO isolates, one was an identical match for the CEO LT BLEN® vaccine known to revert to virulence, and another was a CEO like strain (MD/ch151/11) not matched in GenBank. The CEO-like flock was also found to be

currently infected with the S6 strain of MG. Multiple flocks were found to be exposed or vaccinated for ND, ILT, and MG. Nine flocks were AI seropositive, most likely from exposure to LPAI as subtypes H5, H7, nor H9 were not detected. While no risk factors were identified as statistically significant after multivariate logistic regression, proportional observations were analyzed and reported with strengths of association as addressed in the remaining five objectives, furthering the knowledge of disease prevalence in a population subjected to few inspections. Disease surveillance will always remain a necessity and will further improve disease management programs alongside with prevention and education strategies.

5.2 Online Biosecurity Education

In addition to the surveillance study, online Extension Moodle™ courses on avian influenza and biosecurity were developed for audiences including backyard flocks owners, youth and 4-H members, as well as emergency responders. These self-directed certification courses provide information consolidated from multiple government organizations on avian influenza and how it may affect backyard flocks, as well as details on prevention, response, and recovery. Since the launch of the Backyard Flock course, almost 100 registered users from all over the world including HPAI affected countries such as Egypt, Indonesia, and Iraq have participated. Course impact has been evaluated showing user's knowledge on avian influenza and biosecurity before and after completing the course had increased from 13% to 61%. On a scale of one to five, users rated ease of use as 4.5, information clarity as 4.6, content level appropriate 4.5, and graphics as 4.5. Several owners reported that they plan on changing their biosecurity

practices after taking the course. Supplemental to the courses is a virtual hands on backyard flock designed to test participants' knowledge on biosecurity using an avatar in Second Life©.

As demonstrated in this study, education is essential for backyard flock ownership as the majority of flock owners sampled had kept poultry for five years or less. Many flocks did not practice "good" biosecurity, as previously described, many of which are simple, practical, and affordable. Biosecurity factors associated with disease prevalence identified in this study will be highlighted in the biosecurity courses, such as protecting birds from wild birds and waterfowl, particularly during the spring and summer months when migration season is at its peak and implementing pest control. Education programs, such as these, provide a large impact and are more cost effective than surveillance as they aim to prevent disease closer to the source.

Extension educational sources may be found below:

Backyard Flocks: http://campus.extension.org/course/view.php?id=423

Youth and 4-H: http://campus.extension.org/course/view.php?id=285

Emergency Responders: http://campus.extension.org/course/view.php?id=422

Second Life

Appendices

Appendix A:

INFORMATION / CONSENT FORM

Surveillance Research:
Researchers from the Veterinary Medical Sciences Department at the University of Maryland – College Park are undertaking a research project to determine biosecurity practices and prevalence of poultry diseases in backyard flocks located on the Delmarva Peninsula and surrounding areas. If you are interested in participating please read, initial, date, and sign this information/consent form and return in the self addressed prepaid envelope. A copy of this form will then be sent to you.

Project Title: Epidemiological Analysis of Biosecurity Practices and Associated Prevalence of Diseases in Non-commercial Poultry Flocks

Why is this research being done?
Poultry disease can have a detrimental effect on the health of flock. However, using appropriate biosecurity practices, practices used to prevent the introduction and spread of diseases into a flock, can greatly reduce the incidence of infected birds. No previous information on the uses of these practices in correlation with disease in backyard flocks of Maryland has been published. Dr. Nathaniel Tablante and Mrs. Jenny Madsen would like to invite you to participate in this research study if you own chickens, turkeys, ducks, geese, quail, or pheasants.

What are you looking for?
- Avian Influenza
- Infectious Laryngotracheitis
- Newcastle Disease Virus
- *Salmonella* Enteritidis
- Mycoplasma gallisepticum

To find out more about these diseases please visit: http://partnersah.vet.cornell.edu/avian-atlas/lists/disease and http://www.cdc.gov/nczved/divisions/dfbmd/diseases/*Salmonella* enteritidis/

What will I be asked to do?
It is entirely your choice as to whether you wish to take part in the study. You may withdraw from the study at any time. Participants will be asked to:
- Complete a biosecurity survey (You may chose to only complete the survey)
- Allow trained researchers to take tracheal/cloacal swab samples and drag swabs from backyard flocks
- Allow trained researchers to take blood samples from backyard flocks

How long will it take?
The survey and sample collection will begin in June 2011 and continue till September 2011.
- The survey should take no more than 30 minutes. You may complete the survey online at http://www.kwiksurveys.com/online-survey.php?surveyID=NKEDLJ_ba2f8673
- If owners allow sampling (blood, tracheal, and cloacal) on their birds, collection times would be obtained at a time of their choosing. Sampling time will vary on how many birds are to be sampled; however, one bird will take no longer than approximately 10 minutes. Only one visit will be made per location for sample collections.

What are the benefits of this research?
- Owners will obtain a free diagnostic health status report on their backyard poultry.

- Owners will have access to specialists who can answer questions about the animal and human health aspects of the different pathogens.
- If a disease is detected early it may help prevent of the further spread to the rest of the flock. Participating in this study will also help the owner determine if the biosecurity practices they are using are effective at preventing disease.
- Owners will also be given free access to online biosecurity courses for avian influenza.

What are the risks of this research?
Subjects will be exposed to no or minimal risk by participating in the survey. No physical or invasive procedures will be conducted on human subjects. While extremely unlikely, if birds are found to have these reportable diseases, with the exception of mycoplasma gallisepticum, the state veterinarian will be notified within 48 hours of identifying the pathogen. Any findings will be explained to participants. The state veterinarian will discuss the situation and may arrange to visit the property. A follow up investigation may be required to confirm findings.

How is my privacy maintained?
All personal information collected will be kept strictly confidential and will be locked in files and in password-protected computers. Data on each individual will remain anonymous, unless a reportable disease is present in which case is notifiable to the state veterinarian by Maryland State law. Contact information will be given out for no other reason. Information will only used for research studies at the University of Maryland.

Further Questions:
This research has been reviewed according to the University of Maryland, College Park IRB procedures for research involving human subjects. If you have questions about your rights as a research subject, please contact:
Institutional Review Board Office, University of Maryland, College Park, MD 20742
Email: irb@umd.edu Phone: 301-405-0678

You may contact the researchers at any time if you have any questions about participating.
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Field Investigator
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Statement of Age of Subject and Consent	*Your signature indicates that you are at least 18 years of age;, the research has been explained to you; your questions have been fully answered; and you freely and voluntarily choose to participate in this research project.*	
Signature and Date	**NAME OF SUBJECT**	
	SIGNATURE OF SUBJECT	
	DATE	

Appendix B:

BACKYARD FLOCK BIOSECURITY QUESTIONNAIRE
Virginia-Maryland Regional College of Veterinary Medicine

Thank you for taking the time to fill out the survey, a graduate student appreciates it. Please complete the backyard biosecurity survey and return it in the self addressed prepaid envelope. You may also choice to complete the survey online at http://www.kwiksurveys.com/online-survey.php?surveyID=NKEDLJ_ba2f8673.

Personal Information:

Name: _____ Address: _____

Phone #: __(_____)_____ _____

Species Information:

1. Please fill in the number of each species of bird you currently own, the primary use and type of housing.

Species	Number of Birds	Primary Use: (*choose only one*) 1. SALE-live birds 2. SALE-meat 3. SALE-eggs 4. FOR EGGS-home consumption 5. SHOWS/EXHIBITIONS 6. PETS	Type of Housing *(choose only one)* 1. Coup, fenced in and covered 2. Coup, fenced in but not covered 3. Free range over property 4. Free range, having access to ponds or streams.
Chickens			
Turkeys			
Ducks			
Geese			
Pheasants			
Quail			
Chuckers			
Pigeons			
Other *(please specify)*			

2. How long have you kept backyard poultry?

3. Do you separate your birds by species?	YES NO	☐ ☐
4. Do you separate your birds by age?	YES NO	☐ ☐

5. Where did you get your birds? (*Please check all that apply and specify the name of the source).*

MAIL ORDER HATCHERY	☐
AUCTION MARKET	☐
COMMERCIAL FARM	☐
OTHER (please specify)	☐

Other Animals:

6. Please check yes or no about you and your birds contact with:

	You:		**Poultry have contact with:**	
Wild Waterfowl: (Ducks , geese, etc)	YES NO	☐ ☐	YES NO	☐ ☐
Other Wild Birds: (Crows, sparrows, owls, raptors, etc)	YES NO	☐ ☐	YES NO	☐ ☐
Pet Birds: (Parrots, parakeets cockatiels, etc)	YES NO	☐ ☐	YES NO	☐ ☐
Neighbors' Birds: (Chickens, turkeys, etc)	YES NO	☐ ☐	YES NO	☐ ☐
Pets: (Dogs, cats, etc)	YES NO	☐ ☐	YES NO	☐ ☐
Rodents: (Rats, mice, etc)	YES NO	☐ ☐	YES NO	☐ ☐
Wild carnivores: (foxes, raccoons, etc)	YES NO	☐ ☐	YES NO	☐ ☐
Livestock: (Pigs, sheep, cattle, goats, etc)	YES NO	☐ ☐	YES NO	☐ ☐

Exposure Risks:

7. How many commercial poultry flocks are within 1/4 mile from your flock?		
8. How many backyard flocks are within 1/4 mile from your flock?		
9. Do you or anyone in your household work in the poultry industry outside of your farm?	YES NO	☐ ☐
10. In the average year how many times do you or anyone in your household visit commercial poultry premises?		
If >0, do the visitors come in contact with the birds (*e.g. enter the house, touch the birds*)?	YES NO	☐ ☐
11. In the average year how many times do you or anyone in your household visit backyard poultry premises?		
If >0, do the visitors come in contact with the birds (*e.g. enter the house, touch the birds*)?	YES NO	☐ ☐
12. Do you allow business visitors (vet, extension agent, customers, service personnel) in your poultry area?	YES NO	☐ ☐
If yes, do the visitors come in contact with the birds (*e.g. enter the house, touch the birds*)?	YES NO	☐ ☐
13. Do you allow non-business visitors (school groups, friends, neighbors) in your poultry area?	YES NO	☐ ☐
If yes, do the visitors come in contact with the birds (*e.g. enter the house, touch the birds*)?	YES NO	☐ ☐
14. Do you SELL birds in live bird auction markets?	YES NO	☐ ☐
15. Do you BUY birds from live bird auction markets?	YES NO	☐ ☐
16. Have you purchased new birds in the past 12 months?	YES NO	☐ ☐
18. Do you isolate new birds before introducing them to your flock? If YES, for how long? _____	YES NO	☐ ☐

Poultry Health:

19.	Once the birds are on your premises do you vaccinate your birds for any diseases?	YES NO	☐ ☐

If yes, please specify which diseases or vaccines.

20.	Over the past 2 years, how many of your birds died from:	
	Predators?	
	Disease?	
	Injury?	
	Other causes (unknown)?	

21. Please check all the signs of disease that you have seen in your birds within the past 6 months.

Diarrhea	☐
Respiratory signs *(cough/sneeze, nasal secretions, swollen sinuses)*	☐
Neurological *(lack of coordination, weakness)*	☐
Weight loss/ decreased appetite	☐
Decreased egg production or soft/misshapen eggs	☐
Swelling *(head, comb, wattles, hocks)*	☐

22. Please list any medications you administered to the birds within the last 6 months.

23.	Have you ever sent any **ILL** birds to the diagnostic lab for disease diagnosis or had a vet exam?	YES NO	☐ ☐
	If YES, what did they have?		

Sanitation/Decontamination:

24.	How often do you wash your hands before and after handling your birds?	Always Sometimes Never	☐ ☐ ☐
25.	How often do you put on separate clothes for handling your birds?	Always Sometimes Never	☐ ☐ ☐

26. Do you routinely use a footbath at the entrance to your poultry premises?	YES NO	☐ ☐
If NO, do you wear a different pair of shoes when entering your poultry area?	YES NO	☐ ☐
27. How often do you make visitors wash their hands before and after handling your birds?	Don't allow visitors Always Sometimes Never	☐ ☐ ☐ ☐
28. How often do you make visitors wear separate clothing and/or shoes?	Don't allow visitors Always Sometimes Never	☐ ☐ ☐ ☐
29. Do you clean and disinfect birdcages, floors, and equipment?	YES NO	☐ ☐
If YES, how often?	MORE THAN ONCE A WEEK ONCE A WEEK ONCE A MONTH ONCE A YEAR ONCE A YEAR	☐ ☐ ☐ ☐ ☐
30. Do you dispose of used litter or bedding?	YES NO	☐ ☐
If YES, how do you dispose of used litter or bedding between clean-outs?	MANURE SHED OR COMPOSTER OUTDOOR MANURE PILE SPREAD ON FIELD AT THIS LOCATION HAULED AWAY	☐ ☐ ☐ ☐
31. How do you dispose of dead birds? *Please check all that apply.*	COMPOSTED LANDFILL / TRASH BURIED TAKEN BY PREDATORS HAULED AWAY	☐ ☐ ☐ ☐ ☐
32. Do you use pest control such as mouse traps or insecticides?	YES NO	☐ ☐
33. Would you like to receive educational materials on bird health and management?	YES NO	☐ ☐
34. Would you be interested in participating in the sample collection phase of the study?	YES NO	☐ ☐

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